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The Parasitoid *Hyposoter didymator* Can Transmit a Broad Host Range Baculovirus in a Two Host System

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Abstract: *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae) and baculovirus (BV) might be used jointly to provide effective control of the *Spodoptera* genus. The literature has mostly covered the safe compatibility between natural enemies and BV-based insecticides, but research on the potential dispersal of BV by natural enemies is lacking. Thus, the goal of this manuscript was to ascertain if *H. didymator* was able to disperse the broad host range of *Autographa californica* nucleopolyhedrovirus (AcMNPV) to *Spodoptera littoralis* and *Spodoptera exigua* in choice and non-choice conditions and whether the preference of the parasitoid by one of these noctuids could mediate this dispersion. It was previously needed to improve the rearing of the parasitoid in the laboratory, concerning the optimal host age and length of parasitization, parasitoid competition, and influence of parasitization on the longevity of females. The best rearing conditions for *S. littoralis* are collective parasitization of mature L3 larvae for 24 h, after at least one day of copulation. *Hyposoter didymator* transmits AcMNPV to both lepidopterans, but its efficiency is mediated by host preference and the pathogenicity of the BV in each host. In this particular case, *H. didymator* as well as AcMNPV showed a clear preference towards *S. exigua*.

Keywords: biological control; noctuid; *Spodoptera littoralis*; *Spodoptera exigua*; AcMNPV

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1. Introduction

Caterpillars of moths belonging to *Spodoptera* genus are of primary concern for the agricultural industry in many countries of the world [1]. Traditionally treated with a broad range of insecticides, it is well known that chemical pest control treatments, though extensively used worldwide, result in pest resistance, environmental contamination, and toxicity to non-target organisms. Furthermore, the implementation of European legislation restricting residue levels of most synthetic chemical pesticides and the banning of many others has decreased the availability of effective compounds, driving altogether to boost biological control techniques [2–5].

Hyposoter didymator (Thunberg) (Hymenoptera: Ichneumonidae) is a larval endoparasitoid of the cosmopolitan and polyphagous key pests beet armyworm *Spodoptera exigua* (Hübner) and cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) [6,7] and is among the most promising parasitoids to be used in IPM programs against these noctuids [8,9]. Nevertheless, available larval parasitoids often fail to provide fully effective control of the aforementioned pests by themselves, thus the implementation of additional biocontrol agents may represent an opportunity to significantly reduce the risks associated with the use of synthetic chemical insecticides [10–17]. Baculoviridae (BV), a large family of double-stranded DNA pathogenic viruses, have a proven track record as effective and highly selective microbial insecticides [18,19]. Despite some disadvantages in relation to chemical pesticides, such as slower speed of kill, narrow host specificity, short field stability, susceptibility to UV light, short shelf life, or high production costs, the use of

BV as insecticides presents many advantages: they are natural pathogens that are persistent in the environment, can be marketed in a similar way to chemical pesticides [20], and are traditionally considered amongst the safest pesticides, with no or negligible effects on non-target organisms, including beneficial insects, vertebrates, and plants [21].

Despite the fact that the literature has mostly covered the safe compatibility between natural enemies and BV-based insecticides [22–32], research on the potential dispersal of BV by non-target insects, which would synergistically contribute to their joint use, is lacking. Predators contribute to the dispersal by two means: contamination of their body surfaces during consumption of infected prey or excretion of viable occlusion bodies (OB, resistant forms of transmission containing virions) alongside their body waste products after feeding on infected prey, which does not cause a negative effect on their survival [22–27].

Parasitoids can mechanically transmit lepidopteran BV between hosts. The transmission occurs when their ovipositor, body surface, or gut becomes contaminated [28,29,33]. Parasitoids and BV establish a within-host competition, which leads to more complicated consequences. On one hand, BV infections may have a deleterious impact on parasitoids, such as reduced body size of parasitoids developing inside infected hosts, or death due to the premature BV-induced mortality of the host [28,34,35]. On the other hand, parasitoids suppress the immune responses of the host, favoring BV to infect formerly resistant hosts [30,36]. At the same time, successful parasitism may also weaken or reduce the replication, genetic composition, and virulence of BV [31,32,35,36].

Compatibility between parasitism and BV infection remains a little-known process, and information on their joint application is completely absent for *H. didymator*. The simultaneous use of BV and *H. didymator* would imply a practical combination of macro- and microbiological control against *Spodoptera* spp., as it has been previously explored with the entomopathogenic fungus *Metarhizium brunneum* (Petch) [37].

BV belonging to the genus Alphabaculovirus (lepidopteran-specific nucleopolyhedroviruses, NPV) show important variation in their host range, from viruses that are highly host specific, such as *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV), to viruses that can productively infect multiple hosts, such as *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which can infect at least 15 families of Lepidoptera, including both hosts, *S. exigua* and *S. littoralis* [38]. Moreover, broad host range BV are not equally infective to all host species. In fact, the host range in BV is characterized by high variation in the susceptibility of different hosts to a particular virus [39].

It is relatively frequent that *S. exigua* and *S. littoralis* appear simultaneously in Spanish greenhouses, which can advise applying a broad host range BV together with the parasitoid, in order to enhance the control measure profitability. To our knowledge, the role of parasitoids in the transmission of a BV in a multi-host system has never been examined. Therefore, the main goal of this manuscript was to ascertain the preference of the parasitoid *H. didymator* between *S. exigua* and *S. littoralis* larvae, whether it is able to transmit AcMNPV to both hosts, and how the parasitoid preference can have an effect on this transmission.

In order to achieve this main goal, it was needed to increase scientific knowledge on the rearing of this parasitoid. Obtaining females constitutes the greatest challenge for laboratory rearing since unfavorable conditions cause the population to bias towards males in a few generations [40]. Rearing methods found in the scarce literature to overcome this problem are contradictory in some aspects, with regard to the optimal host age, appropriate time of parasitization, or competition among females [40,41].

2. Materials and Methods

2.1. Insect Rearing

Spodoptera exigua was provided by Dr. Malo (Probelte S.A.U., Murcia, Spain) from a population collected in Murcia (Spain). *Spodoptera littoralis* was collected on *Medicago sativa* L. in 2018 in Los Palacios (Sevilla, Spain). Both populations had not been previously exposed to insecticides and were continuously reared before experiments. Larvae were mass-reared in ventilated transparent plastic containers (30 × 20 × 10 cm) on a semi-solid

wheat germ-based semi-synthetic diet [42] inside a walk-in chamber (4.25 × 2 × 2.5 m) at 24.5 ± 1.8 °C, 56.1 ± 10.2% RH (relative humidity), and 16L:8D photoperiod. Once the larvae reached the last instar, containers were filled with vermiculite so the larvae could form pupae. Then, the pupae were transferred to a ventilated methacrylate cage (40 × 30 × 30 cm). Emerged male and female adults were fed with a 50% (*v*/*v*) honey solution. Filter paper was placed inside the cage to provide an easily extractable surface for the adults to deposit their eggs. For experiments, synchronized eggs layered on filter paper for 24 h were reared under the same conditions as the rearing.

A laboratory colony of *H. didymator* was initiated with specimens kindly provided by Dr. Meelad Yousef from the Universidad de Córdoba (Spain) and continuously mass-reared on *S. littoralis* larvae inside the walk-in chamber previously described. Larvae exposed to parasitism were individually transferred to blister packs and reared ad libitum on the semi-synthetic diet. Larvae were checked three times a week until pupation (ca. 14 days). Pupae were placed in a ventilated cage until emergence. Adults were sexed immediately after emergence and fed with honey droplets.

2.2. Baculovirus Isolates

Autographa californica multiple nucleopolyhedrovirus (AcMNPV C6) isolate was purified, and OB concentrations were determined at the Dr. Primitivo Caballero laboratory in the Universidad Pública de Navarra (Spain). Suspensions of the purified virus were produced in fourth instar S. exigua larvae. Virus-killed larvae were homogenized in distilled water, filtered through muslin, and centrifuged in plastic vials at 3245× *g* for 5 min with sodium dodecyl sulfate (0.1% *w*/*v*) to eliminate insect debris. The resulting pellet was washed in distilled water and re-suspended in Milli-Q water. OB concentration was determined using an improved hemocytometer (Hawksley Ltd., Lancing, UK) under phase contrast microscopy. Suspensions were stored at 4 °C until the experiments were conducted.

2.3. Improvements of H. didymator Rearing on S. littoralis Host in Laboratory

To optimize *H. didymator* mass rearing under laboratory conditions, we studied the (1) optimal age of *S. littoralis* host larvae at the time of parasitization, (2) optimal length of parasitization, (3) individual or collective parasitization, and (4) influence of parasitization on the longevity of females. In the bioassays described hereafter, the repetitions with females that did not produce offspring were not included in the analysis.

For the age of host larvae, one newly emerged female and two males of *H. didymator* were allowed to mate for 24 h inside ventilated cages of 7 cm diameter by 3.5 cm height. Four different age groups of *S. littoralis* larvae were tested: young second instar (L2, newly molted), mature L2 (molted two days prior to their offering), young third instar (L3), or mature L3. A new set of seven larvae of the corresponding age was offered in 24 h periods over 3 consecutive days (n = 20 females per treatment). Food was supplied for both insects. Larvae were individualized and reared as described in Section 2.1. The proportions of parasitized larvae, parasitoid pupae, females, and males with regard to the number of larvae offered were annotated to assess reproductive efficiency. The sex ratio of the offspring was calculated (number of females/number of adults). The preferred host age, mature L3, was selected for the subsequent assays.

For the length of parasitization, one newly emerged female and two males were allowed to mate for 24 h. Then, the pairings were provided with seven mature L3 larvae over 24 h to train the females. The following day, oviposition on a new set of seven larvae was allowed during either 2, 24, or 48 h (n = 23 females per treatment). Food was supplied for both insects. Offspring was evaluated as previously described.

For individual or collective parasitization, newly emerged adults were allowed to mate for 24 h under two conditions, either individually (pairings of one female and two males in ventilated cages of 7 cm diameter by 3.5 cm height) or collectively (10 females and 20 males in ventilated cages of 12 cm diameter by 20 cm height). Seven or 70 mature L3

larvae were offered to individual or collective females, respectively. A new set of larvae was offered in 24 h periods over 2 consecutive days (n = 30 individual females and n = 3 collective cages with 10 females each). Food was supplied for both insects. Offspring was evaluated as previously described.

For the influence of parasitization on the longevity of females, pairings of one newly emerged female and two males were offered a new set of seven mature L3 larvae in 24 h periods during 7 consecutive days since the day of their birth (n = 20 females). Pairings under the same conditions but without exposure to host larvae were included (n = 21 females). Food was supplied for both insects. The lifespan of females was evaluated until death. Offspring were evaluated as previously described to assess reproductive efficiency over the female's first week of life.

2.4. Preference of H. didymator for S. exigua and S. littoralis Hosts

The preference of *H. didymator* was studied under choice and non-choice conditions (Figure 1a). Newly emerged adults were allowed to mate for 24 h. Then, females were trained to oviposit with the corresponding host (either *S. exigua* or *S. littoralis* L3 larvae) for 24 h to avoid host bias. Then, in non-choice experiments, 20 *S. exigua* or 20 *S. littoralis* L3 larvae were offered to one female for 24 h inside ventilated cages of 7 cm diameter by 3.5 cm height (n = 21 females for *S. exigua* and n = 16 females for *S. littoralis*). Food was supplied for both insects. Then, larvae were individualized and reared as described in Section 2.1. The proportions of natural mortality of larvae (non-attributable to parasitism), parasitoid pupae, and adults with regard to the number of larvae offered were annotated to assess reproductive efficiency. Length from parasitization until adult emergence and the sex ratio of the offspring were calculated. In choice experiments, 10 *S. exigua* and 10 *S. littoralis* L3 larvae were offered to one female (n = 16 females). Offspring were evaluated as previously described.

2.5. Transmission of AcMNPV by H. didymator to S. exigua and S. littoralis Hosts

The transmission of AcMNPV by *H. didymator* was studied under choice and non-choice conditions, similar to the previous experiment (Figure 1b). First instar (L1) *S. exigua* larvae were starved for 16 h. Newly molted L2 larvae were prompted to drink 4 µL droplets containing sucrose (15% w/v), blue food-grade dye (0.001% w/v, ProGel®, Preston, UK), and the 95% lethal concentration (LC_{95} = 1.7 × 10^4 OB × mL^{-1}) of AcMNPV in distilled water during 10 min. The LC_{95} had previously been calculated based on second instar *S. exigua* larvae [43]. Previous studies showed that our AcMNPV isolate was not effective against *S. littoralis* as a much higher concentration was needed to kill 50% of the population; therefore, it was already known that AcMNPV was more effective on *S. exigua* [43]. Previous bioassays determined that the food dye was harmless to larvae. Larvae that ingested this solution turned blue, and only these larvae were reared for three days post-inoculation (d.p.i). Then, 20 of these AcMNPV-infected larvae were offered to one parasitoid female for 24 h, which had previously been allowed to mate and train as described in Section 2.4. This procedure was repeated for all females used in the experiment. After this acquisition period by parasitoids, in non-choice experiments, 20 healthy *S. exigua* or 20 *S. littoralis* L3 larvae were offered to one female for 24 h inside ventilated cages of 7 cm diameter by 3.5 cm height (n = 16 females for *S. exigua* and n = 15 females for *S. littoralis*). The parameters described in Section 2.4 and the proportion of larvae killed by AcMNPV were evaluated. Death by AcMNPV was determined by the observation of symptoms (pale yellow/oily spots on the tegument, climbing to the upper lid of the blister to die, or the complete disintegration/liquefaction of the larvae) and the presence of OB inside cadavers under a microscope. Infected larvae from which parasitoids acquired AcMNPV were reared until verification of the symptoms of the infection as well. In choice experiments, 10 healthy *S. exigua* and 10 *S. littoralis* L3 larvae were kept in the same cage described above and offered to one female (n = 16 females). Offspring were evaluated as previously described.

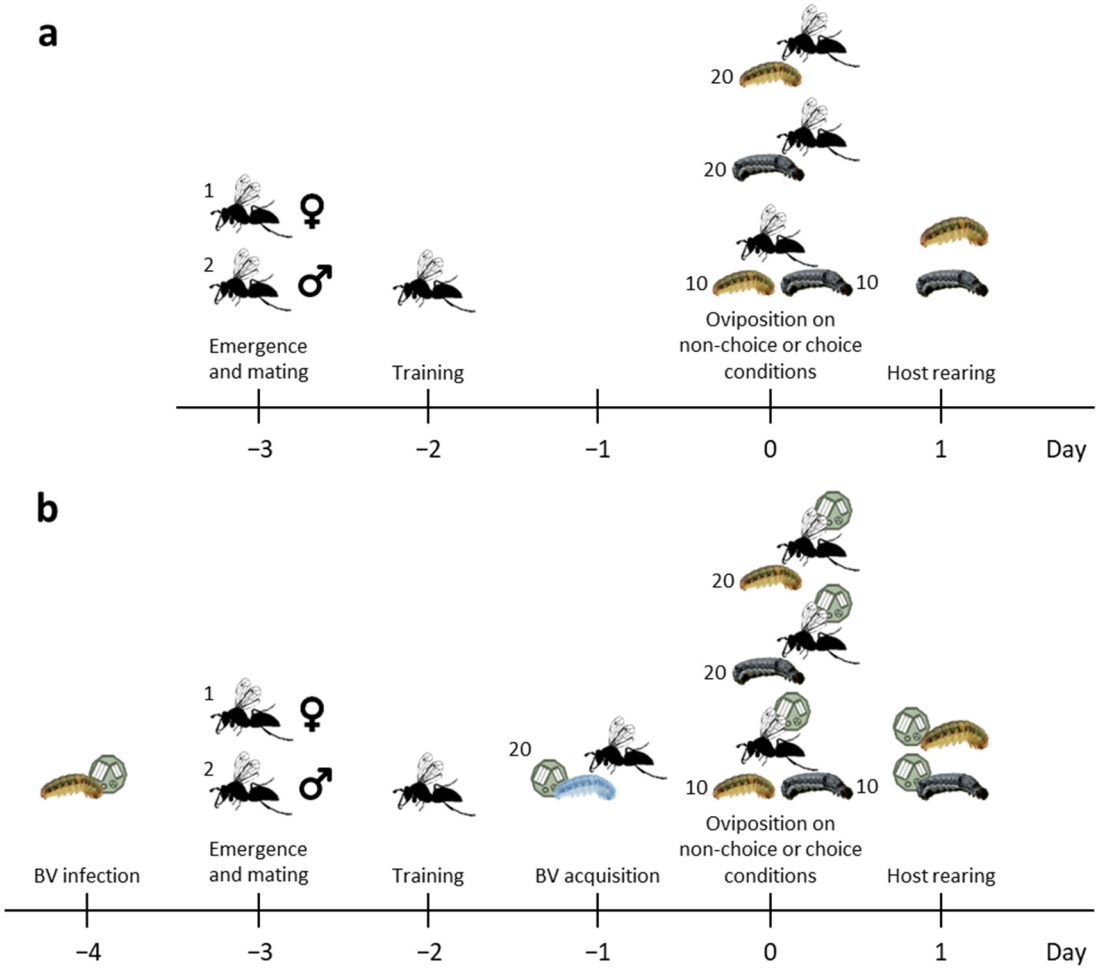

Figure 1. Timeline of the experiments. (**a**) Preference of *Hyposoter didymator* for *Spodoptera exigua* and *Spodoptera littoralis*; (**b**) transmission of AcMNPV by *Hyposoter didymator* to *Spodoptera exigua* and *Spodoptera littoralis*.

2.6. Statistical Analysis

Data were presented as the mean ± SEM. The studies on the optimal age of host larvae and the length of parasitization were analyzed by unifactorial ANOVA ($p \leq 0.05$). Studies on parasitoid competition and the influence of parasitization on the longevity of females were analyzed by Student's *t*-test ($p \leq 0.05$). Values were previously transformed if the requirements for parametric analysis were not reached. Non-parametric Mann–Whitney *U* or Kruskall–Wallis *H* tests ($p \leq 0.05$) were applied if neither the raw nor transformed data met the requirements for parametric analysis. Choice and non-choice experiments were separately analyzed by Student's *t*-test ($p \leq 0.05$). Besides, in non-choice conditions, the effect of the two factors (host species: *S. exigua* or *S. littoralis*; and status of the parasitoid: after oviposition on AcMNPV-infected larvae or not) was analyzed with a general linear model ($p \leq 0.05$). In choice conditions, the factor host species does not fulfill the independence assumption, and GLM does not seem appropriate. The significance of the effects was determined by the *F* test statistic, and the estimated marginal means were compared with the LSD test. All analyses were performed using Statgraphics Centurion 18 [44].

3. Results

3.1. Improvements of H. didymator Rearing on S. littoralis Host in Laboratory

For the effect of the age of *S. littoralis* host larvae at the time of parasitization, there was a significantly higher *H. didymator* female offspring when the host was mature L3 ($H = 8.64$; $p = 0.03$) (Table 1). No statistically significant differences were observed in other parameters,

yet mature L3 showed a tendency to produce more parasitized larvae (H = 3.82; p = 0.28), more *H. didymator* pupae (H = 2.48; p = 0.48), more *H. didymator* adults (H = 0.37; p = 0.95), lowest male offspring (H = 3.49; p = 0.32), and the best sex ratio (H = 5.88; p = 0.12) (Table 1). Therefore, the potentially ideal host age, mature L3, was selected for the subsequent assays.

Table 1. Influence of *Spodoptera littoralis* host larvae age, length of parasitization, and type of parasitization on *Hyposoter didymator* developmental and reproductive parameters, in relation to the number of host larvae offered to the parasitoid. Sex ratio of the offspring refers to the number of females in relation to the number of adults). Lowercase letters stand for significant differences.

***S. littoralis* Age**	***S. littoralis* Parasitized Larvae (%)**	***H. didymator* Pupae (%)**	***H. didymator* Adults (%)**	***H. didymator* Females (%)**	***H. didymator* Males (%)**	**Sex Ratio [3]**
Young L2 [1]	48.85 ± 3.52	47.80 ± 3.53	42.24 ± 3.56	7.98 ± 1.59 a	34.26 ± 3.68	0.24 ± 0.05
Mature L2	51.70 ± 3.22	50.51 ± 3.17	42.79 ± 3.01	10.44 ± 1.79 a	32.36 ± 2.82	0.25 ± 0.04
Young L3 [2]	52.10 ± 3.54	50.67 ± 3.55	42.02 ± 2.82	13.65 ± 2.30 ab	28.37 ± 2.70	0.30 ± 0.04
Mature L3	58.17 ± 3.38	55.31 ± 3.34	44.60 ± 3.09	17.70 ± 2.44 b	26.90 ± 3.02	0.39 ± 0.05
Length of parasitization (h)						
2	42.14 ± 4.90 a	37.14 ± 4.68 a	30.71 ± 4.18 a	5.71 ± 2.41 a	25.00 ± 4.38	0.20 ± 0.08
24	72.86 ± 4.90 b	66.43 ± 5.70 b	42.14 ± 5.02 ab	17.14 ± 4.22 b	25.00 ± 4.13	0.37 ± 0.08
48	64.08 ± 4.57 b	60.97 ± 4.45 b	43.27 ± 4.10 b	15.37 ± 3.78 ab	27.90 ± 4.70	0.34 ± 0.07
Type of parasitization						
Individual	58.93 ± 4.37 a	57.01 ± 4.28 a	39.00 ± 3.60 a	7.53 ± 1.82	31.47 ± 3.07	0.38 ± 0.12
Collective	70.82 ± 3.14 b	69.39 ± 3.26 b	48.76 ± 3.04 b	10.97 ± 1.60	37.79 ± 2.91	0.34 ± 0.07

[1] Second instar larvae, [2] third instar larvae, [3] number of females/number of adults.

The impact of the length of parasitization (2, 24, or 48 h) on *H. didymator* progeny was evaluated (Table 1). While there was not a statistically significant difference between a 24 and 48 h parasitization session, they both returned substantially higher percentages of parasitized larvae ($F_{2,60}$ = 10.49; $p \leq 0.001$), *H. didymator* pupae ($F_{2,59}$ = 9.59; $p \leq 0.001$), *H. didymator* adults (H = 5.83; df = 2; p = 0.05) and female offspring (H = 5.81; df = 2; p = 0.05) than the 2 h parasitization group. No statistically significant differences were found for the percentage of males (H = 0.17; df = 2; p = 0.92) and sex ratio (H = 3.23; df = 2; p = 0.20). The 24 h parasitization treatment group returned similar numbers of *H. didymator* adults, females, and males to those obtained for mature L3 larvae in the first bioassay (Table 1).

The effect of females parasitizing collectively or individually with the same ratio of female to male *H. didymator* adults showed significant differences between the two treatments (Table 1). There was a significant tendency for collective parasitization to produce a higher level of parasitized larvae (t = −2.25; p = 0.03), more *H. didymator* pupae (t = −2.34; p = 0.02), and more *H. didymator* adults (t = −2.09; p = 0.04). No differences were found for female offspring (t = −1.43; p = 0.16), male offspring (t = −1.49; p = 0.14), or sex ratio (t = 0.24; p = 0.81). The percentage of parasitized larvae, parasitoid pupae, adults, males, and sex ratio in individual parasitization conditions were in line with those from previous bioassays (Table 1). However, individual parasitization of mature L3 larvae during 24 h returned a low percentage of female offspring (7.53 ± 1.82%), which contrasts the results of the first (17.70 ± 2.44%; Table 1) and second (17.14 ± 4.22%, Table 1) bioassays under similar conditions.

This bioassay examined how the efficiency of the parasitoid female changed over the course of its lifespan. When *H. didymator* was parasitized daily since its emergence, the percentage of parasitized larvae started low (25.86 ± 6.31%) before rising to its peak on the third day (59.88 ± 6.41%) (Figure 2a). This parameter began to decrease from the fourth day onwards before reaching comparable levels to the beginning of the experiment on the seventh day. A similar trend was observed with the parasitoid pupae formed, which

displayed a peak on the third day (56.19 ± 5.99%) (Figure 2a). After that, pupae began to decrease gradually. Male progeny showed a similar increase to the first two parameters and reached its peak on the third day (27.50 ± 5.01%) (Figure 2b). This parameter then gradually began to fall until leveling out on the sixth day. Female progeny began very low (3.57 ± 1.99%), but gradually rose on the fifth day (16.14 ± 4.38%) (Figure 2b). After this peak, female offspring were slightly reduced by the sixth day but fell drastically on the seventh day (6.78 ± 2.04%). The sex ratio gradually increased until the fifth day (0.51 ± 0.10) (Figure 2c). Despite the drastic reduction in female progeny on the seventh day, *H. didymator* still maintained a relatively high sex ratio throughout the bioassay. The last bioassay also showed how the lifespan of an *H. didymator* parasitizing wasp (7.40 ± 1.22 days) was significantly shorter than that of a non-parasitizing one (13.66 ± 2.25 days; $W = 46.5$; $p \leq 0.001$).

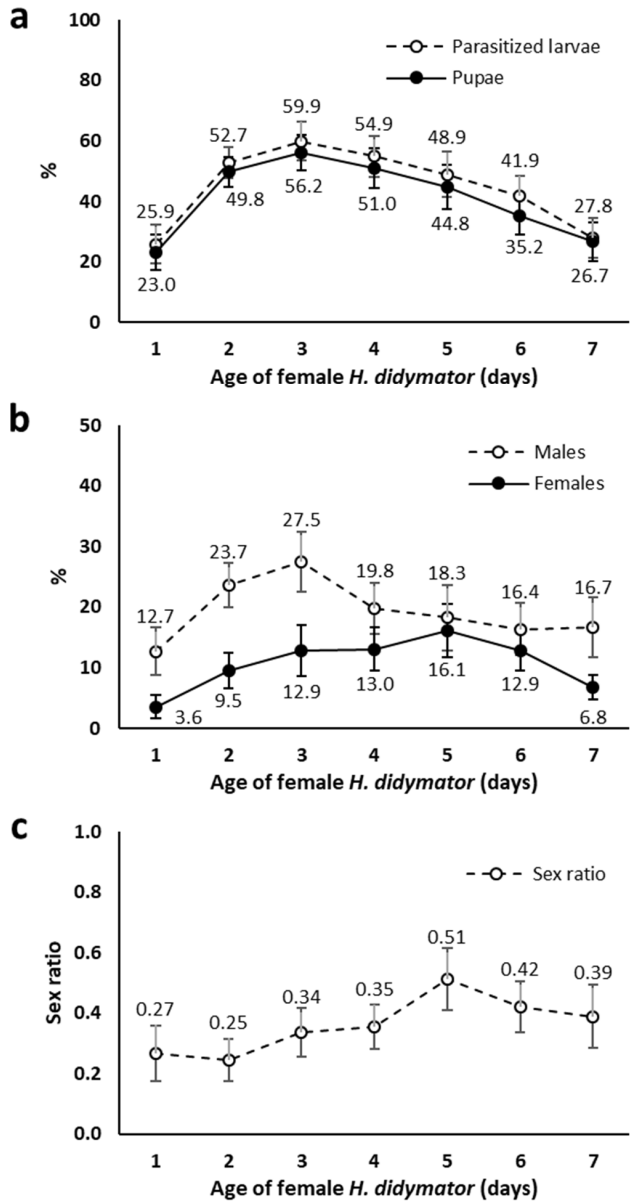

Figure 2. Efficiency of *Hyposoter didymator* females over the course of their lifespan when parasitizing daily since emergence over seven consecutive days. (**a**) Parasitized larvae and parasitoid pupae in relation to the number of larvae offered to the parasitoid; (**b**) male and female adult offspring in relation to the number of larvae offered to the parasitoid; (**c**) sex ratio of the offspring (number of females/number of adults).

3.2. Preference of *H. didymator* for *S. exigua* and *S. littoralis* Hosts and Transmission of AcMNPV

Host preference was tested under choice and non-choice conditions. When analyzing the effect of the two factors (host species and status of the parasitoid) in non-choice arenas, we observed a significantly higher percentage of host larvae of both species dead by parasitism and natural causes when the parasitoid females had not previously acquired AcMNPV by exposure to infected larvae as it could be expected (Table 2). Moreover, a significantly higher percentage of *S. littoralis* completed their development whether *S. littoralis* was parasitized by non-contaminated or AcMNPV-contaminated *H. didymator* in comparison with the percentages observed when the host was *S. exigua*. The proportion of parasitoid adults was significantly higher when the parasitoids had not previously acquired AcMNPV. The sex ratio was significantly higher when the parasitoid females previously acquired AcMNPV (Table 2). The length from parasitization to adult emergence did not differ among treatments, with values that ranged from 16.54 ± 0.39 to 17.61 ± 0.24 days.

Table 2. Preference of *Hyposoter didymator* females for *Spodoptera exigua* and *Spodoptera littoralis* larvae under non-choice conditions. Influence of host species (HS; *Spodoptera exigua* or *Spodoptera littoralis*) and status of the parasitoid *Hyposoter didymator* (*Hd*; which were or were not contaminated by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) after previous exposure to infected larvae) on *H. didymator* reproductive parameters in relation to the number of larvae offered to the parasitoid: Percentages of larvae dead by natural causes non-attributable to parasitism (natural mortality), healthy *Spodoptera* adults that completed their development (noctuid adults), larvae dead by parasitism (parasitoid pupae), parasitoid adults, and sex ratio of the parasitoid offspring. Uppercase letters in the same row stand for significant differences between the status of the parasitoid. Lowercase letters in the same column stand for significant differences between the host species.

Parameter (%)	Host Species	*Hyposoter didymator*				HS	*Hd*	HS × *Hd*
		Non-Contaminated	AcMNPV-Contaminated	Mean				
Natural mortality [1]	***S. exigua***	7.60 ± 1.31	2.22 ± 0.92	5.27 ± 0.94	*F*	1.99	16.22	0.31
	S. littoralis	5.29 ± 1.54	1.38 ± 0.61	3.40 ± 0.91	*p*	0.16	<0.001	0.58
	Mean	6.60 ± 1.00 A	1.81 ± 0.55 B					
Noctuid adults [2]	***S. exigua***	36.27 ± 6.28	28.87 ± 7.20	33.07 ± 4.71 a	*F*	21.01	0.49	3.81
	S. littoralis	51.83 ± 4.33	67.50 ± 5.45	59.41 ± 3.69 b	*p*	<0.001	0.49	0.06
	Mean	43.00 ± 4.18	47.57 ± 5.70					
Parasitoid pupae [3]	***S. exigua***	56.13 ± 6.02	35.67 ± 8.25	47.28 ± 5.15	*F*	2.68	8.04	0.32
	S. littoralis	42.88 ± 3.91	29.12 ± 5.36	36.22 ± 3.47	*p*	0.11	0.01	0.57
	Mean	50.40 ± 3.92 A	32.50 ± 4.94 B					
Parasitoid adults [4]	***S. exigua***	48.75 ± 5.53	26.03 ± 7.69	37.39 ± 3.80	*F*	2.80	7.08	2.32
	S. littoralis	31.37 ± 2.77	25.21 ± 4.61	28.29 ± 3.87	*p*	0.10	0.01	0.13
	Mean	40.06 ± 3.58 A	25.61 ± 4.09 B					
Sex ratio [5]	***S. exigua***	0.13 ± 0.04	0.38 ± 0.10	0.26 ± 0.05	*F*	0.63	22.17	0.41
	S. littoralis	0.14 ± 0.05	0.48 ± 0.08	0.31 ± 0.04	*p*	0.43	<0.001	0.53
	Mean	0.13 ± 0.04 A	0.43 ± 0.05 B					

All parameters have the same degrees of freedom: HS = 1, *Hd* = 1, Interaction = 1, error = 66. [1] GLM: $F = 6.65$; $p < 0.001$; [2] GLM: $F = 8.32$; $p < 0.001$; [3] GLM: $F = 4.06$; $p = 0.010$; [4] GLM: $F = 4.77$; $p = 0.005$; [5] GLM: $F = 8.92$; $p < 0.001$.

Hyposoter didymator females always preferred to parasitize on *S. exigua*, but significant differences were only found under choice conditions, and when the parasitoids had previously acquired AcMNPV by exposure to infected larvae ($t = 2.134$; $p = 0.041$) (Figure 3b). *Hyposoter didymator* successfully dispersed AcMNPV to both hosts, to a significantly greater extent than *S. exigua* under non-choice conditions (W = 47.0; $p = 0.002$) (Figure 3a). Thus, a significantly higher percentage of *S. littoralis* completed their development under non-choice conditions [non-contaminated parasitoids: W = 243.0; $p = 0.022$. AcMNPV-contaminated parasitoids: W = 204.5; $p < 0.001$ (Figure 3a)] and choice con-

ditions [non-contaminated parasitoids: W = 198.5; p = 0.008. AcMNPV-contaminated parasitoids: t = −2.955; p = 0.006) (Figure 3b)]. Surprisingly, under choice conditions, and when the parasitoids had not previously acquired AcMNPV, *S. exigua* had significantly more developmental mortality due to natural causes (W = 57.0; p = 0.005) (Figure 3b).

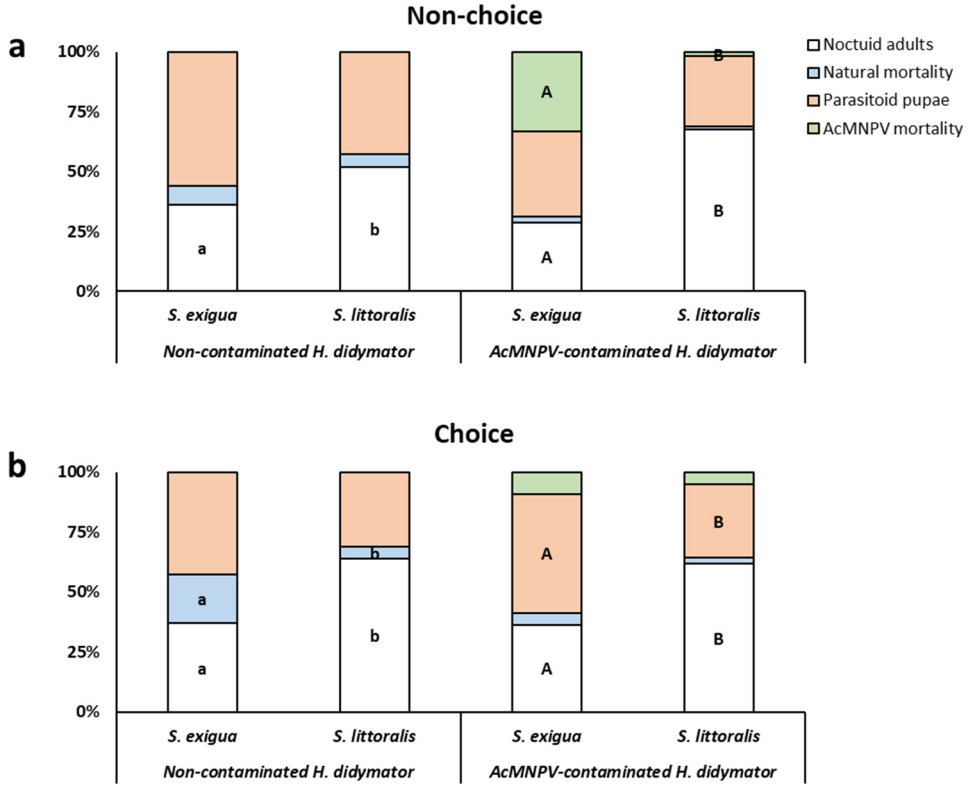

Figure 3. Preference of *Hyposoter didymator* females for *Spodoptera exigua* and *Spodoptera littoralis* larvae, when non-contaminated females and females contaminated by Autographa californica multiple nucleopolyhedrovirus (AcMNPV) after previous exposure to infected larvae. Cumulative bars show the following parameters: Percentages of larvae dead by AcMNPV infection (green), larvae dead by parasitism (orange), larvae dead by natural causes non-attributable to the aforementioned (blue), and healthy Spodoptera adults that completed their development (white). Lowercase letters stand for significant differences between the host species within a parameter, in the case of non-contaminated *H. didymator* females. Uppercase letters stand for significant differences between the host species within a parameter, in the case of AcMNPV-contaminated *H. didymator* females. (**a**) Non-choice conditions; (**b**) choice conditions.

4. Discussion

4.1. Improvements of H. didymator Rearing on S. littoralis Host in Laboratory

Our research started by determining the appropriate rearing conditions for *H. didymator* in a laboratory setting. This included the determination of the ideal *S. littoralis* larval instar, length of parasitization, and parasitization method. Additionally, both the effect of parasitization on the lifespan of a female and its effectiveness over time were analyzed.

Mature L3 *S. littoralis* is the most suitable host for laboratory rearing as it guarantees the largest production of female offspring. These findings mean that *H. didymator* not only parasitizes this instar well but also chooses it to reproduce sexually and produce diploid females. Our results are in accordance with Schneider and Viñuela [40]. Their values were higher than those obtained in the present investigation, probably due to the reintroduction of wild *H. didymator*, which increased allelic variability and female progeny [40]. Further support comes from the investigations of Bahena et al. [45] and Miranda-Fuentes et al. [37,46]. Various investigations have demonstrated the aptitude of mature L3 larvae of other Lepidoptera of similar size, such as *Helicoverpa armigera* Hübner

(Lepidoptera: Noctuidae) [47]. The most probable reason is that a larger host size is preferred because parasitoids recognize that there are more nutrients available for their progeny [48,49], yet there is a maximum host size at which parasitization is not possible due to either an immunological response of the host or its size preventing the parasitoid's exit. Schneider [49] demonstrated that the parasitoid prefers L4 to L3 to parasitize, but *S. littoralis* encapsulates 59% of parasitoid eggs layered in L4 because the noctuid produces larger masses of hemocytes. The percentage of encapsulation is only 22.65% in L3, most likely because the parasitoid more successfully utilizes polydnaviruses to evade the immune responses of the Lepidoptera [40,50], concluding that the poorer encapsulation compensates for the lesser parasitization in L3.

A 24 h period of parasitization is the most efficient. The additional costs associated with an extra day in the insectarium and, more importantly, with missing an extra day of *H. didymator* production (in the form of parasitization) do not outweigh the slight benefits of a 48 h session. These results are consistent with the majority of other studies where a parasitization time of 24 h returned more larvae parasitized than after a few hours [37,46,51]. The poorer results after a 2 h parasitization can be most likely explained because *H. didymator* does not have enough time to parasitize all larvae [52]. Conversely to our findings, Schneider and Viñuela [40] obtained a better percentage of parasitized larvae and sex ratio after 2 h with an improved rearing that included the reintroduction of wild *H. didymator*. The current investigation tried to avoid sibling mating, but family lines were probably crossed, which could have produced lower results.

Collective parasitization is also preferable because it saves time and resources at all stages of rearing. Additionally, it is more appropriate for large-scale commercial rearing. Harrington et al. [51] provide a great example of the success of mass rearing in large cages, as they were able to produce an impressive 80,000 wasps in 18 months with 25% females. The lack of significant differences between collective and individual parasitization is seen in various *H. didymator*-rearing method investigations [37,40,41,46,52].

The lifespan of a parasitizing wasp was reduced by 6.26 days when compared to a non-parasitizing wasp that only expended energy through somatic maintenance, locomotion, or mating, but not in egg laying, in agreement with *Microplitis rufiventris* Kokujev (Hymenoptera: Braconidae) parasitizing *S. littoralis* [53]. The first day of parasitization produced very low numbers of females (3.57%) and a low rate of parasitized larvae (25.86%), which can be explained by the fact that newly emerged females do not contain mature eggs until they are 12 h old [54]. Such details would therefore support the recommendation to provide newly emerged wasps with a day of copulation prior to beginning to parasitize.

The peak in parasitized larvae, number of pupae, and male progeny was obtained on the third day of parasitization as females gained experience. Interestingly, the maximums for the sex ratio and female progeny were reached on the fifth day. Hatem et al. [41] observed a similar gradual increase and then a gradual decline until the seventh day. Bahena et al. [52] witnessed the same with *M. umbrigera*. Khatri et al. [54] also concluded that a significantly greater number of mature eggs were found in the ovaries of the Ichneumonidae parasitoids 72 h after emergence. Thus, it is recommended to parasitize through the fifth day of the parasitoid's life. When the female died before the end of the seventh day, a notable decrease in parasitization was observed, with many failing to parasitize a single larva the day prior to their death. The same has been seen in other Ichneumonidae, with productivity gradually declining around the halfway point of their lives until they stopped reproducing shortly before their deaths [55,56]. The transition from pupa to emerged adult was a second critical period in the rearing; the first being the initial parasitization on the first day. Almost all parasitized larvae were able to form a pupa (average loss of 3.6% in 7 days), but 11.0% of larvae that pupated were unable to emerge as adults. One last important consideration is that inbreeding can lead to a reduction in female progeny over time [40]. Periodic reintroductions of wild *H. didymator* would be essential to introduce allelic variability in the offspring.

4.2. Transmission of AcMNPV by H. didymator to S. exigua and S. littoralis Hosts

The focus on the transmission of BV in our study was in large part due to the importance of these pathogens in controlling globally important lepidopteran pests such as *Helicoverpa* spp. and *Spodoptera* spp., which have a marked propensity to rapidly develop resistance to conventional chemical insecticides, providing potential market niches for BV in fields and protected crops [4]. Both *Spodoptera* species, the cotton leaf worm and the armyworm, might be controlled with their specific and highly pathogenic BV, SpliMNPV [57,58] and SeMNPV [59], respectively, and *S. exigua* can also be infected by SpliMNPV [60]. The use of BV pesticides in conjunction with indigenous or introduced parasitoids could be an alternative in integrated pest management programs [29,32]. There are multiple examples in the literature, as their interaction within common hosts is inevitable [31]. These studies commonly explored the dynamics of BV transmission in a population of a given host species. However, many parasites are generalists and infect multiple host species. The ability to infect different hosts predetermines the epidemiology and pathogenicity of generalist pathogens and, therefore, is highly relevant for pathogen management [61]. These multi-host networks may offer a better framework for investigating parasite dynamics.

In our work, we have demonstrated that even though BV-contaminated *H. didymator* is not able to fully control *S. exigua* and *S. littoralis*—at least under laboratory conditions since the concentrations of BV used are low in comparison with the commercial formulations—the parasitoid can transmit the generalist BV AcMNPV to both lepidopterans, from infected larvae to healthy ones. However, the parasitoid transmits AcMNPV more successfully to *S. exigua* since it seems to have a preference for this host over *S. littoralis,* and/or AcMNPV is more efficient on *S. exigua*. Belda et al. [39] reported that a host species that dies following inoculation with an OB dose similar to that of the homologous host in the same developmental stage is usually classified as a permissive species. In contrast, a host species that requires a much larger inoculum or in which the virus replicates poorly and OB yields are low is usually classified as a semi-permissive species. Thus, *S. exigua* can be considered a permissive species for AcMNPV (the amount of OB needed to kill the host is similar to that needed with SeMNPV, the specific BV) [62], whereas *S. littoralis* would be a semi-permissive species as very high concentrations of AcMNPV are needed to kill larvae [43].

More parasitoids were obtained from L3 *S. exigua* than *S. littoralis*, regardless of whether *H. didymator* was contaminated by AcMNPV or not, in choice and non-choice conditions (not always significant differences were found). It has been reported that this parasitoid prefers L2 over L3 when the host is *S. exigua* [63]. Even with L3 not being its preferred instar, taken together, this can lead to the conclusion that *H. didymator* prefers *S. exigua* over *S. littoralis*. From our bioassay, it was not possible to ascertain if one host was more attacked than the other, as *H. didymator* parasitizes very quickly and cannot be visually differentiated. Bahena et al. [45] compared the optimal host of *H. didymator* among L3 larvae of different Lepidopteran species, and *Helicoverpa armigera* (Hübner) yielded the highest ratio of parasitism (78%) and number of progeny (56%). There were no significant differences between *S. littoralis* and *S. exigua*, well down in comparison with *H. armigera*. According to Frayssinet et al. [64], *H. didymator* parasitizes more often on *H. armigera* although it can occasionally occur on non-preferred noctuids, which demonstrated that the parasitoid can switch hosts in nature due to fluctuating densities of the preferred host over the year. Choice tests showed that infection with AcMNPV did not affect the host preference of the parasitoid. It is possible that the higher ability of *S. littoralis* to encapsulate the parasitoid eggs allowed it to overcome the parasitization of *H. didymator* [49]. The history of exposure to parasitoids may also influence the susceptibility of host populations to BV. Encapsulation is used by Lepidopteran larvae as a defense against both BV and parasitoids. Thus, populations that invest in a strong encapsulation response as a result of selection pressure may be more resistant to BV, as we see in our results [62].

Strikingly, *H. didymator* progeny were less male-biased after escaping BV infection. In arrhenotokous hymenopterans such as *H. didymator,* unbalanced sex allocation of the

progeny occurs because mated females produce a diploid female or a haploid male by opting to fertilize an egg or not [65]. It has been reported that the primary sex ratio determined by sex allocation (a trend toward more males than females in non-infected hosts) may then be distorted by sex-biased mortality due to abiotic conditions, declines in food quality, interactions with conspecifics, and mortality induced by pathogens, predators, and parasitoids [66]. In the case of infection with BV, the sex ratio of parasitoids emerging from the infected host may be biased towards males if the BV kills the host before most of the female individuals complete their development [67], as usually female development of most parasitoids such as *H. didymator* takes slightly longer than male development at laboratory temperature. However, we just observed the opposite. We have not found any references supporting our bias towards females under AcMNPV infection. This result should be taken cautiously, as one plausible explanation could be that the numbers of parasitoids that emerged were so low that it was casually due to fate and not to BV infection.

5. Conclusions

In conclusion, *H. didymator* can contribute to transmitting the extended host range of BV AcMNPV from infected larvae to healthy *S. exigua* and *S. littoralis* host larvae. This parasitoid showed such a clear preference for *S. exigua* that it is maintained even if the host is infected with AcMNPV. Moreover, AcMNPV was more pathogenic to *S. exigua* than to *S. littoralis*. Thus, the transmission of a BV by a parasitoid when more than one host is involved will depend on both, the preference of the parasitoid and the pathogenicity of the BV in each host. Moreover, the infection improved the natural sex ratio observed in laboratory rearing, bettering the female numbers of the progeny. All these findings should be confirmed in the field, but the use of this generalist BV in conjunction with *H. didymator* might be more economically profitable than using the specific BV of these noctuids, SeMNPV and SpliMNPV, at the same time when both pests appear together in protected crops.

When BV and parasitoids coexist, the relative competence between agents will determine the success of each strategy. Research has traditionally focused on the impact that baculoviral host infections have on parasitoids. Applications of BV for biocontrol may lead to substantial detrimental effects on immature parasitoids [33–35], although field experiments have not yet demonstrated such an effect [25,26,28]. In our results, there was a significant reduction in parasitoid offspring from the AcMNPV-contaminated females compared to the non-contaminated. On the other hand, parasitoids can transmit BV, as shown in our findings, and, in this way, increase the efficacy of BV. Moreover, some parasitoids are able to avoid hosts infected with BV, minimizing interference. Thus, the final effect of the interaction (antagonism, addition, or synergy) will counterbalance all the effects. Further research needs to be done to maximize the efficacy of both parasites.

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