

Few indirect effects of baculovirus on parasitoids demonstrate high compatibility of biocontrol methods against *Tuta absoluta*

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Abstract

BACKGROUND: Combining different biocontrol agents, particularly micro- and macroorganisms, can contribute to new and sustainable pest control approaches. *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most destructive pests of solanaceous crops. An emerging management strategy consists of biological control using microbial insecticides such as baculoviruses, but with limited efficacy. Thanks to their high target specificity, baculoviruses can be used simultaneously with natural enemies such as parasitoids for improved control of *T. absoluta*. However, potential indirect nontarget effects of baculoviruses on parasitoids can result from overlapping resource requirements. We assessed whether ovipositing parasitoid females discriminated against virus-treated hosts and examined the outcome of within-host competition between the hymenopteran parasitoids *Necremnus tuta* (Reuter) (Eulophidae) and *Dolichogenidea gelechiidivoris* Marsch (Braconidae), and the Phthorimaea operculella granulovirus (PhopGV, Baculoviridae) that infects *T. absoluta* larvae.

RESULTS: Female *D. gelechiidivoris* discriminated against virus-treated hosts, whereas *N. tuta* did not. We found few indirect virus-related effects depending on the species, the sex, and the time of virus treatment. Effects were ambivalent for *D. gelechiidivoris* offspring and ranged from increased male longevity when infection occurred before parasitization to reduced emergence and male longevity when infection occurred after parasitization. *N. tuta* offspring showed a longer development time and shorter male longevity when they developed in virus-treated hosts.

CONCLUSION: The virus had a low impact on parasitoid offspring. In rare cases, adverse effects were detected; however, the low magnitude of these effects is unlikely to reduce the fitness of parasitoid offspring, therefore both parasitoids seem compatible with the baculovirus for control of *T. absoluta*.

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Keywords: nontarget effect; baculovirus; Braconidae; Eulophidae; tomato leafminer; host discrimination; compatibility

1 INTRODUCTION

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is a destructive pest of tomato plants that also attacks other solanaceous species such as eggplant or tobacco. The pest has become a serious threat to tomato production worldwide,^{1–5} invading more than 90 countries outside of its endemic region (EPPO, <https://gd.eppo.int>). *T. absoluta* is very difficult to control due to the cryptic behavior of its larvae, the increasing resistance to synthetic insecticides, its high reproduction rate with many generations per year, and its high dispersal capacity.^{6–8} Therefore, new management tools that are safe for human health and the environment are urgently needed for managing this invasive pest.⁹

As biological insecticides, baculoviruses can help control lepidopteran pests.¹⁰ The baculovirus family includes a group of arthropod-specific viruses that infect insects from the orders Lepidoptera, Hymenoptera, and Diptera. These invertebrate pathogens are characterized by the presence of two different phenotypes during their infection in susceptible hosts, budded viruses (BVs) and occlusion derived viruses (ODVs). Infection occurs when a susceptible host feeds on plants contaminated with the ODVs that are occluded

within occlusion bodies (OBs). After ingestion, the OBs are dissolved in the gut and the ODVs infect the midgut epithelial cells. BVs then disperse and replicate in other susceptible tissues. When a lethal infection occurs, the tissue of the host larva disintegrates, releasing new ODVs on the plant surface.^{11,12} Baculoviruses have been used very successfully in the past, such as against the soybean pest *Anticarsia gemmatilis* (Hübner) (Lepidoptera: Noctuidae) in South America and in Europe against the codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), a pest of apple and walnut.^{13,14}

A member of the genus *Betabaculovirus* (lepidopteran granuloviruses), Phthorimaea operculella granulovirus (PhopGV), has been

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considered for the management of different lepidopteran pest species of the Gelechiidae family such as *Phthorimaea operculella* (Zeller), *Tecia solanivora* (Povolny), and *T. absoluta*.^{15–19} Recently, a microbial insecticide based on PhopGV was commercially developed against *T. absoluta* (Tutavir©, Andermatt Biocontrol, Switzerland). The product is registered for use in Brazil and has been granted emergency approval in different European countries such as Greece, Cyprus, Germany, and Switzerland (Andermatt, personal communication). When ingested by *T. absoluta* neonates, the PhopGV induces mortality and sublethal effects such as delayed development, pupation failure, and reduced fecundity.^{15–17} Nevertheless, high doses (weekly application of the highest recommended concentration) are required to lethally infect the most damaging older larvae, so that complementary control measures are required.

The use of baculoviruses for pest control takes place within a wider agricultural system, which may include biological control agents that are present naturally or mass-released. Due to the high target specificity of baculoviruses,²⁰ natural enemies such as hymenopteran parasitoids are not susceptible to infection. Yet, negative fitness consequences have been observed, such as reduced size or weight in parasitoid offspring that emerge from virus-infected hosts.^{21,22} These are indirect consequences of an altered quality of the infected hosts. Deleterious effects of viruses on parasitoids can also result from the viral infection killing the host larva before the parasitoid has completed its development or,²³ more generally, from overlapping resources requirements of immature parasitoid stages and the replicating virus.¹¹

Many larval parasitoids have been found attacking *T. absoluta* in the Mediterranean region, among them *Necremnus tutae* (Reuter) (Hymenoptera: Eulophidae) is particularly abundant and efficient.^{24–29} While several companies have attempted to mass-produce this species, the utilization of *N. tutae* is now restricted to conservation biological control due to an unfavorable cost–benefit balance.⁹ More recently, *Dolichogenidea gelechiidivoris* (Marsh) (Hymenoptera: Braconidae), which originates from the Neotropics, has established in Spain and Algeria.^{30,31} In South America, it is considered an essential agent against *T. absoluta*,³² and in 2017 it was imported to Kenya from Peru to contribute to the control of *T. absoluta* in Africa.³³ These parasitoid species have very distinct life history traits. *D. gelechiidivoris* is a solitary koinobiont larval endoparasitoid that prefers to parasitize early larval instars of *T. absoluta*,³³ whereas *N. tutae* is an idiobiont ectoparasitoid, which preferably parasitizes later instars.³⁴ As a koinobiont parasitoid, *D. gelechiidivoris* develops alongside its host, whereas the idiobiont *N. tutae* paralyzes the host and stops its development. Whether virus applications might have adverse effects on the parasitoids attacking *T. absoluta* and how these effects relate to the parasitoid's life strategy is currently unknown. Understanding these interspecific interactions will better predict the compatibility of PhopGV applications and larval parasitoids to control *T. absoluta*.

We conducted laboratory experiments to investigate interactions between PhopGV and the parasitoids *N. tutae* and *D. gelechiidivoris*. In a first step, we assessed the parasitoids' capacity to discriminate between virus-treated and healthy host larvae. In a second step, we evaluated parasitoid development in larvae that had been treated with the virus before and after parasitization.

2 MATERIAL AND METHODS

2.1 Insect and plant material

The study was conducted jointly at the biosafety laboratory of Agroscope in Switzerland (*N. tutae*) and the sustainable plant

protection laboratory of IRTA in Spain (*D. gelechiidivoris*). The tomato plant varieties used in Switzerland and Spain were *Solanum lycopersicon* cv Rentita and Simona, respectively. Plants were grown in greenhouses.

All insect rearing and experiments in both countries took place in climate chambers at 25 ± 1 °C, $70 \pm 10\%$ relative humidity, and a 16:8 h light:dark photoperiod. In Switzerland, *T. absoluta* was provided by Andermatt Biocontrol; in Spain, it was obtained from an established laboratory colony that originated from field-sampled adults from the Barcelona region. Adults were kept in mesh cages (50 × 50 × 50 cm) (bug dorm; MegaView Science Co. Ltd., Taiwan) and provided with cotton soaked in honey-water (10% v/v) placed on the top of the cage and tomato plants for egg-laying. After 7 days, plants with eggs and young larvae were moved to another cage to start a new colony.

N. tutae and *D. gelechiidivoris* rearing was initiated with individuals collected in commercial tomato fields in El Maresme county, Barcelona, Spain. Adult parasitoids were kept in mesh cages and provided with cotton soaked in honey-water and tomato plants with *T. absoluta* second to third larval instar for parasitization. After emergence, adult parasitoids were collected and stored at 12 °C with honey and water. Every 10 days, a new parasitoid generation was started. All female parasitoids used in the experiments were naive, mated (stored with males for at least 2 days), and less than a week old.

2.2 Virus application

The virus suspension Tutavir© (Andermatt Biocontrol, 6146 Grossdietwil, Switzerland) containing a minimum of 2×10^{13} OB/l of PhopGV was diluted with tap water to the highest recommended rate for field application, 0.02% v/v (Andermatt Biocontrol, personal communication). Thus, the suspension used in the experiments contained at least 4×10^9 OB/l. The suspension was sprayed on the tomato plants to obtain infected larvae for experiments. A hand sprayer was used and attention was paid to covering all parts of the leaves. The plants were sprayed with the virus suspension twice with an interval of 4 days, i.e., inoculation occurred in first larval instar (L1) larval instar for all experiments except for experiment 2.4.2, in which L2–L4 larval instars were inoculated.

2.2.1 Virus effect on *T. absoluta* larvae mortality and weight

Mortality in *T. absoluta* larvae caused by PhopGV infection usually increases after incubation for 9–11 days.¹⁹ However, after six days, inoculated larvae showed the typical signs of baculovirus infection, such as loss of mobility, swollen body, decreased feeding, and change in color from green to white. In the late stages, larvae with lethal infection become sluggish and flacid before complete lysis of the body and death.^{17,19} Sublethal effects in the late stage were visible when the larvae were characteristically swollen with a bright white-orange color and did not pupate even after several weeks.

To assess mortality, experiments were conducted in plastic containers (10 cm diameter, 15 cm height) covered with a mesh. Five eggs of *T. absoluta* (less than 24 h old) were placed on a tomato leaf using a fine brush (one leaf per container with five fully grown leaflets, each with one egg). In each container, the leaf stem was placed in a solution of agar water (8 g L^{-1}) to provide moisture. At days one and four after egg hatching, leaves in half of the containers ($n = 18$) were treated with the virus (inoculation of L1 larval instar) and leaves in the other half of the containers ($n = 18$) were left untreated. Fifteen days after the start of the experiment, the

number of healthy pupae (natural shape and color) was compared between the control group and the virus group.

To assess the effect of virus treatment on larval weight after different incubation times, another experiment was conducted. On days 1 and 4 after egg hatching, the tomato plants in the treatment group were sprayed with the virus suspension to infect L1 larvae. Then, healthy and virus treated larvae were weighted after different incubation times. Groups of 6- and 12-day-old healthy and treated larvae were randomly selected and killed in ethanol. The ethanol was then evaporated on a paper towel and the fresh weight of each insect was determined ($n = 40$ larvae per group).

2.3 Parasitoid host choice

A choice test was performed in the laboratory to determine whether infection with PhopGV affected parasitoid host preference (see Fig. 1). On days 1 and 4 after egg hatching, the tomato plants in the treatment group were sprayed with the virus suspension to infect L1 larvae. At 24 h prior to each experiment, larvae were placed on clean leaflets (not sprayed with the virus) to allow time to burrow into a mine. Tomato leaflets (5 ± 2 cm length, freshly cut) with either a healthy or a virus-treated host larva were offered to female parasitoids at equal distances in a 15-cm diameter Petri dish covered with a mesh.

2.3.1 *Necremnus tuta*

For the parasitoid *N. tuta*, six leaflets with a healthy or a virus-treated host larva were offered for 24 h to single naive females in a Petri dish ($n = 27$ replicates). Host larvae were 8 days old (L3 larval instar), corresponding to the parasitoids' preferred host instar.³⁴ Subsequently, females were removed, and each host larva was observed under a stereomicroscope and classified as (i) parasitized (paralyzed and presence of *N. tuta* eggs), (ii) host-fed (dead and presence of biting or stinging marks), (iii) dead without any attack mark, or (iv) alive. An additional experiment was conducted with 12-day-old host larvae (L4 larval instar) to evaluate the host choice with older larvae. For this experiment,

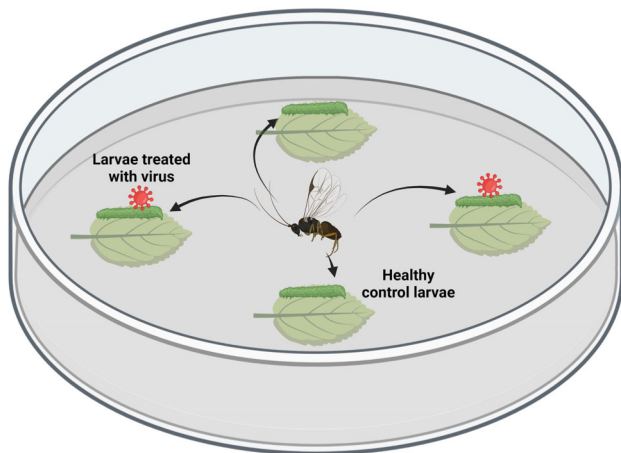


Figure 1. Experimental study on parasitoid host choice. *Tuta absoluta* larvae were treated with PhopGV on days 1 and 4 after hatching, untreated larvae served as control. An equal number of tomato leaflets with healthy or treated larvae were offered to female parasitoids at equal distances in a Petri dish. Leaflets were offered to naive females *Necremnus tuta* and *Dolichogenidae gelechiidivoris* for 24 h and 30 min, respectively. For *D. gelechiidivoris*, behavioral observations were conducted during the 30 min to assess first and second choice (first host that was stung by the wasp) and record all stung larvae. Successful parasitization was assessed for both parasitoids.

only four leaflets (carrying healthy or virus-treated host larvae) were offered to single *N. tuta* females ($n = 27$).

2.3.2 *Dolichogenidae gelechiidivoris*

The same experimental set-up was used for *D. gelechiidivoris*. Since preliminary experiments showed that female *D. gelechiidivoris* are much more active than *N. tuta*, they were only allowed to parasitize for 30 min ($n = 15$ replicates). Host larvae were 6 days old (L2 larval instar), corresponding to the preferred host instar of *D. gelechiidivoris*.³³ Behavioral observations were conducted during the 30-min period to assess first and second choice (first host that was stung by the wasp) and record all stung larvae. Subsequently, the females were removed, and each larva was individualized in a Petri dish with fresh tomato leaflets. Since *D. gelechiidivoris* is an endoparasitoid, parasitization is only recognizable when larvae leave the host for pupation, approximately 12 days after parasitization (J. Gonthier, personal observation). After 14 days, each larva was observed under a stereomicroscope and compared with the behavioral data to assess if it was (i) stung and parasitized with the presence of an external cocoon, (ii) stung but rejected (host larvae emerged as an adult), (iii) stung but host larvae died, (iv) not stung and host larvae emerged, or (v) not stung but host larvae had died. An additional experiment was conducted with 12-day-old host larvae to evaluate the host choice with older larvae. For this experiment, only four leaflets per Petri dish (carrying healthy or virus-treated host larvae) were offered to the *D. gelechiidivoris* females ($n = 19$ replicates).

2.4 Host quality

2.4.1 Virus treatment before parasitization

Healthy and virus-treated host larvae were parasitized to assess the effect of host-virus infection on parasitoid offspring. Treated larvae were parasitized at different time intervals after the virus treatment, considering the long incubation time. The plants were sprayed with the virus suspension twice with a 4-day interval, i.e., on days 1 and 4 after egg hatching (inoculation of L1 larval instar). Two treatments, each with a control, were compared: (i) parasitized host larvae 6 days after virus treatment (6V) and (ii) parasitized host larvae 12 days after virus treatment (12V). Parasitized 6- and 12-day-old host larvae served as control (6C and 12C) (Fig. 2(A)).

2.4.1.1. *Necremnus tuta*. Six days after *T. absoluta* larva emergence, a few leaflets with about 20 larvae (treated or healthy as control) were placed in a Petri dish for parasitization by individual females for 24 h. Leaflets were kept fresh throughout the experiments by placing the stem in a piece of wet cotton, and a droplet of honey was available to the parasitoid females during parasitization. The experiments were conducted in three runs, resulting in a total of $n = 31$ replicates for 6V and $n = 38$ for 6C. Each female was considered as one replicate. Five days after parasitization, the leaflets were dissected under a stereomicroscope.

For each female, the first five parasitoid pupae that were found were individualized in small tubes (1 cm diameter, 7 cm height) covered with a mesh. Development time, emergence rate, sex ratio, adult size, fertility, and survival of parasitoid offspring were monitored. Survival was measured daily from emergence until death or up to 35 days, and parasitoids were fed every other day with a drop of honey and water. The tibia length of individuals was measured under a stereomicroscope as a proxy for body size. When possible (more than one female emerged in a replicate),



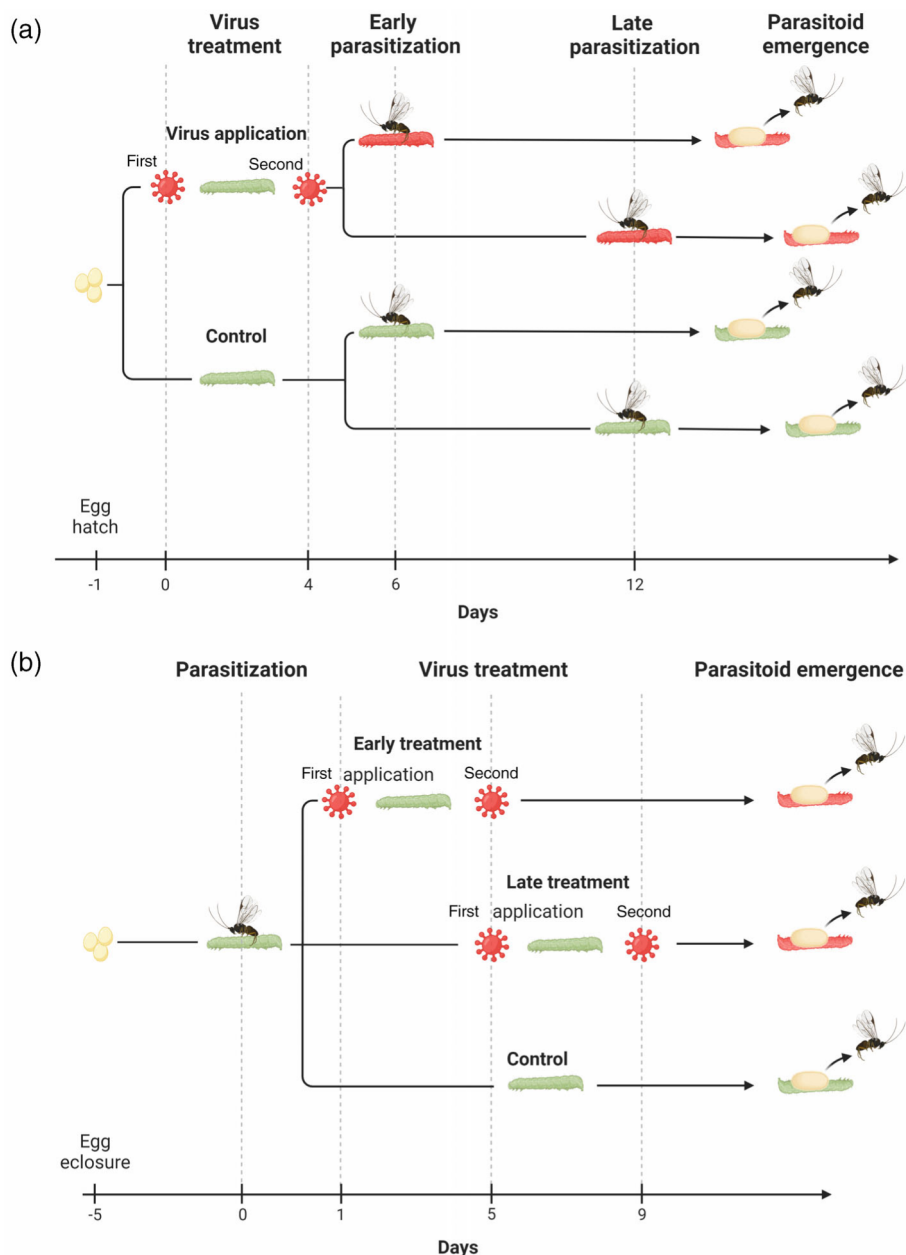


Figure 2. (A) Experimental study set-up for virus treatment before parasitization. The experiment was conducted separately with the parasitoids *Necremnus tutae* and *Dolichogenidae gelechiidivoris*. Half of the *Tuta absoluta* larvae were treated with PhopGV 1 day after emergence. Half of the treated larvae were subsequently parasitized 6 days after virus treatment (6V) and the other half 12 days after infection (12V). The untreated larvae served as the control and were similarly parasitized after 6 days (6C) and 12 days (12C). (B) Experimental study on virus treatment after parasitization. Larvae of *Tuta absoluta* were parasitized by the parasitoid *Dolichogenidae gelechiidivoris* 5 days after emergence. One-third of the larvae were treated with PhopGV 1 day after parasitization, one-third were treated 5 days after parasitization, and one-third remained untreated (control).

one female was killed in ethanol on the third day after emergence to measure its egg load as a proxy for fecundity. The mean of the male and female offspring for each replicate was calculated for the measured parameters to avoid pseudo-replication (from one to four individuals). The experiment was repeated with 12-day-old host larvae (healthy control 12C $n = 34$, treated 12V $n = 21$).

parasitoid, new leaflets were placed on top of the older ones as an additional food source for further development of host larvae after removing the female parasitoids. Fourteen days after parasitization, the leaflets were dissected under a stereomicroscope. The first five parasitoid pupae that were found were individualized in small tubes and monitored as described above.

2.4.1.2. *Dolichogenidae gelechiidivoris*. The same experiment (Fig. 2(A)) was conducted for *D. gelechiidivoris* over two runs (6C $n = 24$, 6V $n = 23$, 12C $n = 18$, 12V $n = 9$). Since it is a koinobiont

2.4.2 Virus treatment after parasitization

As host larvae parasitized by *D. gelechiidivoris* continue feeding and can become infected by the virus after parasitization, this

experiment was performed to evaluate the virus' impact on the parasitoid offspring when infection occurs after parasitization (Fig. 2(B)). Two virus treatments were compared with healthy larvae. For the first group, virus treatment occurred 1 day after parasitization (V1). For the second group, virus treatment occurred 5 days after parasitization (V5). The leaflets were sprayed with the virus suspension twice with a 4-day interval, i.e., for V1 starting on day 1 after parasitization (inoculation of L2–L3 larval instars) and for V5 starting on day 5 (inoculation of L3–L4 larval instars). Parasitized larvae without virus treatment served as the control.

For each group (control and treatment), the following procedure was performed. Five days after the emergence of host larvae, a few leaflets with about 20 untreated *T. absoluta* larvae were placed in Petri dishes and offered to individual females for parasitization as described in the previous experiment. After removing the females, new leaflets were placed *ad libitum* on the older leaflet as additional food for the host larvae. Fourteen days after parasitization, the leaflets were dissected under a stereomicroscope and the first 10 parasitoid pupae that were found were individualized in small tubes covered with a mesh. For each replicate and each measured parameter, the means of male and female offspring were calculated to avoid pseudoreplication (ranging from one to 10 individuals). Survival and the different fitness traits in the offspring were monitored as described above. Between 20 and 21 replicates (control = 20, V1 = 21, V5 = 21) were conducted over two experimental runs.

2.5 Data analysis

The software NCSS (2020) (NCSS, LLC, US) was used for statistical analysis. Data were tested for normal distribution using the Shapiro–Wilk's test, and visual inspections of the data were made using Q–Q plots. The data were mostly not normally distributed (Shapiro–Wilk's test, $P < 0.05$). The first and second host choices (healthy vs. treated) in the behavioral observation with *D. gelechiidivoris* were compared to the unbiased proportion of 50% with a binomial test. A chi-square test on contingency tables (2×4 for *N. tutae* and 2×5 for *D. gelechiidivoris*) was used to compare the category ratio (alive, dead, parasitized, host-fed, etc.) between healthy and treated larvae. A Wilcoxon signed-rank test was used as a *post hoc* test. The effect of virus treatment on the weight and mortality of *T. absoluta* larvae was assessed with a Mann–Whitney *U*-test. The same test was used for the host quality experiments to compare the emergence rate, development time, sex ratio, egg load, and tibia length between healthy and treated larvae at the two different time intervals with their respective control. Survival analyses were conducted using Kaplan–Meier survival curves and log rank tests (Mantel–Haenszel test) with pairwise comparison. *P* values lower than 0.05 were considered statistically significant.

3 RESULTS

3.1 Effect of the virus on the weight and mortality of *T. absoluta* larvae

Virus treatment significantly decreased the larval survival of *T. absoluta* from $76 \pm 6.2\%$ (mean \pm SE, $n = 18$) in the control to $47 \pm 6.0\%$ in the treated group ($n = 18$, $U = 70.5$, $P = 0.003$, Mann–Whitney *U*-test).

The weight of *T. absoluta* larvae was affected by the virus at 6 days but not at 12 days after virus treatment. Six days after virus treatment, the treated larvae were lighter [$1046 \pm 117.2 \mu\text{g}$ (mean \pm SE), $n = 42$] than the control larvae ($1420 \pm 130.8 \mu\text{g}$,

$n = 41$, $U = 541$, $P = 0.003$). Twelve days after virus treatment, no significant differences were found between the control larvae ($4106 \pm 189 \mu\text{g}$, $n = 43$) and the treated larvae ($4508 \pm 291.5 \mu\text{g}$, $n = 41$, $U = 795$, $P = 0.439$).

3.2 Parasitoid host choice

3.2.1 *Necremnus tutae*

Eight days after virus treatment, the proportions of larvae parasitized, host-fed, alive and dead were not significantly different from the healthy larvae ($\chi^2 = 0.015$, $df = 3$, $P = 0.999$, chi-square test; Fig. 3(A)). Each female parasitized $11 \pm 5\%$ vs. $16 \pm 6\%$ and host-fed on $36 \pm 5\%$ vs. $31 \pm 5\%$ of healthy or virus-treated larvae, respectively (mean \pm SE, all *P* values for paired comparisons > 0.870 , Wilcoxon signed-rank test; Fig. 3(A)). Twelve days after virus treatment, the proportions of larvae parasitized, host-fed, alive, and dead did not differ significantly between treated and healthy larvae ($\chi^2 = 0.031$, $df = 3$, $P = 0.998$, chi-square test; Fig. 3(B)). Females parasitized $19 \pm 5.0\%$ vs. $11 \pm 4.0\%$ or used larvae for host feeding $26 \pm 5.0\%$ vs. $31 \pm 7.0\%$ for healthy and virus-treated larvae, respectively (all *P* values for paired comparisons > 0.206 , Wilcoxon signed-rank test; Fig. 3(B)).

3.2.2 *Dolichogenidae gelechiidivoris*

Behavioral observations revealed that female *D. gelechiidivoris* differentiated between healthy and treated host larvae. Six days after virus treatment, significantly more healthy larvae were stung as first choice, 66% (38.3–88.1%, 95% confidence interval, $n = 15$, $P = 0.031$, binomial test). However, no preference was detected in the second choice, 40% (12.1–73.7%, $n = 10$, $P = 0.612$). Twelve days after virus treatment, significantly fewer healthy larvae were stung as first choice, 31% (12.5–56.5%, $n = 19$, $P = 0.001$), but, no significant preference was observed in the second-choice 43% (17.6–71.1%, $n = 14$, $P = 0.513$).

Six days after virus treatment, the proportion of larvae parasitized, rejected (stung but host larvae emerged), dead (stung and dead), alive (not stung), and dead (not stung) did not differ significantly between treated and healthy larvae ($\chi^2 = 0.096$, $df = 4$, $P = 0.998$; chi-square test; Fig. 3(C)). Females parasitized $24 \pm 8\%$ vs. $31 \pm 9\%$ or rejected $12 \pm 5\%$ vs. $13 \pm 6\%$ of healthy and virus-treated larvae, respectively (mean \pm SE, all *P* values for paired comparisons > 0.263 , Wilcoxon signed-rank test; Fig. 3(C)). Twelve days after virus treatment, the proportion of larvae parasitized, rejected, dead (stung), alive (not stung), and dead (not stung) was also not significantly different from that of the healthy larvae ($\chi^2 = 0.038$, $df = 4$, $P = 0.998$; chi-square test; Fig. 3(D)). However, females rejected significantly more virus-treated larvae ($34 \pm 9.0\%$) than healthy larvae ($3 \pm 3.0\%$) (mean \pm SE, $Z = 2.737$, $P = 0.006$, Wilcoxon signed-rank test; Fig. 3(D)). Nevertheless, they successfully parasitized (progeny successfully developed and emerged) $24 \pm 8.0\%$ vs. $26 \pm 9.0\%$ of healthy and virus-treated larvae, respectively (all *P* values for paired comparisons > 0.146 ; Fig. 3(D)).

3.3 Virus treatment before parasitization

3.3.1 *Necremnus tutae*

Three of the fitness traits assessed for *N. tutae* offspring developing in virus-treated (6V) larvae were affected, but these effects differed for males and females. The development time of immature males was extended compared to the control group ($U = 172.5$, $P = 0.030$; Mann–Whitney *U*-test; Table 1), whereas no difference was observed in the immature female development time. In female offspring, the tibia length was reduced compared to the



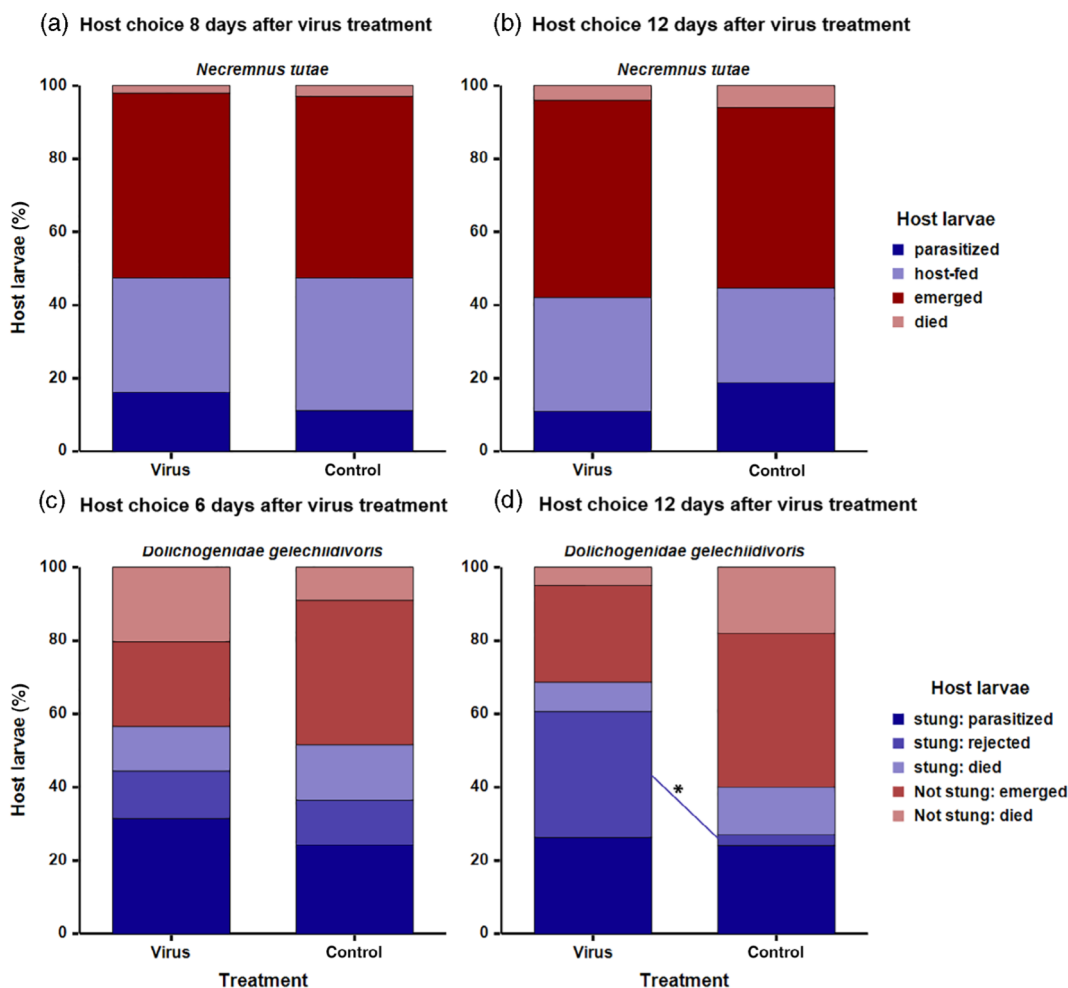


Figure 3. Host preference of female *Necremnus tutae* and *Dolichogenidae gelechiidivoris* (% of host larvae). Single females were offered the same number of healthy and virus-treated hosts. (A) Female *N. tutae* were offered for 24 h three healthy and three larvae treated with the virus 8 days prior to the experiment ($n = 27$). (B) Female *N. tutae* were offered for 24 h two healthy and two larvae treated with the virus 12 days previously ($n = 27$). (C) Female *D. gelechiidivoris* were offered for 30 min three healthy and three larvae treated with the virus 6 days before ($n = 15$). (D) Female *D. gelechiidivoris* were offered for 30 min two healthy and two larvae treated with the virus 12 days previously ($n = 19$) (* $P < 0.05$, Wilcoxon signed-rank test).

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Table 1. Measures of fitness traits of *Necremnus tutae* offspring developing in healthy or virus-treated hosts

		6 days			12 days		
		Control (6C)	Virus (6V)		Control (12C)	Virus (12V)	
Emergence (%)		83.6 ± 5.7 (30)	90.3 ± 4.5 (38)	ns	65.1 ± 9.8 (34)	80.6 ± 6.2 (21)	ns
Development (d)	F	12.0 ± 0.2 (13)	12.0 ± 0.2 (15)	ns	13.0 ± 0.3 (27)	13.2 ± 0.2 (12)	ns
	M	11.9 ± 0.1 (20)	12.3 ± 0.2 (27)	*	12.3 ± 0.3 (9)	12.3 ± 0.1 (4)	ns
Sex ratio (male %)		67.4 ± 7.0 (29)	64.4 ± 8.2 (33)	ns	23.3 ± 10.8 (29)	18.7 ± 6.0 (15)	ns
Egg load		5.1 ± 0.3 (14)	5.8 ± 0.5 (13)	ns	5.0 ± 0.4 (15)	5.6 ± 0.3 (15)	ns
Tibia length (µm)	F	672.5 ± 23.7 (10)	525.3 ± 40.9 (11)	**	714.7 ± 36.4 (15)	635.1 ± 32.7 (11)	ns
	M	451.0 ± 13.5 (9)	475.1 ± 23.9 (13)	ns	435.0 ± 46.1 (12)	447.3 ± 21.2 (15)	ns

Two different treatments were compared to the respective controls: 6V, host larvae parasitized 6 days after virus treatment; 6C, control group with healthy host larvae of the same age; 12V, host larvae parasitized 12 days after virus treatment; 12C, control group with healthy host larvae of the same age. All data are shown as means ± SE (sample size). F, females; M, males; ns, not significant. * $P < 0.05$, ** $P < 0.01$, Mann–Whitney U -test.

control group ($U = 18.5$, $P = 0.010$), whereas male tibia length was similar in treated and control hosts ($U = 41$, $P = 0.384$). The male offspring's survival was reduced compared to the control group

($P = 0.031$, log-rank test; Fig. 4(A)), although no difference was observed in female survival ($P = 0.882$, log-rank test; Fig. 4(A)). Offspring were otherwise not affected when developing in 6V



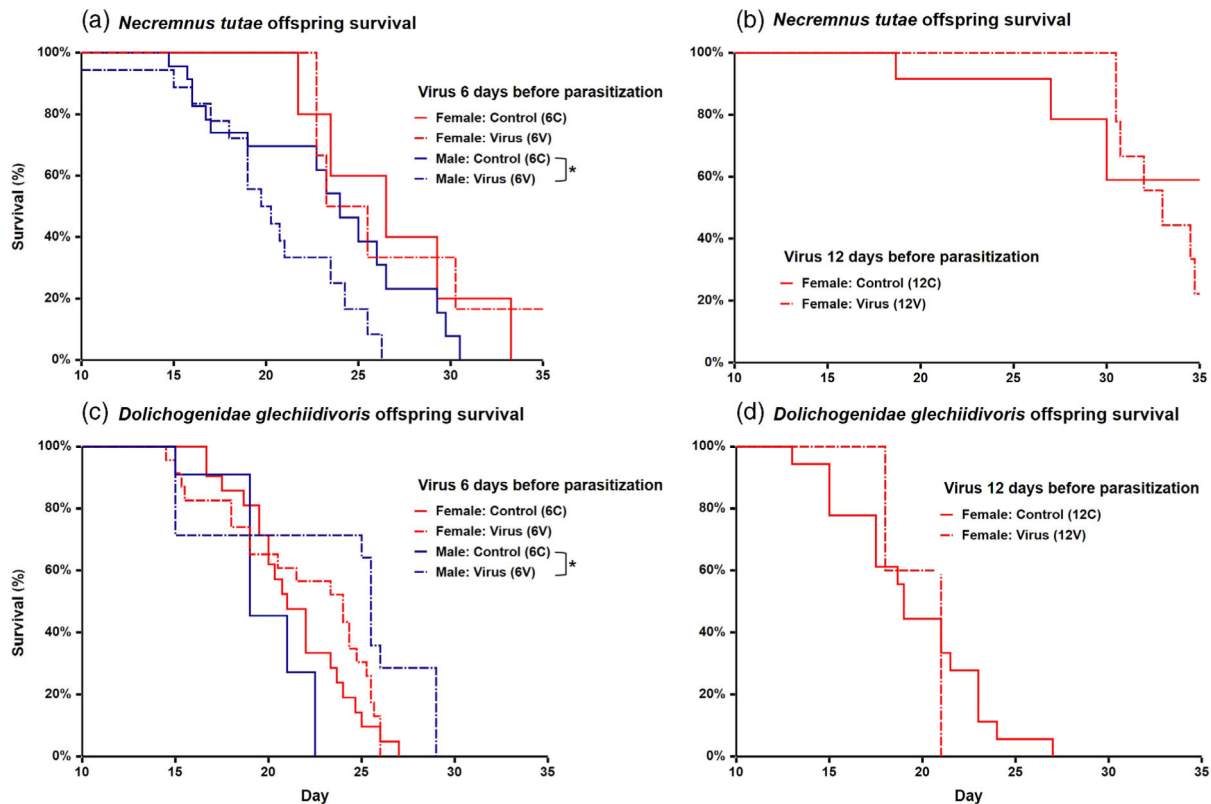


Figure 4. Kaplan–Meier survival curves of *Necremnus tutae* and *Dolichogenidae gelechiidivoris* offspring that developed in healthy (control) or virus-treated host larvae of *Tuta absoluta*. (A) Male and female *N. tutae* offspring that developed on larvae 6 days after virus treatment. (B) Female *N. tutae* offspring that developed on larvae 12 days after virus treatment. (C) Male and female *D. gelechiidivoris* offspring that developed in larvae 6 days after virus treatment. (D) Female *D. gelechiidivoris* offspring that developed in larvae 12 days after virus treatment. Significant differences between groups are indicated (* $P < 0.05$, log-rank test: Mantel–Haenszel).

Table 2. Measures of fitness traits of *Dolichogenidae gelechiidivoris* offspring developing in healthy or virus-treated hosts

		6 days			12 days		
		Control (6C)	Virus (6 V)		Control (12C)	Virus (12 V)	
Emergence (%)		57.8 ± 5.2 (23)	50.4 ± 5.7 (24)	ns	52.5 ± 7.4 (9)	38.9 ± 11.5 (18)	ns
Development (d)	F	20.9 ± 0.2 (21)	21.6 ± 0.5 (19)	ns	19.9 ± 0.2 (6)	19.3 ± 0.4 (13)	ns
	M	20.8 ± 0.4 (10)	22.1 ± 0.6 (12)	ns	19.9 ± 0.5 (3)	19.3 ± 0.3 (8)	ns
Sex ratio (male %)		31.1 ± 8.1 (22)	23.1 ± 6.4 (24)	ns	34.7 ± 10.3 (7)	28.6 ± 15.3 (16)	ns
Egg load		107.3 ± 3.3 (9)	102.1 ± 2.6 (11)	ns	111.2 ± 3.6 (4)	118.3 ± 4.9 (9)	ns
Tibia length (µm)	F	752.8 ± 11.5 (10)	811.3 ± 15.6 (11)	**	721.2 ± 17.2 (11)	837.5 ± 38.9 (15)	*
	M	743.2 ± 13.1 (9)	766.3 ± 13.7 (13)	ns	780.0 ± 9.4 (4)	750.0 ± 75 (12)	ns

Two different treatments were compared to the respective controls: 6V, host larvae parasitized 6 days after virus treatment; 6C, control group with healthy host larvae of the same age; 12V, host larvae parasitized 12 days after virus treatment; 12C, control group with healthy host larvae of the same age. All data are shown as means ± SE (sample size). F, females; M, males; ns, not significant. * $P < 0.05$, ** $P < 0.01$, Mann–Whitney *U*-test.

larvae and had a similar emergence rate, egg load, and sex ratio to that of offspring emerging from 6C larvae (all P values > 0.195 , Mann–Whitney *U*-test; Table 1). No significant differences in the fitness traits of offspring developing in 12C and 12V larvae were detected (all P values > 0.126 ; Table 1 and Fig. 4(B)).

3.3.2 *Dolichogenidae gelechiidivoris*

Two of the fitness traits assessed for *D. gelechiidivoris* offspring developing in virus-treated (6V) larvae were increased, but the

effects depended on the sex. The tibia length of female offspring from 6V larvae was increased compared to 6C larvae offspring ($U = 74$, $P = 0.009$, Mann–Whitney *U*-test; Table 2), while male tibia length was similar in treatment and control insects ($U = 41$, $P = 0.296$). The survival of males in the virus group was increased compared to the control group ($P = 0.001$, log-rank test; Fig. 4(C)), whereas no significant difference was observed in female survival ($P = 0.431$; Fig. 4(C)). The offspring of 6V larvae were otherwise unaffected and had a similar development time, emergence rate,



Table 3. Measures of fitness traits of *Dolichogenidae gelechiidivoris* offspring developing in healthy and virus-treated hosts

	Control (C)	Virus (V1)	Virus (V5)	C vs V1	C vs V5
Emergence (%)	59.3 ± 3.7 (20)	41.8 ± 3.6 (21)	49.1 ± 4.1 (21)	**	ns
Development (d)	F	22.0 ± 0.3 (14)	22.7 ± 0.5 (17)	ns	ns
	M	21.7 ± 0.5 (12)	21.0 ± 0.6 (12)	ns	ns
Sex ratio (male %)	48.5 ± 10.6 (20)	41.2 ± 9 (20)	60.9 ± 10.2 (21)	ns	ns
Egg load	101.8 ± 4.7 (8)	110.0 ± 4.3 (10)	107.5 ± 7.1 (6)	ns	ns
Tibia length (µm)	F	735.3 ± 10.4 (12)	756.0 ± 18.7 (11)	ns	ns
	M	749.5 ± 25.3 (12)	741.8 ± 10.4 (14)	ns	ns

Comparisons were made between two treatments and a control group: V1, host larvae treated by the virus 1 day after parasitization; V5, host larvae treated by the virus 5 days after parasitization; C, healthy host larvae. All data are shown as means ± SE (sample size). F, females; M, males; ns, not significant. ** $P < 0.01$, Mann–Whitney U -test.

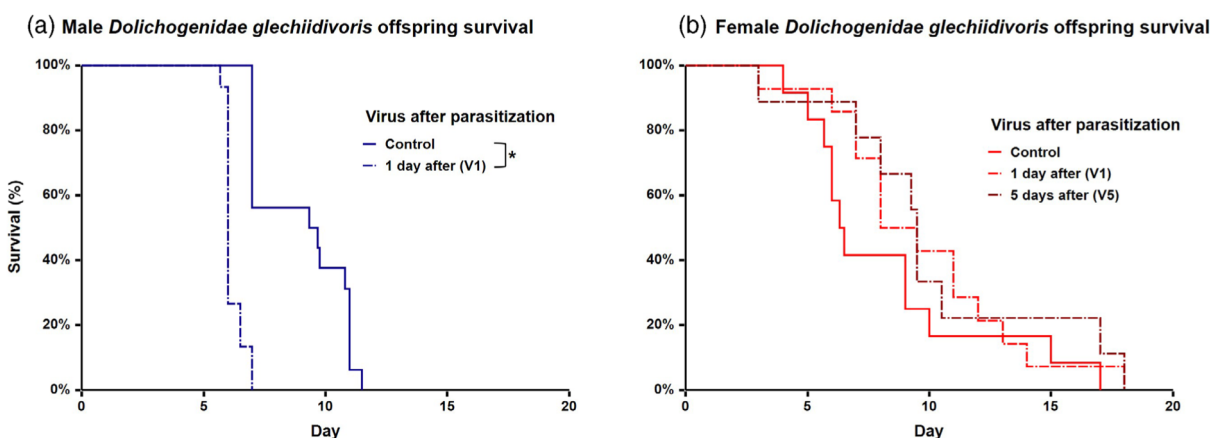


Figure 5. Kaplan–Meier survival curves of *Dolichogenidae gelechiidivoris* offspring that developed in healthy (control) or virus-treated host larvae of *Tuta absoluta*. (A) Male offspring of *D. gelechiidivoris* from hosts treated by the virus 1 day after parasitization. (B) Female offspring of *D. gelechiidivoris* from hosts treated with the virus 1 and 5 days after parasitization. Significant differences between groups are indicated ($*P < 0.05$, log-rank test: Mantel–Haenszel).

egg load, and sex ratio compared to the offspring of 6C larvae (all P values > 0.086 , Mann–Whitney U -test; Table 2). Twelve days after virus treatment, the tibia length of female offspring that developed in 12V larvae was also increased compared to offspring that developed in 12C larvae ($U = 5.5$, $P = 0.031$). No other significant differences in the fitness traits were found between offspring developing in 12C and 12V larvae (all P values > 0.109 ; Table 2 and Fig. 4(D)).

3.4 Virus treatment after parasitization

Two out of the six fitness traits assessed for *D. gelechiidivoris* developing in host larvae treated by the virus after parasitization were adversely affected compared to the control. When the treatment occurred 1 day after parasitization (V1), the emergence rate of offspring (male and female mixed) was reduced ($U = 92$, $P = 0.001$, Mann–Whitney U -test; Table 3) as well as the survival of the males ($P = 0.001$, log-rank test; Fig. 5(A)). None of the other parameters (i.e., development time, sex ratio, egg load, tibia length, and female survival) were significantly affected, and no differences were observed when host larvae were treated 5 days after parasitization (V5) (Table 3 and Fig. 5(B)).

4 DISCUSSION

The research presented here is among the first to examine the effects of a baculovirus on two parasitoids with very distinct life histories. The results of these laboratory experiments suggest that the baculovirus would pose negligible risks to the fitness of both the ectoparasitoid and the endoparasitoid of *T. absoluta*. The few observed effects on some fitness parameters were ambivalent (positive and negative) and species- and sex-specific. They depended on the virus incubation time (days since treatment) and whether treatment occurred before or after parasitization.

The observed effects on the parasitoids were likely mediated by the virus effects on the host larvae. Virus treatment significantly increased mortality and decreased the weight gain of *T. absoluta* larvae over time. Six days after virus treatment, the treated larvae were lighter than the control larvae, confirming earlier studies reporting that first larval instar are highly susceptible to virus infection, which typically slows down the growth of the host.³⁵ Nonetheless, no differences between healthy and treated larvae were found at 12 days after treatment. Probably, the most susceptible or most heavily infected larvae had already died at that time, dissolving in a pool of virus OBs, and therefore were



indiscernible at the second sample time on day 12. Usually, the susceptibility of larvae is a function of the virus' virulence, the developmental status of the insect, the stress imposed by the environment, and the dose of the viral inoculum.¹¹ The prevalence of infection can therefore greatly vary in the same population. Moreover, frequent sublethal symptoms were observed in the older larvae, which points to covert infections of the remaining larvae.¹²

The result of the host choice experiment showed that *N. tutae* did not discriminate between host larvae irrespective of the time since the virus treatment (8 or 12 days). These results are consistent with those of a previous study³⁶ that demonstrated the inability of the hymenopteran parasitoids *Chelonus insularis* Cresson (Hymenoptera: Braconidae) and *Campoletis sonorensis* Cameron (Hymenoptera: Ichneumonidae) to discriminate between nucleopolyhedrovirus-infected and healthy hosts. Host discrimination in parasitic wasps can be mediated by cues from antennal contact with hosts.³⁷ Since the larvae of *T. absoluta* feed inside mines, no direct antennal contact with the host is possible, which might explain why this parasitoid species did not discriminate. Nevertheless, parasitoids may also detect infected hosts on insertion of the ovipositor, so-called probing behaviour.³⁸ Unfortunately, due to the slow parasitization behavior of this species, direct observations were not feasible. Nonetheless, the female wasps killed the same number of treated and healthy larvae at the end of the experiment. Since *N. tutae* is known for host-killing *T. absoluta* during probing,³⁹ this supports the conclusion that the females did not probe more frequently on virus-treated larvae.

Interestingly, behavioral observations during the host choice experiment with *D. gelechiidivoris* showed contrasting results. When virus treatment occurred 6 days before parasitization, females preferred as their first choice to sting healthy larvae. In contrast, when virus treatment occurred 12 days before parasitization, females preferred to sting treated larvae as their first choice. Conversely, no preferences were observed in the second choice, therefore discrimination for stinging does not seem to be consistent over time in this species. We cannot, however, rule out the possibility that the apparent inconsistency in response to 6- and 12-day-old treated larvae is due to the relatively small sample size of females responding ($n = 15$).

Comparison of the larvae stung and successfully parasitized by *D. gelechiidivoris* showed that treated larvae were rejected more often. Even more interesting, rejection was found only with older larvae after an incubation period of 12 days, so that the ability to discriminate likely increases with the severity of the infection. Similar results were reported in a previous study,⁴⁰ in which female *Meteorus gyrator* (Thunberg) (Hymenoptera: Braconidae) reduced the number of eggs inserted into granulovirus-infected Indian meal moth, *Lacanobia oleracea* (L) (Lepidoptera: Noctuidae) larvae, based on the level of host infection. In fact, host discrimination is a well-known phenomenon and numerous hymenopteran parasitoids can discriminate against infected larvae to varying degrees.⁴¹ Consequently, by discriminating between healthy and treated hosts, *D. gelechiidivoris* would be less susceptible than *N. tutae* to the adverse effects of virus-infected hosts. In terms of pest control efficacy, the combination could even be beneficial since the parasitoid can minimize harmful interference and control those hosts that have escaped the virus. Moreover, the wasps can spread infection by transmitting budded virions when stinging the larvae.¹¹

Unexpectedly, even though female *D. gelechiidivoris* rejected more treated than healthy larvae 12 days after virus treatment, the same number of hosts was successfully parasitized. There are several possible explanations. First, treated host larvae could be more attractive at first (as shown by the first-choice preference), with more volatile cues being emitted by treated larvae, but considered less suitable after closer examination, i.e. through probing. Second, female *D. gelechiidivoris* are very efficient in host finding and handling (J. Gonthier, personal observation). Even though they rejected many larvae, they could have managed to locate and parasitize enough hosts with a low infection level during the time allowed (30-min period). It is important to bear in mind that we cannot exclude that hosts that were classified as rejected (stung but *T. absoluta* emerged) could also be hosts in which the parasitoid egg did not survive (e.g., egg encapsulation by the host). However, this is unlikely since the same number of parasitoid offspring successfully pupated in treated and healthy larvae.

The host quality experiment showed varying results. When virus treatment occurred before parasitization, offspring of the parasitoid *N. tutae* that developed in larvae 6 days after treatment (6V) were negatively affected in comparison to offspring from healthy control larvae (6C). Specifically, males had a longer development time and shorter adult lifespan, and females were smaller. In parasitoid wasps, fitness is often correlated with body size,⁴² thus the smaller body size might negatively affect the fitness of the females. However, the increase in development time by less than a day is unlikely to affect male fitness. Similarly, the reduced male lifespan is unlikely to affect reproduction since more than 90% of males developing in virus-treated larvae survived until day 20. In fact, mating occurs in the first few days of adult life as oviposition by *N. tutae* females begins no later than 4 days after emergence.³⁴ A possible explanation for these negative effects might be that by paralyzing the host larvae, the wasp weakened the host's immune response against the virus, which could have favored virus replication and reduced host quality. It is important to note that the reduced lifespan might be exacerbated under field conditions due to harsh environmental conditions and other stress factors.

Increased parasitoid development time due to virus infection of the host has been documented previously. For instance, the development of *Ascogaster reticulata* Watanabe (Hymenoptera: Braconidae) was longer in infected hosts.⁴³ Virus infection also resulted in reduced offspring body weight in *Apanteles glomeratus*.²¹ The differences between the sexes in our study might be explained by the marked sexual dimorphism of *N. tutae*, with particularly large females and small males. Females might have chosen the larger, lightly infected hosts for production of female offspring. Smaller hosts, with a high virus infection level, might have been used for male offspring. Males developing on those hosts compensated for the lower host quality by slower development to attain a minimum size necessary for emergence. It is interesting to note that no differences were observed between offspring developing in host larvae 12 days after virus treatment (12V) or healthy control larvae (12C). As already explained, a possible explanation might be that host larvae had mostly mild or sublethal infections at this infection stage, whereas the most heavily infected larvae had already died. Those infections seem not to have affected the developing parasitoids. An alternative explanation might be that the late-instar 12-day-old larvae may have been large enough for the parasitoids, which naturally prefer



the third instar,³⁴ therefore host resources might not have been a limiting factor.

Koinobiont endoparasitoids such as *D. gelechiidivoris* spend most or all of their larval development inside an actively feeding host insect, which provides the sole source of nutrition for the immature parasitoid and the environment in which parasitoids develop.⁴⁴ One could expect endoparasitoids to be more susceptible to virus-related indirect effects than ectoparasitoids. In contrast, the host quality experiment showed that PhopGV infection even improved some fitness traits of *D. gelechiidivoris* offspring developing in hosts 6 days after treatment (6V): females were larger and males had a longer adult lifespan. Likewise, female offspring were larger when developing in hosts 12 days after treatment (12V). Because of the evolutionary history with *D. gelechiidivoris*,³² *T. absoluta* has likely evolved a solid immune response against this parasitoid, which might have been compromised by virus infection,¹¹ to the benefit of the parasitoid. The granulovirus might act similarly to endosymbiotic polydnviruses of parasitoids, which cause suppression of host cellular immune responses and inhibit feeding or prolong or arrest development, among others.^{11,45}

Interestingly, when virus treatment occurred after parasitization, the offspring of *D. gelechiidivoris* were negatively affected. Offspring had a lower emergence and males had a shorter lifespan when treatment occurred 1 day after parasitization. As discussed, one possible explanation for this result is that infection may cause a general decrease in host quality. In this case, the virus could have the opposite effect by boosting the host's immune response. A previous study reported that infection with a baculovirus expressing a protease decreased the survival of the braconid parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) emerging from tobacco budworm larvae *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) if the host was infected with the virus less than 72 h post parasitization.⁴⁵

No effects were visible in offspring that developed in treated hosts 5 days after parasitization. This result corroborates the idea that postponing the exposure of parasitized larvae to a baculovirus increases the percentage of successful parasitoid development.¹¹ Overall, these results must be interpreted cautiously because it is impossible to detect parasitization by *D. gelechiidivoris* before pupation without dissecting the host. Therefore, assessment of the impact of the virus on the young parasitoid immature stages of *D. gelechiidivoris* (egg to pupae) was not possible.

In this study, we examined the relative importance of indirect effects of a granulovirus infecting *T. absoluta* on two of its parasitoids with very distinct life-history traits. We found that *D. gelechiidivoris* could discriminate against treated larvae after prolonged incubation and is likely to be less affected by baculovirus applications to crops. However, *N. tutae* did not discriminate and suffered more than *D. gelechiidivoris* from virus treatment of the host after parasitization. Overall, both parasitoids were negligibly affected by the virus treatment, indicating that they can be combined with the baculovirus for control of *T. absoluta* and that the virus is unlikely to impact naturally occurring parasitoid populations. Research is currently underway to assess the efficiency of such a combination, and modeling studies will be conducted to understand the underlying mechanism at the population level.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at <https://doi.org/10.6084/m9.figshare.20557488>.

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