

Offspring From Sequential Matings Between *Bacillus thuringiensis*-Resistant and *Bacillus thuringiensis*-Susceptible *Heliothis virescens* Moths (Lepidoptera: Noctuidae)

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ABSTRACT The tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), is an economically important pest of the Americas. Females of this species copulate multiple times during their lifetimes, and the presence of sperm from multiple males inside them could allow for a diversity of paternal genotypes in the offspring, unless there was complete precedence of sperm from the first mating. If a female copulates with a male that is insecticide-susceptible and another male that is insecticide-resistant, her progeny could vary in their resistance phenotypes. In some cases, this could impact the evolution of insecticide resistance in a population. We designed a series of experiments to determine whether *Bacillus thuringiensis* susceptibility is maintained when an *H. virescens* female that is homozygous for a genetically recessive form of *B. thuringiensis* resistance copulates with a Cry1Ac-susceptible and a Cry1Ac-resistant males. During the lifetime of double-copulated females, a proportion of F₁ progeny were Cry1Ac-resistant. This indicates that when a *B. thuringiensis*-resistant *H. virescens* female copulates with two males, with one male being resistant to Cry1Ac, some of the progeny will carry resistance to this insecticide. Due to the polyandrous nature of this species, the above-mentioned scenario is not unrealistic; therefore, results from this study may help understand and manage the evolution of *B. thuringiensis*-resistance in field populations.

KEY WORDS tobacco budworm, insecticide resistance management, YHD2, sperm precedence, reproductive biology

The tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), is an economically important pest of the Americas. It is particularly injurious to cotton, *Gossypium hirsutum* L., and tobacco, *Nicotiana tabacum* L., where multiple applications of insecticides have been commonly made to control this spe-

cies (Fitt 1989). Due to the intensive selection pressure exerted by these applications, this pest has become resistant to a variety of insecticides in different regions (Sparks 1981, Terán-Vargas et al. 2005, Zenner de Polanía et al. 2008). The use of *Bacillus thuringiensis* (Bt)-expressing cotton has been added to the multiple approaches to control *H. virescens*. This effective technology has alleviated the need for the multiple insecticide applications aimed for the control of some Lepidoptera, but the possibility that this insect might develop resistance to Bt exists. One of the measures to delay the development of Bt resistance on targeted Lepidoptera has been the implementation of refuges (Roush 1997a,b; Andow and Hutchinson 1998, Gould 1998, Gould and Tabashnik 1998, Matten and Reynolds 2003). These areas or different crops can produce tobacco budworm moths (Blanco et al. 2007b, 2008c), assumed to be Bt-susceptible, that could have the opportunity to copulate when Bt-resistant moths putatively emerge from Bt cotton. Because Bt resistance is typically a recessive trait (Gould 1998), the offspring of Bt-resistant mated with Bt-susceptible moths is often assumed to be Bt-susceptible, thereby reducing the frequency of Bt-resistant individuals in the population compared with cases in which resistance is dominant or additive.

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Another approach taken to control the tobacco budworm was the release of moths that induced sterility during the production of F_1 offspring (Hardee and Laster 1996). Among the main obstacles that prevented the successful implementation of this technique on a large scale was the fact that females that copulated with sterile males produced fertile offspring when they had also mated with wild fertile males (Flint and Kressin 1968, Pair et al. 1977), ruling out the possibility of complete sperm precedence (Flint and Kressin 1968, Pair et al. 1977, LaMunyon 2001, Simmons 2005, Blanco et al. 2008a). The interaction of different males' sperm in *H. virescens* indicates that it would be expected that a Bt-resistant female that copulates with a Bt-susceptible as well as with a Bt-resistant male, can produce offspring carrying Bt-resistant and Bt-susceptible alleles.

Females of this species that have copulated multiple times during their lifetimes with field-captured females contain up to seven spermatophores (median, 2) inside their bursa copulatrix (Raulston et al. 1975). Under laboratory conditions the maximum number of spermatophores is 11 or 12 (Flint and Kressin 1968, Blanco et al. 2009a). Finding more than one spermatophore inside a female indicates that she is likely to have copulated with more than one male (polyandry), although multiple copulations with the same male are possible. Because the sex ratio in *H. virescens* is $\approx 50\%$ females:50% males, it is feasible that a male can also copulate with different females (polygyny).

By conducting eight different mating experiments we assessed whether the ratio of Cry1Ac-resistant offspring differed, depending on the order of males with which the females had mated. The fact that 1) females and males emerge from the field almost simultaneously (a possibility that they can concur with Bt-resistant moths); 2) the possibility that during that time Cry1Ac-susceptible moths emerging from non Bt cotton areas can occur sympatrically with those Cry1Ac-resistant moths emerging from Bt crops; and 3) the fact that *H. virescens* mates multiple times, creates the possibility of a Bt-resistant female carrying sperm of a Bt-susceptible and a Bt-resistant male.

Materials and Methods

***H. virescens* Colonies.** Two *H. virescens* colonies with different susceptibility to Cry1Ac from *B. thuringiensis* (Gould et al. 1995, Blanco et al. 2008b) were used: 1) the YHD2 is a Bt resistant colony maintained at the North Carolina State University and at the USDA-ARS in Stoneville, MS; and 2) the USDA-ARS is a homozygous Bt-susceptible colony maintained at Stoneville, MS. The two colonies are maintained in separate buildings at the Mississippi location.

Initial Moth Crosses. YHD2 moths are significantly (38%) lighter than ARS moths. Due to the fact that this intrinsic difference in weight and/or the Bt-resistance mutation present in YHD2 influences the amount of offspring production between YHD2 and ARS colonies (a reduction of $\approx 40\%$ on egg production, Blanco et al. 2008b), we introgressed YHD2's Cry1Ac-resis-

tance into the ARS colony to have similar moth sizes and offspring production. Reciprocal individual pairs of YHD2 and ARS P_0 moths (54 pairs of ARS ♀ and YHD2 ♂ and 54 pairs of YHD2 ♀ and ARS ♂) were held individually in 500-ml containers (no. 42505LY, Consolidated Plastics Co., Twinsburg, OH), covered the top with cloth (batist, Zweigart, Piscataway, NJ), given free access to 10% sucrose solution in a plastic cup (37-ml [no. T-125] Solo plastic soufflés, Urbana, IL) with a paper tissue (Kleenex, Kimberly-Clark, Roswell, GA) stuffed in it to prevent moths from drowning. Pairs were maintained in an incubator at $27 \pm 0.4^\circ\text{C}$, $75 \pm 10\%$ RH, and a photoperiod of 14:10 [L:D] h. Except where noted otherwise, these conditions were maintained for the rest of the study. Cloths with eggs were removed daily and placed in individual plastic bags (no. 94600, Ziploc, Crawfordsville, IN), and F_1 neonates were reared individually on ≈ 10 ml of insect artificial diet (Blanco et al. 2009 a) in plastic cups covered with paper lids. Thirty-six sets of three pairs each of F_1 moths belonging to each cross (ARS ♀-YHD2 ♂ [cross 1] or YHD2 ♀-ARS ♂ [cross 2]) were enclosed in 36 500-ml containers and their ≤ 24 -h-old F_2 neonates were exposed for 7 d to ≈ 1.0 ml of insect artificial diet containing $10 \mu\text{g}$ of Cry1Ac toxin (MVP II insecticide, Mycogen Corporation, San Diego, CA) per ml, incorporated into the diet (Blanco et al. 2007a). The Cry1Ac-containing diet in bioassay trays (BAW-128, C-D International, Pitman, NJ) was infested with an F_2 neonate per cell and covered with self-adhesive covers (BIO-CV-16, C-D International). F_2 larvae that developed to third instar or older in the Cry1Ac diet after the 7-d exposure were transferred to untreated (control) insect artificial diet to obtain homozygous Cry1Ac-resistant F_2 moths. This approach was used because YHD2 resistance to Cry1Ac toxin in MVP II is inherited as a recessive trait. Thus, 25% of the F_2 offspring would be homozygous for individual resistance genes.

To confirm that the *B. thuringiensis*-resistant genotype was incorporated into the two F_2 crosses (ARS ♀-YHD2 ♂ and YHD2 ♀-ARS ♂), 36 cohorts of three pairs of moths of each cross were enclosed in 500-ml containers. In addition, ARS moths in three-pair moth cohorts were also held in separate 500-ml containers to serve as a comparison. F_3 neonates of these three different crosses were exposed to Cry1Ac-incorporated diet ($10 \mu\text{g}$ of Cry1Ac per ml of diet) for 7 d in bioassay trays as described above. In this F_3 neonate screening, one fourth of the bioassay tray cells contained control insect artificial diet ($0 \mu\text{g}$ of Cry1Ac) that served to evaluate larval development of the three crosses without exposure to *B. thuringiensis* toxin to assess natural larval mortality. F_3 larvae of each line containing the YHD2 genotype (crosses 1 and 2) that developed to third instar or older on Cry1Ac diet were individually transferred to cups containing control diet to obtain F_3 Cry1Ac-resistant moths.

Experiment 1. Mating of *B. thuringiensis*-Resistant Females With *B. thuringiensis*-Resistant and *B. thuringiensis*-Susceptible Males in Reciprocal Orders. This experiment was designed to determine the proportion

Table 1. Production of Cry1Ac-resistant offspring (\pm SE), fertile egg production and female longevity of Cry1Ac-resistant *H. virescens* females mated with a Cry1Ac-resistant and a Cry1Ac-susceptible males in a determined order

Female	Treatment		% resistant offspring	No. fertile eggs per female	Female longevity (d)	Sample size	
	First male	Second male				Females	Tested larvae
1. ARS ♀-YHD2 ♂ ^a	ARS ♂	ARS ♀-YHD2 ♂ ^{a,b}	19.1 \pm 5.9Aa	572 \pm 55Aa	10.7 \pm 0.5Aa	21	4068
2. ARS ♀-YHD2 ♂ ^a	ARS ♀-YHD2 ♂ ^a	ARS ♂ ^b	24.2 \pm 5.0Aa	657 \pm 53Aa	10.6 \pm 0.5Aa	29	5632
3. ARS ♀-YHD2 ♂ ^a	ARS ♂ ^b	ARS ♀-YHD2 ♂ ^a	15.7 \pm 5.4Aa	773 \pm 54Aa	11.5 \pm 0.5Aa	25	4720
4. ARS ♀-YHD2 ♂ ^a	ARS ♀-YHD2 ♂ ^{a,b}	ARS ♂	28.2 \pm 5.5Aa	718 \pm 56Aa	10.8 \pm 0.5Aa	24	4820
5. YHD2 ♀-ARS ♂ ^a	ARS ♂	YHD2 ♀-ARS ♂ ^{a,b}	15.7 \pm 4.5AB	579 \pm 55Aa	8.2 \pm 0.5Ba	36	8350
6. YHD2 ♀-ARS ♂ ^a	YHD2 ♀-ARS ♂ ^a	ARS ♂ ^b	41.8 \pm 4.3Aa	624 \pm 49Aa	8.1 \pm 0.4Ba	40	9770
7. YHD2 ♀-ARS ♂ ^a	ARS ♂ ^b	YHD2 ♀-ARS ♂ ^a	20.9 \pm 4.3Ab	596 \pm 47Aa	8.4 \pm 0.4Ba	39	9816
8. YHD2 ♀-ARS ♂ ^a	YHD2 ♀-ARS ♂ ^{a,b}	ARS ♂	33.3 \pm 4.3Aa	614 \pm 57Aa	8.0 \pm 0.5Ba	40	9888

Means in columns followed by a common uppercase letter are not significantly different ($t < 0.05$) between the Cry1Ac resistance origin of the female. Means in columns followed by a common lowercase letter are not significantly different ($P < 0.05$) among the order in which the males were introduced for a particular cross (treatments 1–4 and treatments 5–8).

^a Cry1Ac-resistant parent.

^b Marked with rhodamine.

of Bt-resistant offspring produced over the lifetime of a Bt-resistant female that mates in sequential order with a Bt-resistant male and then with a Bt-susceptible male or in reverse order (e.g., sperm precedence). Newly emerged (≤ 24 -h-old) F₃ moths from crosses 1 and 2 that were confirmed to be Cry1Ac-resistant from the F₃ larval screening were divided into six groups: groups 1 and 2) females of each of the crosses 1 and 2 were held at a density of 15 moths per cross in 500-ml containers with free access to sucrose solution for 2 d; groups 3 and 4) half of the males of both crosses (in groups of 15 moths for each cross) were held in 500-ml containers with free access to sucrose solution for 2 d (unmarked ARS ♀-YHD2 ♂ [cross 1] or YHD2 ♀-ARS ♂ [cross 2] males). The other half of the males (groups 5 and 6) had free access to rhodamine B (no. 234141, Sigma-Aldrich, St. Louis, MO) dissolved in 10% sucrose solution for 2 d (marked cross 1 and 2 males). ARS male moths were obtained from the ARS colony and were fed in the same manner. Rhodamine presence in marked male spermatophores provides a measure to determine the origin of these marked reproductive structures and confirm that a female has copulated with two different males (Blanco et al. 2006a).

After the 2-d feeding period, a female of cross 1 was enclosed in a 500-ml container, as previously described with an ARS unmarked male for 24 h. The ARS unmarked male was removed and a marked male of cross 1 was introduced for another 24 h. After the second male was removed the female was left alone in the 500-ml container until her death. Cloths with eggs from every female were removed daily (oviposition days), the number of eggs was estimated (Blanco et al. 2009a), and cloths were individually placed in plastic bags to obtain F₄ neonates. These enclosing conditions were constantly maintained throughout the rest of the experiment 1 for the eight different treatments (see Table 1 for a graphic representation of these treatments):

1. As described above. Cross 1 (ARS ♀-YHD2 ♂) female enclosed with an unmarked ARS male for 24 h

and then with a marked cross 1 (ARS ♀-YHD2 ♂) male for another 24 h.

2. A cross 1 female enclosed with an unmarked cross 1 male for 24 h and then with a marked ARS male for 24 h.
3. A cross 1 female enclosed with a marked ARS male for 24 h and then with an unmarked cross 1 male for another 24 h.
4. A cross 1 female enclosed with a marked cross 1 male for 24 h and then with an unmarked ARS male for 24 h.
5. A cross 2 (YHD2 ♀-ARS ♂) female enclosed with an unmarked ARS male for 24 h and then with a marked cross 2 (YHD2 ♀-ARS ♂) male for 24 h.
6. A cross 2 female enclosed with an unmarked cross 2 male for 24 h and then with a marked ARS male for 24 h.
7. A cross 2 female enclosed with a marked ARS male for 24 h and then with an unmarked cross 2 male for 24 h.
8. A cross 2 female enclosed with a marked cross 2 male for 24 h and then with an unmarked ARS male for 24 h.

Eighteen females were set up for each of the eight treatments for every replication. Fertile egg production was assessed daily (oviposition day) for each female. Neonates of each female from each oviposition day were exposed to control (25%) and Cry1Ac (75%) diets for 7 d. The number of neonates from a given female and oviposition day exposed to these diets varied between 16 and 128, depending on the availability of larvae. The proportion of larvae belonging to each female that developed to third instar or older on the Cry1Ac diet within 7 d were recorded and a sample of them transferred to untreated diet. Moths that emerged from this screen were used to set up the next replication of the experiment. The Cry1Ac-susceptible males (ARS colony) were obtained from the main large colony at each replication. When females used in the experiment died they were dissected to extract the spermatophores and these reproductive structures were placed in 1.5-ml centrifuge tubes (no.

Table 2. Egg production (\pm SE), percentage of resistant offspring, and longevity of Cry1Ac-resistant *H. virescens* females mated with a Cry1Ac-resistant or a Cry1Ac-susceptible male

Treatment		% resistant offspring	No. fertile eggs per female	Female longevity (d)	Sample size	
Female	Male				Females	Tested larvae
1. ARS♀-YHD2♂ ^a	ARS♂	0Ac	780 \pm 45Aa	9.2 \pm 0.5Aa	14	1616
2. ARS♀-YHD2♂ ^a	ARS♀-YHD2♂ ^a	96.6 \pm 2.6Aa	529 \pm 46Ab	7.4 \pm 0.5Ab	13	1600
3. ARS♀-YHD2♂ ^a	ARS♂ ^b	0Ac	717 \pm 63Aa	8.8 \pm 0.7Aab	7	944
4. ARS♀-YHD2♂ ^a	ARS♀-YHD2♂ ^{a,b}	87.4 \pm 2.4Ab	675 \pm 43Aa	8.3 \pm 0.4Aab	15	1696
5. YHD2♀-ARS♂ ^a	ARS♂	0Bc	373 \pm 50Bb	5.9 \pm 0.5Ba	11	1136
6. YHD2♀-ARS♂ ^a	YHD2♀-ARS♂ ^a	27.6 \pm 3.3Bb	662 \pm 59Ba	7.1 \pm 0.6Ba	8	640
7. YHD2♀-ARS♂ ^a	ARS♂ ^b	0Bc	570 \pm 53Ba	6.6 \pm 0.6Ba	10	944
8. YHD2♀-ARS♂ ^a	YHD2♀-ARS♂ ^{a,b}	78.9 \pm 3.0Ba	599 \pm 53Ba	6.6 \pm 0.6Ba	10	1184

Means in columns followed by a common uppercase letter are not significantly different ($t < 0.05$) between the Cry1Ac resistance origin of the female. Means in columns followed by a common lowercase letter are not significantly different ($P < 0.05$) among the presence of rhodamine in males of a particular cross (treatments 1–4 and treatments 5–8).

^a Cry1Ac-resistant parent.

^b Marked with rhodamine.

RU-06333-50, Cole-Parmer Instrument Co., Vernon Hills, IL) and stored frozen (-13°C). When two or more spermatophores were present in a female's bursa copulatrix, these structures were inspected under fluorescent light (Blanco et al. 2006a) to confirm the presence of a marked and an unmarked spermatophore. Only females that had marked and unmarked spermatophores were used for data analysis.

Experiment 2. Mating of *B. thuringiensis*-Resistant Females With Either *B. thuringiensis*-Resistant or *B. thuringiensis*-Susceptible Males. To document the offspring production and the proportion of larvae that develop to third instar or older in Cry1Ac diet when a Bt-resistant female mated only with one type of male, a cross 1 (ARS♀-YHD2♂) or a cross 2 (YHD2♀-ARS♂) female was enclosed with the first male described previously for treatments 1–8 of experiment 1, for 48 h. This served to compare the offspring production parameters of only one type of mating as compared with the double mating of experiment 1. See Table 2 for a graphical representation of the treatments.

Eighteen pairs of each of the eight treatments were set up and evaluated as described above. Fertile eggs on cloths were collected and assessed daily until each female died, and only neonates from the second oviposition day were exposed to Cry1Ac diet, due to the fact that on this day the average number of eggs is greater than any other day (Blanco et al. 2006b,

2008b), as well as the possibility of detecting resistant offspring (Blanco et al. 2008b).

Experiment 3. Simultaneous Mating of *B. thuringiensis*-Resistant Females With *B. thuringiensis*-Resistant and *B. thuringiensis*-Susceptible Males. To assess the proportion of larvae that develop to third instar or older in Cry1Ac diet when females might have an opportunity to choose between two males of different Bt susceptibility, or the order in which they mated with both males, sets of 18 ARS♀-YHD2♂ (cross 1) or a YHD2♀-ARS♂ (cross 2) females were enclosed individually with one male of her respective cross and an ARS male for 48 h (see Table 3 for a graphical representation of these treatments). After the 2-d enclosure, both males were removed and the female was left alone in the container until her death. Cloths with eggs were removed daily, the number of eggs was estimated, and the female dissected to analyze her spermatophores. A sample of neonates produced from each oviposition day per female was exposed to Cry1Ac-containing diet as described for experiment 1. This experiment was repeated two times with 18 females of each of the eight treatments in different dates.

Statistical Analysis. The experimental design in experiment 1 was a split plot where the main unit was moth female resistance source (paternal treatments 1–4, maternal treatments 5–8). The subunits were a factorial treatment structure of two sources of male

Table 3. Egg production (\pm SE), percentage of resistant offspring, and longevity of Cry1Ac-resistant *H. virescens* females mated with a Cry1Ac-resistant and a Cry1Ac-susceptible males in an unknown order

Treatment		% resistant offspring	No. fertile eggs per female	Female longevity (d)	Sample size	
Female	Males introduced simultaneously for 48 h				Females	Tested larvae
1. ARS♀-YHD2♂ ^a	ARS♂ and ARS♀-YHD2♂ ^{a,b}	41.4 \pm 6.6Aa	511 \pm 34Aa	7.0 \pm 0.3Aa	39	6216
2. ARS♀-YHD2♂ ^a	ARS♂ ^b and ARS♀-YHD2♂ ^a	49.1 \pm 5.9Aa	571 \pm 30Aa	7.3 \pm 0.3Aa	47	6696
3. YHD2♀-ARS♂ ^a	ARS♂ and YHD2♀-ARS♂ ^{a,b}	36.4 \pm 8.4Aa	359 \pm 44Ba	5.3 \pm 0.4Ba	24	3492
4. YHD2♀-ARS♂ ^a	ARS♂ ^b and YHD2♀-ARS♂ ^a	27.8 \pm 8.1Aa	354 \pm 41Ba	6.2 \pm 0.4Ba	26	3840

Means in columns followed by a common uppercase letter are not significantly different ($t < 0.05$) between the Cry1Ac resistance origin of the female. Means in columns followed by a common lowercase letter are not significantly different ($P < 0.05$) among the presence of rhodamine in males of a particular cross (treatments 1 and 2 and treatments 3 and 4).

^a Cry1Ac-resistant parent.

^b Marked with rhodamine.

resistance/susceptibility by two male markings. The replications of main unit consisted of seven sets (replications) of female resistance source. Within each set of each female resistance source, each female was considered as a replication of subunit treatments; therefore, there was an additional level of replication for subunit treatments. Data were analyzed for all experiments by analysis of variance by using SAS version 9.1 (SAS Institute 2001). Least significant differences at $P \leq 0.05$ were used to determine significant differences in means between treatments.

Results

Experiment 1. Mating of *B. thuringiensis*-Resistant Females With *B. thuringiensis*-Resistant and *B. thuringiensis*-Susceptible Males in Reciprocal Orders. There were no significant differences ($F = 2.23$; $df = 1, 246$; $P = 0.13$) in the proportion of Cry1Ac resistant offspring between treatments that inherited the Bt resistance from the mother side (cross 2, treatments 5–8) or from the father side (cross 1, treatments 1–4). However, when the first male enclosed with the female was Cry1Ac-resistant (treatments 2, 4, 6, and 8) the proportion of resistant progeny ($31.7 \pm 4.2\%$ [mean \pm SEM]) was significantly higher ($F = 18.2$; $df = 1, 241$; $P < 0.0001$) than when the first male was susceptible ($17.2 \pm 4.4\%$). The mark of the males did not have an influence on the proportion of resistant offspring ($F = 0.01$; $df = 1, 241$; $P = 0.93$) (Table 1).

Fertile egg production was not affected ($F = 1.08$, $P = 0.30$, $df = 1, 246$) by the mode of Cry1Ac-resistance inheritance (mother side, treatments 5–8 or father side, treatments 1–4), nor by the male marking ($F = 3.51$, $P = 0.06$) or by the order in which they were enclosed with the females ($F = 2.19$, $P = 0.69$). Female longevity was significantly diminished ($F = 17.49$; $df = 1, 246$; $P < 0.0001$) when Cry1Ac resistance was inherited from the mother's side. Male marking or order of males did not make a difference in female's longevity ($F = 3.55$; $df = 1, 246$; $P = 0.06$ / $F = 0.99$, $df = 1, 246$; $P = 0.32$, respectively) (Table 1). Male moth weights were not significantly different ($F = 0.59$; $df = 2, 211$; $P = 0.55$).

Experiment 2. Mating of *B. thuringiensis*-Resistant Females With Either *B. thuringiensis*-Resistant or *B. thuringiensis*-Susceptible Males. A significantly lower proportion of Cry1Ac-resistant offspring ($F = 51.08$; $df = 1, 80$; $P < 0.0001$) were obtained when Bt resistance was inherited from an initial YHD2 male (cross 2). Male marking significantly lowered the proportion of resistant offspring when rhodamine was given to Cry1Ac-resistant males ($t = 10.15$; $df = 1, 133$; $P < 0.0001$ [treatments 4 and 8]) but not when Bt-susceptible males were treated with rhodamine ($t = 0.00$; $df = 1, 133$; $P = 1.00$) (Table 2).

Fertile egg production was significantly decreased ($F = 9.98$; $df = 1, 80$; $P = 0.002$) when resistance was inherited from an initial YHD2 female (cross 2). Male marking caused a significant reduction of fertile egg production when rhodamine was given to Cry1Ac-resistant males that inherited this trait from an initial

YHD2 male ($t = 18.83$; $df = 1, 80$; $P < 0.0001$ [treatments 2 and 4]), and when it was given to *B. thuringiensis*-susceptible males ($t = 15.76$; $df = 1, 80$; $P < 0.0001$ [treatments 5 and 7]).

Female longevity was significantly reduced ($F = 4.30$; $df = 1, 80$; $P = 0.04$) when *B. thuringiensis*-resistance was inherited from the maternal side (cross 2, treatments 5–8). Females that inherited Cry1Ac-resistance from the paternal side (cross 1) lived significantly shorter lives ($F = 21.4$; $df = 1, 80$; $P < 0.0001$) when they copulated with a marked Cry1Ac-resistant male than when they copulated with a Cry1Ac-susceptible nonmarked male (Table 2).

Experiment 3. Simultaneous Mating of *B. thuringiensis*-Resistant Females With *B. thuringiensis*-Resistant and *B. thuringiensis*-Susceptible Males. When Cry1Ac-resistant females were enclosed with two males simultaneously, the percent of Bt-resistant progeny was not significantly affected (mean = 38.6%) ($F = 3.19$; $df = 1, 133$; $P = 0.27$) by the interaction of all of the above-mentioned factors or by any of them separately. Fertile egg production was significantly reduced ($F = 23.27$; $df = 1, 132$; $P < 0.0001$) as well as female longevity ($F = 12.93$; $df = 1, 133$; $P = 0.0005$) when Cry1Ac-resistance was inherited from the maternal side (cross 2) (Table 3).

Discussion

There were significant differences in many of the parameters evaluated in this study that were affected by the source of Cry1Ac resistance. In the three experiments, the proportion of resistant offspring, the fecundity, or the female longevity was affected by the initial male/female parent from which the *B. thuringiensis* resistance was inherited (also see Blanco et al. 2008b). When Cry1Ac-resistance was acquired from the maternal side, the percentage of offspring with the resistance phenotype was lower (Tables 1–3). However, there was a similar overall trend obtained from two of the main experiments in terms of Cry1Ac offspring production. A Cry1Ac-resistant tobacco budworm female that copulated with a Cry1Ac-resistant and a Cry1Ac-susceptible male, produced Cry1Ac progeny every day of their reproductive life, regardless of the order in which she mated with the two males (Figs. 1 and 2). These findings add more information to what has been reported previously (LaMunyon 2001, Blanco et al. 2008a), that the tobacco budworm does not exhibit sperm precedence in the strict sense of the concept; that is, one of the male's sperm displaces the other almost entirely.

These findings corroborate one of the obstacles found in the successful implementation of the tobacco budworm sterile male release technique (Flint and Kressin 1968, Pair et al. 1977, Hardee and Laster 1996). The lack of strict sperm displacement made it possible for multiple-mated females to produce viable offspring when sperm of a sterile and a nonsterile male made it to their reproductive track. The new information generated from this study is also relevant to the efficacy of resistance management programs for Bt

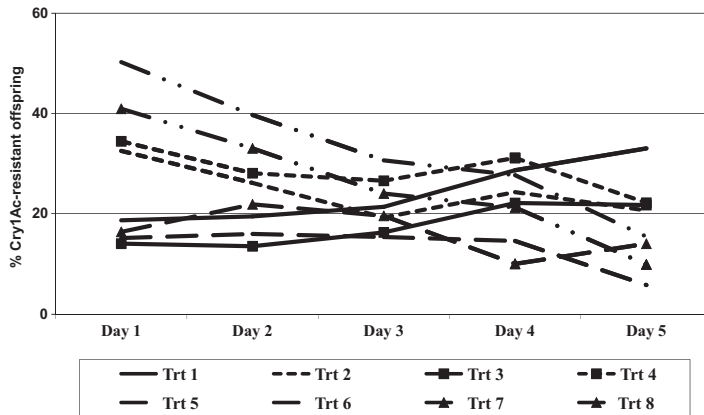


Fig. 1. Percentage of *B. thuringiensis*-resistant *H. virescens* offspring produced by a *B. thuringiensis*-resistant female that mated with a *B. thuringiensis*-resistant male and a *B. thuringiensis*-susceptible male in an arranged mating order.

crops that use a high-dose/refuge approach. There are at least two implications of this study. First, it is clear from experiments 1 and 3 that when there is competition between sperm from resistant and susceptible males, there is a strong trend for the sperm from susceptible males to be competitive. If this is the case for field-generated resistance mechanisms, it constitutes a fitness cost for Bt resistance alleles and could slow the evolution of Bt-resistant insect populations.

A second implication of these results relates to the fact that when resistance alleles are rare, the chance that a homozygous resistant female (rr) will encounter a male that is not homozygous susceptible (SS) is low. If that rare resistant female only mates once or has complete sperm precedence, the chance that any of her offspring will be homozygous resistant (rr) and thus capable of surviving on Bt crops is equal to the small fraction of males that are rr and rS. If the rr female mates twice and there is not complete sperm precedence, then the chances of some of her offspring being rr is twice the fraction of rr plus rS males in the male population. This stochastic process that is expected to start the formation of a pocket of Bt resis-

tance alleles can have a significant impact of the rate of resistance evolution (Peck et al. 1999). The results generated here indicate that production of *B. thuringiensis*-resistant (rr) *H. virescens* offspring by an rr female copulated by an rr and an SS male is approximately $\leq 40\%$.

When Cry1Ac-resistant females are copulated by two types of males (Bt-resistant and Bt-susceptible), their progeny is sired by one of the males in approximately the same proportion over most of their reproductive life (Fig. 1). The proportion of resistant offspring over a five-day oviposition period (constituting $\approx 95\%$ of their reproductive output) changed very little over her reproductive life, this could indicate that sperm mixing occurs inside the bursa copulatrix and is used in not under female or male control, as is perceived under sperm precedence of a female choice mechanism (Eberhard 1996). When females were presented with two males simultaneously, this trend was very similar in three of the four treatments of experiment 3 (Fig. 2), except in one instance where the proportion of Cry1Ac-resistant offspring continued increasing for the first 4 d, but continuously producing

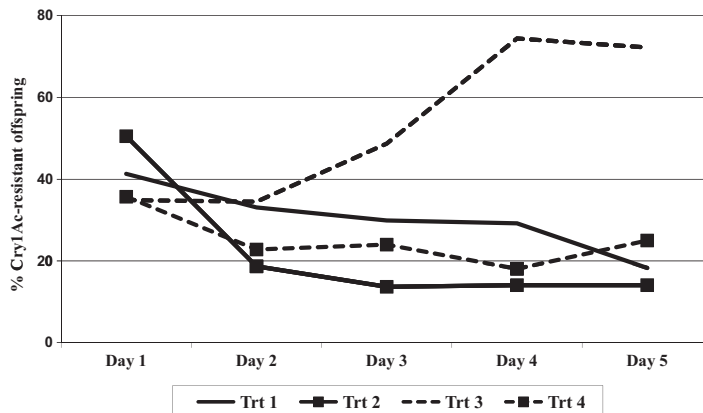


Fig. 2. Percentage of *B. thuringiensis*-resistant *H. virescens* offspring produced by a *B. thuringiensis*-resistant female that mated with a *B. thuringiensis*-resistant male and a *B. thuringiensis*-susceptible male in an unknown mating order.

F₁ larvae with the two parental genotypes. It is interesting to note that the number of copulations varied ($F = 29.55$; $df = 2, 331$; $P < 0.0001$.) between experiment 1 (2.0 ± 0.05 [mean \pm SEM] spermatophores per female), experiment 2 (1.77 ± 0.06) and experiment 3 (2.38 ± 0.05), indicating that the copulations increased as the number of males increased (experiments 1 and 3 versus 2), regardless of the fact that a female was only with a male(s) for 2 d.

Lack of complete sperm precedence could affect the evolution of resistance to the high dose/refuge strategy used for Bt crops. An important part of the preservation of this effective technique relies on the potential benefit that the structured and natural refuges might have in preserving populations susceptible to *B. thuringiensis*. Under this concept it is assumed that when Bt-resistant moths emerge, the potential mating of a Bt-resistant female with a Bt-susceptible male will produce Bt-susceptible offspring, therefore preserving Bt-susceptibility in the next generation. An aspect that has not been entirely addressed by this assumption has to do with the fact that tobacco budworm (and other Lepidoptera) mate multiple times (Flint and Kressin 1968, Raulston et al. 1975, Blanco et al. 2009b). The fact that $\geq 90\%$ of both sexes of Cry1Ac-resistant tobacco budworm moths emerge within a 3-d period, ≈ 4 d later than the synchronized emergence of a Bt-susceptible colony (C.A.B., unpublished), indicates that there is a greater chance that a Bt-resistant female will encounter a Bt-resistant male than a Bt-susceptible male under field conditions. This creates the possibility of the double-mating referred to herein that might produce a constant proportion of Cry1Ac-resistant offspring.

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