Fitness Costs Associated with Cry1Ac-Resistant *Helicoverpa zea* (Lepidoptera: Noctuidae): A Factor Countering Selection for Resistance to Bt Cotton?

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ABSTRACT The heritability, stability, and fitness costs in a Cry1Ac-resistant *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) colony (AR) were measured in the laboratory. In response to selection, heritability values for AR increased in generations 4–7 and decreased in generations 11–19. AR had significantly increased pupal mortality, a male-biased sex ratio, and lower mating success compared with the unselected parental strain (SC). AR males had significantly more mating costs compared with females. AR reared on untreated diet had significantly increased fitness costs compared with rearing on Cry1Ac treated diet. AR had significantly higher larval mortality, lower larval weight, longer larval developmental period, lower pupal weight, longer pupal duration, and higher number of morphologically abnormal adults compared with SC. Due to fitness costs after 27 generations of selection as described above, AR was crossed with a new susceptible colony (SC1), resulting in AR1. After just two generations of selection, AR1 exhibited significant fitness costs in larval mortality, pupal weight and morphologically abnormal adults compared with SC1. Cry1Ac-resistance was not stable in AR in the absence of selection. This study demonstrates that fitness costs are strongly linked with selecting for Cry1Ac resistance in *H. zea* in the laboratory, and fitness costs remain, and in some cases, even increase after selection pressure is removed. These results support the lack of success of selecting, and maintaining Cry1Ac-resistant populations of *H. zea* in the laboratory, and may help explain why field-evolved resistance has yet to be observed in this major pest of *Bacillus thuringiensis* cotton, *Gossypium hirsutum* L.

KEY WORDS *Helicoverpa zea*, Cry1Ac, heritability of resistance, fitness costs, stability of resistance

The evolution of resistance in target insect populations is the primary concern with the use of crops expressing *Bacillus thuringiensis* (Bt) proteins such as Cry1Ac in Bt cotton (Bollgard), *Gossypium hirsutum* L., in the United States and elsewhere. However, even after 12 yr of commercial use in the United States, there are still no documented cases of field-evolved resistance in Bollgard to any of the three target pests, especially to bollworm, *Helicoverpa zea* (Boddie) (Moar and Anilkumar 2007). *H. zea* is significantly more tolerant to Cry1Ac present in Bollgard than other target pests (MacIntosh et al. 1990, Ali et al. 2006, Sivasupramaniam et al. 2008) and can survive on Bollgard late season (Jackson et al. 2004a). Although resistance management strategies such as "high dose plus refuge" have been used to delay resistance development (Gould 1998), the use of these tactics alone cannot fully explain the total lack of field-evolved resistance. Factors such as fitness costs, stability, and the genetics of resistance may play a significant role in delaying or mitigating resistance evolution (Tabashnik 1994, Gould 1998). Many models have predicted the delay in resistance development due to fitness costs (Caprio 2001; Storer et al. 2003a, 2003b; Gustafson et al. 2006). Studies with laboratory-selected Cry1Ac-resistant insects such as *Pectinophora gossypiella* (Saunders), *Helicoverpa armigera* (Hübner), and *Plutella xylostella* (L.) support these model predictions by documenting fitness costs and incomplete resistance to Bt crops (Liu et al. 1999; Carriére et al. 2001a, 2001b, 2006; Sayyed and Wright 2001, Bird and Akhurst 2004, 2005; Higginson et al. 2005).

Selection for Cry1Ac-resistant *H. zea* in the laboratory has been attempted numerous times over a 10-yr period, but all attempts have resulted in colony crashes due to fitness costs (Luttrell et al. 1999; R. Luttrell, personal communication; Jackson et al. 2004b; R. Jackson, personal communication; W.J.M., unpublished data; Anilkumar and Moar 2006; Anilkumar et al. 2008). Additionally, although there have been many attempts to rear field-collected *H. zea* populations, often collected from Bt crops, with relatively high tolerances to Cry1Ac (MVP II) in the laboratory, they typically cannot be maintained for more than five to seven of generations, with many

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populations crashing after one to two generations (R. Luttrell, personal communication; K.J.A. and W.J.M., unpublished results). Anilkumar et al. (2008) reported a stable Cry1Ac-resistant *H. zea* strain (AR) after selection with Bt Cry1Ac toxin for 11 generations (100-fold resistance), and this colony was maintained at this level for 25 generations under continuous selection. Even though AR was relatively stable, fitness costs were observed during selection and when insects were removed from selection. (Note: This colony was crossed to a susceptible population in generation 26 to avoid the total collapse of this strain due to fitness costs). Furthermore, higher fitness costs usually affect the stability of resistance in the population, thereby affecting the heritability of resistance. Therefore, a thorough understanding of the biological traits of *H. zea* affected by fitness costs, heritability and stability of resistance could contribute to the development of more realistic models for predicting the development of resistance and thereby aid in formulating better strategies for effective resistance management. Furthermore, findings in this article could help explain why field-evolved resistance has yet to be observed in this pest to Bollgard after 12 yr of intense use. Therefore, this study investigated the various biological parameters associated with fitness costs, heritability and stability of resistance exhibited by Cry1Ac toxin-resistant *H. zea* (Anilkumar et al. 2008).

Materials and Methods

Insect Strains. A laboratory susceptible colony of H. *zea* (SC) was established in September 2004 from a laboratory colony from Monsanto (Union City, TN). The culture at Monsanto was annually infused with field-collected insects; therefore, the population was heterogeneous and contained Cry1Ac-resistant genes (Anilkumar et al. 2008). One strain (AR) was selected from SC for resistance on artificial diet containing Bt Cry1Ac toxin for 25 generations by exposing individual neonates for 7 d (Anilkumar et al. 2008). Only second and third instars were transferred to 24-well tissue culture plates containing untreated diet and were reared an additional 7 d (Ali et al. 2006, Anilkumar et al. 2008, Sivasupramaniam et al. 2008). Late fourth- to early fifth-instar larvae were transferred to diet cups (30 ml; Bio-serve, Frenchtown, NJ) containing artificial diet and were reared to pupation. Except for selection using Cry1Ac toxin, AR and SC were treated similarly in regards to diet used, number of larvae reared, quality of larvae harvested, and number of adults used for generating subsequent generations.

Resistance Heritability and Resistance Risk Assessment. Heritability of resistance (h^2) and resistance risk (G) (number of generations required for 10-fold increase in resistance) were estimated. LC_{50} values for SC and AR conducted simultaneously (Anilkumar et al. 2008) and percentage of survival in each generation of selection were used for calculating parameters necessary for determining h^2 and G (Tabashnik 1992).

Fitness Costs in AR on Cry1Ac-Treated Diet. While conducting selection experiments, a reduction in egg hatch was observed in AR after nine generations of selection (>36-fold resistance, Anilkumar et al. 2008). Further observations indicated no embryo development, confirming egg infertility. Therefore, both resistant (AR) and control (SC) strains were monitored for mating success during resistance selection, and maintenance, respectively, from generations 9 to 24.

Larvae were selected and reared as discussed above; the resistance ratio of AR (LC_{50} of AR/LC_{50} of SC) exceeded 100-fold. The resulting pupae were sexed and maintained in separate boxes (18 by 18 by 7 cm) for adult eclosion. Pupal sex ratio was recorded for 15 generations (generations 10–24; 6,314 pupae total). Further, pupal mortality (dead pupae and malformed adults) were recorded for 11 generations (generation 15 to generation 25; 4,867 pupae total). The proportions of males were modeled as the number of males in a total population, and were analyzed using a binomial test; the interaction of population and generation were considered as residuals (Hardy 2002, SAS Institute 2003). Pupal mortality was compared between strains using a paired *t*-test (SPSS 2006).

Initially, adults were released into mating cages (34 by 19 by 11 cm) at a 1:1 sex ratio, and 30 moths were maintained per cage. However, additional (maximum of three) moths from either sex were released into mating cages because of premature adult mortality in some of the original 30 moths (within three days). The resultant sex ratio was not significantly different (see below) from 1:1. Mating cages were covered with white cloth for oviposition and moths were fed a 10% sucrose solution. Egg sheets were replaced daily and incubated at 27 ± 2°C until hatching. Adults were maintained in cages until death or when moths quit laying eggs (after 10 d). Dead moths were removed daily from cages, and all surviving moths (after 10 d) were dissected under a stereo microscope to determine mating frequency. Female moths were classified as mated or unmated based on the presence or absence of spermatophore(s) in the spermatheca. Furthermore, females were classified as having mated once, twice, three, four, or five times depending on the number of spermatophores present in the spermatheca. Mean number of spermatophores produced per male was calculated by taking the total number of spermatophores produced in a generation divided by the number of males released into cages (Bird and Akhurst 2004). Observations were made for 14 generations (generation 9 [resistance ratio {RR} >36-fold], and generations 12–24 [RR >100-fold]) from a period spanning nearly 2 yr and from a total of 3,886 moths. Percentage of mating, multiple mating, and mean number of spermatophores between AR and SC were compared by paired *t*-tests (SPSS 2006).

Mating propensity observations indicated a reduction in mating success in AR (see Results; Table 2; Fig. 2). Therefore, reciprocal crosses between AR and SC (AR[♀] × SC[♂] and SC[♀] × AR[♂]) were conducted at an equal sex ratio to test whether the reduction in mating success was sex-linked. Moths were caged, and percentage of mating was ascertained as described above. Reciprocal crosses were conducted

with five male and five female moths spanning three generations, >50 moths (1:1 sex ratio) in two generations (two replicates). Therefore, the total number of moths used in each of the two reciprocal (AR × SC) crosses was 140. Percentage mating, multiple mating, and mean number of spermatophores between AR and SC were analyzed by analysis of variance (ANOVA) (SPSS 2006).

Fitness Costs in AR on Untreated Diet. The relative performances of both susceptible (SC) and >100-fold resistant (AR) strains were measured on untreated artificial diet (referred to hereafter as regular diet) and untreated selection diet (artificial diet diluted with 20% water; used for incorporating Bt proteins in selection experiments, referred to hereafter as selection diet) (Anilkumar et al. 2008). In total, 160 larvae (48, 48, and 64 larvae in replication 1, 2, and 3, respectively) for each treatment were tested. Individual neonates were placed on diet in 128-well CD International bioassay trays (CD International, Pitman, NJ) and reared for 7 d. Larval weight and instar were recorded after 7 d, and larvae were transferred to 30-ml diet cups containing regular diet and reared until pupation. Larval duration and mortality were recorded. All insects were removed from diet on the second day of pupation; weights were recorded and were transferred to new 30-ml cups (containing no diet). Pupae were sexed and observed daily for adult eclosion. Adults failing to eclose and those with fringed wings were considered as malformed adults. Pupae that did not eclose after 15 d were considered dead.

Growth rate (weight gain per day) was calculated for both strains after the first 7 d (on either diet) and at pupation. The growth rate for the first 7 d (when insects were exposed to either selection diet or regular diet) was calculated by dividing larval weight by 7. The growth rate after 7 d (when insects with different exposure background were transferred to regular diet) was calculated by the following equation:

$$\text{Growth rate (weight gain per day)} = (\text{Pupal weight} - \text{larval weight at 7 d}) / (\text{larval duration} - 7 \text{ d}).$$

Furthermore, the difference in growth rates was calculated by subtracting the growth rate during the first 7 d from that determined after seven days. Insects that died prematurely were not included in the analysis.

Thirty adults were released into mating cages and maintained as explained above. Total number of eggs laid was recorded daily, and mean number of eggs per female was calculated. Eggs were incubated for 4 d at 27 ± 2°C, and hatching percentage was calculated. Each experiment on selection and regular diets was considered as a block, each insect as a replicate and the entire test as a randomized complete block design for analysis. Larval and pupal periods were log-transformed to stabilize variance. Larval weight, duration and mortality; pupal weight and duration; and percentage of malformed adults were analyzed using two-way ANOVA, and means were separated using Tukey's least significant differences (LSDs) (SPSS Inc. 2006). Growth rates during initial 7 d, after 7 d, and their difference were analyzed using ANOVA and means were separated using Fisher LSDs (SPSS Inc. 2006).

Crosses with the Susceptible Strain. AR was crossed with a new susceptible strain (SC1) resulting in AR1, to avoid complete loss of the strain due to fitness costs (see Results) associated with Cry1Ac resistance selection and maintenance. As discussed above, the laboratory colony at Monsanto is infused annually with field-collected insects; therefore, SC1 is a derivative of SC from the most recent infusion in 2007. SC1 had increased tolerance to Cry1Ac toxin (LC_{50} = 31.25 μg Cry1Ac/g diet) compared with SC (LC_{50} = 9–15 μg Cry1Ac/g diet; Anilkumar et al. 2008). Even though both reciprocal crosses were attempted, only AR[♀] × SC1[♂] yielded a F_1 population due to mating costs associated with AR males (see Results). AR1 was selected at the regular selection concentration of Cry1Ac (500 μg Cry1Ac/g diet) for two generations. Furthermore, fitness parameters (discussed above) were measured only on regular diet. Three experiments were conducted with 32 larvae each per strain per replication and data were analyzed as discussed above.

Stability of Resistance. The desired number of larvae could not be obtained for bioassays when AR was reared on untreated diet for two generations due to extremely high pupal mortality (discussed in Results). Therefore, bioassays were conducted immediately after one generation. Neonates (130) were tested in two replications at 500 μg Cry1Ac/g diet (concentration used in resistance selection/maintenance experiments) compared with an untreated control. Parallel tests were conducted on AR subjected to continued selection. Paired *t*-tests were conducted to compare the survivorship of AR on 500 μg Cry1Ac/g diet when AR was continuously selected at 500 μg Cry1Ac/g diet, and after AR had been reared one generation on regular diet.

Statistical Analysis. All statistical tests were conducted at the 0.05 level of significance by using either SPSS or SAS (SAS Institute, Cary, NC) statistical programs, and for those parameters that required transformations for stabilizing the variance, data are presented as nontransformed arithmetic means.

Results

Heritability and Resistance Risk Assessment

The heritability (h^2) of resistance to Bt Cry1Ac toxin varied at different generations of selection (Table 1). The h^2 was 0.315 after four generations (12-fold resistance), increased to 0.401 after seven generations (36-fold resistance) and decreased to 0.256 and 0.123 after 11 and 19 generations, (>100-fold resistance), respectively. Resistance risk (G) assessment considering heritability values after 19 generations of selection (0.123) indicated that 9.66 generations are required for a 10-fold increase in resistance.

Table 1. Heritability (h^2) and resistance risk assessment for resistance to Cry1Ac in *H. zea*

N[a]	LC_{50} values		RR[b]	R[c]	Slope		S[d]	h^{2e}	G[f]
	Initial[g]	Final[h]			Initial	Final			
4	8.89	107.64	12.11	0.271	1.71	1.42	0.860	0.315	3.69
7	8.94	321.22	35.93	0.222	1.93	1.89	0.554	0.401	4.50
11	11.82	1,450.00	122.7	0.190	1.76	1.76	0.742	0.256	5.27
19	15.00	1,390.00	92.67	0.104	2.31	1.39	0.840	0.123	9.66

LC_{50}, resistance ratio and slope values are from Anilkumar et al. (2008).
[a] Number of generations of continuous selection using Bt Cry1Ac treated diet.
[b] Resistance ratio.
[c] R, response to selection.
[d] Selection differential.
[e] h^2, heritability.
[f] G, resistance risk = number of generations required for 10-fold increase in resistance.
[g] Initial: LC_{50} for unselected parental strain.
[h] Final, LC_{50} for resistant strain measured after number of generations of selection.

Fitness Costs in AR on Cry1Ac-Treated Diet

Pupal Sex Ratio and Mortality. There were significant ($F_{1, 14} = 9.44$; $P = 0.0083$) differences in sex ratios of AR and SC (Fig. 1). In 11 of 15 generations, AR produced more males than females compared with only three of 15 generations of male bias in SC. Results from 11 generations indicated that mean ± SE pupal mortality in AR (24.48 ± 2.47%) was significantly ($t_{10} = 5.244$, $P < 0.001$) higher than SC (11.67 ± 1.16%). However, there were no significant differences in mortality between sexes for either AR ($t_{10} = -1.138$, $P = 0.284$) or SC ($t_{10} = -0.881$, $P = 0.401$).

Mating Studies. Percentage of mating success during generations 9 and 12 was not significantly ($t_1 = -1.963$, $P = 0.30$) different between AR and SC (Fig. 2). Resistance ratios for these generations were 36- and 122-fold, respectively (Anilkumar et al. 2008). Mating success in AR declined after achieving >100-fold resistance and there was always a 1.5- to 3-fold decrease in mating success for AR compared with SC. Furthermore, significantly ($t_{13} = -2.521$, $P = 0.026$) more SC females (26%) had multiple mating compared with AR (17%) (Table 2). SC males (1.23 ± 0.10) produced significantly ($t_{13} = -5.058$, $P = 0.001$) more spermatophores compared with AR (0.58 ± 0.08).

Reciprocal Crosses. Mating success between AR and SC was significantly ($F_{3, 19} = 14.29$; $P = 0.000$) different (Table 3). Reciprocal crosses (AR ♀ × SC ♂, SC ♀ × AR ♂), with SC as male had significantly ($P = 0.046$) higher mating compared with AR as male. There were no significant differences in mating between AR and the reciprocal cross with AR as male ($P = 0.35$); similarly between SC and SC as male in reciprocal cross ($P = 0.61$). There were no significant differences in multiple mating between either parental strains or their reciprocal crosses ($F_{3, 19} = 0.7$; $P = 0.566$). The number of spermatophores produced per male was significantly ($F_{3, 19} = 3.804$; $P = 0.028$) different in AR and SC strains. However, when AR males were used in the reciprocal cross, no significant differences were observed in mean spermatophore/male

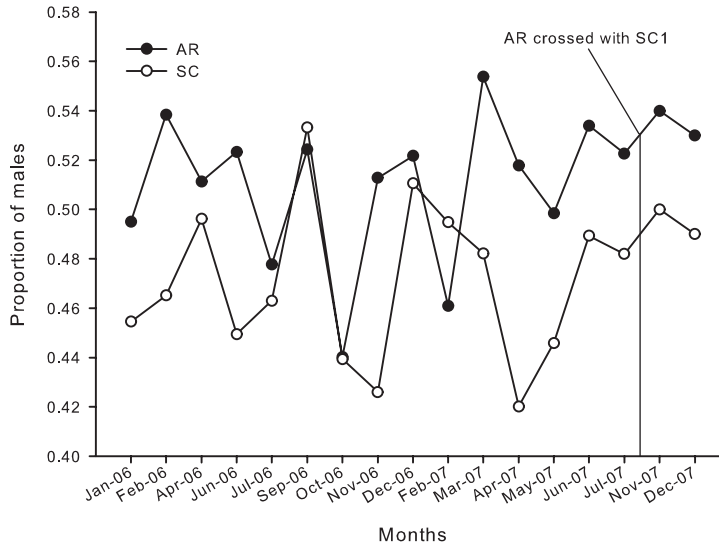

Fig. 1. Pupal sex ratio of AR and SC strains over time with selection and rearing, respectively.

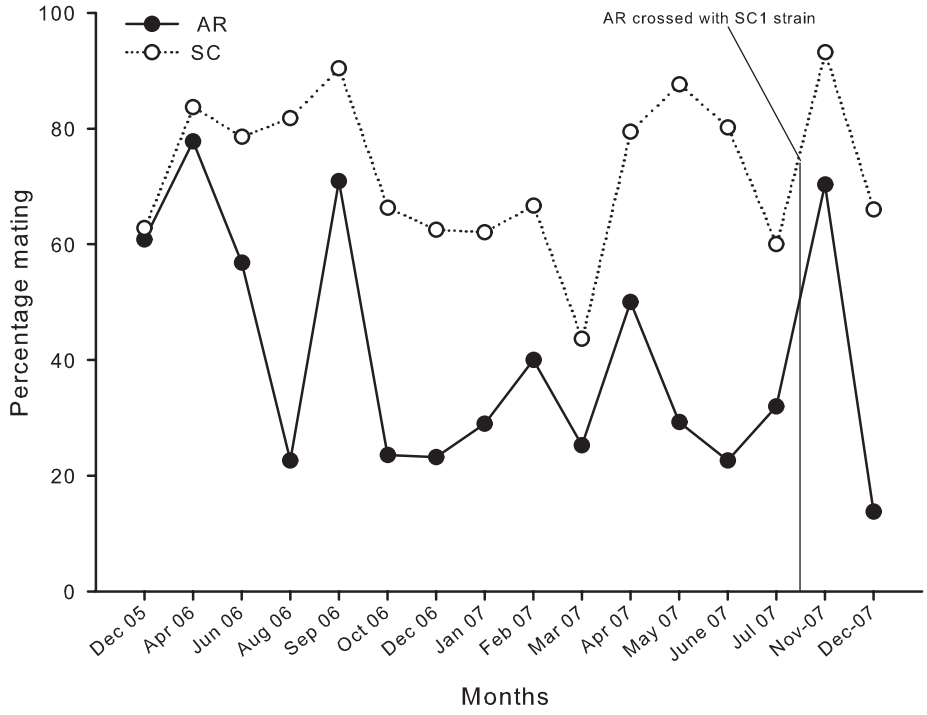

Fig. 2. Mating success in AR and SC over time with selection and rearing, respectively.

compared with SC as male ($P = 0.074$) and/or the SC strain ($P = 0.099$).

Fitness Costs in AR on Untreated Diet

Larval Weight, Duration, and Mortality. SC gained significantly ($F_{3,\ 574} = 48.178$; $P = 0.000$) more weight in 7 d compared with AR. Furthermore, selection diet or regular diet did not affect larval weight in SC (Table 4). In contrast, AR on selection diet had significantly lower weight compared with when reared on regular diet. Significant differences ($F_{3,\ 571} = 124.01$; $P = 0.000$) existed between strains for larval duration regardless of diet tested; AR required one additional day to complete larval development compared with SC. Furthermore, rearing larvae either on selection diet or regular diet for one week did not influence the total larval duration in either AR or SC. It is important to note that significantly ($F_{3,\ 11} = 4.623$; $P = 0.037$) higher larval mortality was recorded in AR compared with SC, although larval mortality did not differ between selection and regular diet in either AR or SC.

Pupal Weight, Duration, and Mortality. Pupal weight of AR on regular diet varied significantly ($F_{3,\ 503} = 25.402$; $P = 0.000$) from SC. Interestingly, AR pupal weight on selection diet was not different from SC. SC on regular diet recorded the shortest pupal duration, which was significantly ($F_{3,\ 492} = 39.425$; $P = 0.000$) different from SC on selection diet and AR on both diets. Production of morphologically abnormal adults in AR was significantly ($F_{3,\ 11} = 14.281$; $P = 0.001$) increased (approximately six-fold) compared with SC, which did not differ between selection diet and regular diet and had the most pronounced effect on fitness in relation to resistance.

Growth Rate. During the initial 7 d, weight gained per day by SC larvae was significantly ($F_{3,\ 471} = 22.70$; $P = 0.000$) higher than AR, but there was no significant (AR: $P = 0.06$, SC: $P = 0.30$) difference between selection diet and regular diet (Table 5). The slowest

Table 2. Reproductive propensity (mean ± SE) of Cry1Ac-resistant (AR) and susceptible (SC) strains of *H. zea* during selection and rearing, respectively[a]

Strain	N^b	% mating	% multiple mating	Spermatophores/male
AR	2,066	40.27 ± 5.18	16.76 ± 3.40	0.58 ± 0.08
SC	1,820	71.85 ± 3.53	26.01 ± 2.34	1.23 ± 0.10
t-test results		$t_{13,1} = -6.468$; $P = 0.000$	$t_{13,1} = -2.521$; $P = 0.026$	$t_{13,1} = -5.628$; $P = 0.000$

Lowercase letters after means within a column indicate significant differences at $P < 0.05$ level by Tukey's test.
[a] Results are from 14 generations of observations.
[b] Number of moths.

Table 3. Reproductive (mean ± SE) success in a Cry1Ac-resistant (AR), susceptible (SC) and their reciprocal crosses[a]

Strain/cross	N^b	% mating	% multiple mating	Spermatophores/male
AR	960	29.48 ± 2.98a	12.04 ± 1.93	0.41 ± 0.13a
SC	815	66.29 ± 3.65b	21.60 ± 4.43	1.01 ± 0.39b
AR ♀ × SC ♂	140	58.48 ± 3.98b	20.19 ± 5.52	1.04 ± 0.32b
SC ♀ × AR ♂	143	40.24 ± 7.38a	16.10 ± 7.25	0.53 ± 0.30ab
F-test results		$F_{3,19} = 14.29$; $P = 0.000$	$F_{3,19} = 0.700$; $P = 0.566$	$F_{3,19} = 5.772$; $P = 0.007$

Lowercase letters after means within a column indicate significant differences at $P < 0.05$ level by Tukey's test.
[a] Results are from five generations of experiments.
[b] Number of moths.

growth rate (15.82 ± 0.54 mg/d) was observed when AR larvae were exposed to selection diet. After 7 d, when both AR and SC were transferred to or continued on regular diet, growth rates were significantly different ($F_{3, 471} = 12.34$; $P = 0.000$). During this time, the growth rate in SC did not differ significantly ($P = 0.684$) based on their previous exposure. However, initial exposure influenced the growth rate of AR larvae significantly ($P = 0.013$). The slowest growth rate (28.89 ± 0.62 mg/d) after 7 d was observed in AR when they were initially exposed to regular diet.

The difference in growth rate (Fig. 3) before and after 7 d was significantly different ($F_{3, 471} = 3.84$; $P = 0.010$), and the highest difference (15.44 ± 0.77 mg/d) was observed when AR from selection diet was shifted to regular diet (Table 5). Considering the change in growth rate on regular diet as 100% when SC was moved from selection to regular diet, growth rate increased by 107.35%. However, in a similar comparison, the growth rate increase in AR was 131.18%.

Fecundity and Fertility. Fertility and fecundity in AR on all types of diet could not be determined due to insufficient number of adults (result of high pupal mortality). In SC, the fecundity and fertility were not influenced by the initial seven days exposure to diet of different strengths (Table 4).

Fitness Values after Crossing AR with SC1. Larval mortality ($F_{1, 5} = 11.148$; $P = 0.029$), pupal weight ($F_{1, 151} = 15.426$; $P = 0.000$), and percentage of malformed adults ($F_{1, 5} = 53.646$; $P = 0.002$) differed significantly between AR1 and SC1 (Table 6). However no significant differences were observed in larval weight after 7 d ($F_{1, 173} = 1.599$; $P = 0.208$), and larval ($F_{1, 151} = 0.003$; $P = 0.957$) and pupal periods ($F_{1, 104} = 0.229$; $P = 0.633$).

Stability of Resistance. Stability of resistance were conducted after only one generation of rearing on regular diet due to extremely high (range, 40–80%) pupal mortality leading to the colony crashing. After removing AR from Cry1Ac selection (referred as AR-Unsel in Fig. 4) for one generation, mean ± SE percentage of survivors (10.2 ± 1.7) was reduced significantly ($t_1 = -7.78$, $P = 0.016$) compared with percentage of survivors (35.4 ± 1.54) when AR was under continuous selection (referred as AR-Sel in Fig. 4).

Discussion

In the current study, heritability (h^2) of resistance, stability of resistance, and fitness were assessed in a laboratory selected Cry1Ac-resistant strain of *H. zea* (AR). Heritability (h^2) values initially increased and then decreased over generations, indicating the increase in the genetic homogeneity of the population and hence, resistance factor. At $h^2 = 0.123$, AR could develop 10-fold resistance to Cry1Ac in 10 generations at 30% selection pressure, which is less than the number of generations predicted for tobacco budworm, *Heliothis virescens* (F.) (Tabashnik 1992); possible reasons for quicker resistance evolution are discussed in Anilkumar et al. (2008).

Fitness costs and the degree of dominance of fitness costs related to resistance determine the rate of resistance development (Carriére et al. 1994). In most studies, fitness costs were usually measured in the

Table 4. Fitness parameters (mean ± SE) for Cry1Ac-resistant (AR) and susceptible (SC) strains of *H. zea*[a]

Life-history trait	AR		SC	
	Regular diet	Selection diet[b]	Regular diet	Selection diet
Larval wt in 7D (mg)	109.48 ± 3.22b	91.04 ± 3.89a	144.58 ± 3.95c	131.99 ± 4.48c
Larval duration (d)	15.01 ± 0.09b	15.20 ± 0.11b	13.94 ± 0.11a	14.09 ± 0.13a
Larval mortality (%)	18.23 ± 2.46b	12.85 ± 2.11ab	5.04 ± 0.63a	10.07 ± 3.91ab
Pupal wt (mg)	347.70 ± 4.22a	355.41 ± 3.37ab	375.32 ± 3.34bc	368.4 ± 3.67bc
Pupal duration (d)	12.02 ± 0.12b	12.19 ± 0.12b	11.39 ± 0.1a	11.82 ± 0.1b
Malformed adults (%)	74.38 ± 13.46b	71.10 ± 9.49b	13.87 ± 1.88a	16.10 ± 6.12a
Number of eggs	NA[c]	NA	653.74 ± 51.58	583.26 ± 68.53
Hatching (%)	NA	NA	85.29 ± 2.61	86.60 ± 3.12

Lowercase letters after means within a row indicate significant differences at $P < 0.05$ level by Tukey's test.
[a] Results are from 160 larvae.
[b] Selection diet (regular diet + 20% water [used for the purpose of adding Bt proteins into regular diet in resistance selection experiments]).
[c] NA, not available, experiments were not continued due to higher percentage of malformed adults.

Table 5. Growth rate (mean ± SE) for Cry1Ac-resistant (AR) and susceptible (SC) strains of *H. zea* on different strengths of diet[a]

Strain	Diet[b]	Growth rate (mg/d)		
		During 7D	After 7D	Difference
SC	RD	21.09 ± 0.53b	32.93 ± 0.56c	11.83 ± 0.81a
	SD	20.39 ± 0.60b	33.09 ± 0.61c	12.70 ± 0.85a
AR	RD	17.12 ± 0.44a	28.89 ± 0.62a	11.77 ± 0.78a
	SD	15.82 ± 0.54a	31.07 ± 0.51b	15.44 ± 0.77b
F-test results		$F_{3,471} = 22.70$; $P = 0.000$	$F_{3,471} = 12.34$; $P = 0.000$	$F_{3,471} = 3.84$; $P = 0.010$

Lowercase letters after means within a column indicate significant differences at the $P < 0.05$ level by Fisher's least significant differences.
[a] Results from 160 larvae.
[b] RD, regular diet; SD, selection diet (RD + 20% water [used for the purpose of adding Bt proteins into regular diet]).

absence of the selection agent, presumably to approximate how long resistance would remain in the absence of field selection (Liu et al. 1999; Carriére et al. 2001a, 2001b; Bird and Akhurst 2004, 2005). This current study shows that under continuous selection AR had significantly higher pupal mortality, a male biased sex ratio, and decreased mating ability of moths compared with SC. Increased pupal mortality for *H. zea* was also reported when larvae originated from Bt corn, *Zea mays* L. (Storer et al. 2001) and Bt cotton (Jackson et al. 2004a) compared with their non-Bt counterparts. The sex ratio in SC (0.47 ± 0.01) was similar to five batches of larvae (0.48 ± 0.01) collected from non-Bt field corn (during 2006 and 2007) (K.J.A. and W.J.M., unpublished data). Furthermore, the sex ratio of AR (0.51 ± 0.01) was similar to 167 larvae collected from a Bt corn field in 2006 (0.51) that were shown to be highly resistant to Cry1Ac toxin in the F_1 generation (K.J.A. and W.J.M., unpublished data). Therefore, the male-biased sex ratio in AR may be a result of resistance selection, suggesting higher susceptibility of females (De Lame et al. 2001, Shearer and Usmani 2001).

The magnitude of mating costs is expected to be influenced by factors such as mating history, life span, current and past population sizes (bottlenecks and founder effects), environmental conditions, and possibly interactions between these factors (Bird and Akhurst 2004). SC is the parental population of AR (Anilkumar et al. 2008), both colonies were reared in parallel; genetic inbreeding independent from Cry1Ac selection seems an unlikely cause. Even under conditions where there were significantly fewer adult AR compared with SC in a particular generation, the reduction in mating success for AR may not be linked to genetic inbreeding; AR in two generations (August 2006 and April 2007) had fewer (61 [30♂:31♀] and 58 [32♂:26♀]) adults but had increased (47 and 25% increase over previous generations) mating success past this potential bottleneck. Percentage of mating for SC was similar to moths collected from light traps (Hendricks et al. 1970) and lower compared with collections made from sweep net and/or blacklight traps (Latheef et al. 1991). Furthermore, mating increased in AR1 F_1 adults, but it was still significantly different from SC1. Additionally, AR1 F_2 adults had reduced mating compared with their parents and the mating success was similar to AR before being crossed to SC1. Therefore, reduced mating in AR may be due to Cry1Ac resistance and not necessarily inbreeding. Furthermore, reciprocal crosses indicate significant

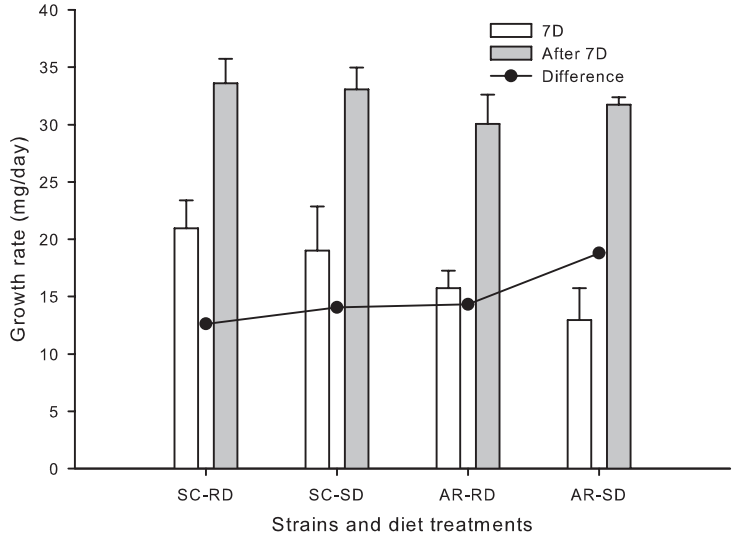

Fig. 3. Growth rate differences in AR and SC, when larvae were reared on regular diet after exposing to regular diet and selection diet (20% diluted regular diet) for initial 7 days.

Table 6. Fitness parameters (mean ± SE) for Cry1Ac-resistant (AR1) and susceptible (SC1) strains of *H. zea* after crossing AR with SC1[a]

Life-history trait	AR1	SC1
Larval wt in 7D (mg)	95.91 ± 6.38	88.99 ± 6.43
Larval duration (d)	15.14 ± 0.17	15.24 ± 0.18
Larval mortality (%)	22.70 ± 3.55b	9.69 ± 1.62a
Pupal wt (mg)	359.06 ± 7.12a	391.72 ± 5.11b
Pupal duration (d)	11.59 ± 0.20	11.72 ± 0.14
Malformed adults (%)	60.81 ± 6.49b	10.77 ± 2.14a

Lowercase letters after means within life history traits indicate significant differences at the $P < 0.05$ level by Tukey's test.

[a] Results are from 96 larvae.

mating costs in males as against females. Reduced mating, mainly because of mating problems in males was also observed in Bt-resistant (selected using Dipel 2X) *Plutella xylostella* (L.) moths (Groeters et al. 1993).

Fitness costs associated with resistance in AR have been demonstrated in many life history traits when reared on untreated diet. Insects adopt different feeding strategies depending on the nutritional quality of the diet or host plants (Woods 1999). Here, AR larvae exposed to selection diet had increased growth rate when shifted to regular diet, and with an additional day they achieved pupal weights similar to SC. The increase in growth rate suggests increased feeding and/or higher assimilation rate, both of which may be due to an increased titer of digestive enzymes (Woods 1999). Interestingly, AR produced a higher percentage of normal adults when exposed to toxin in selection experiments than when reared on untreated diet. This may be due to 1) in the absence of selection, average fitness of individuals may decline due to the accumulation of deleterious mutations (Lynch et al. 1999); 2) elimination of higher percentage of insects with lower fitness (W.J.M., unpublished data); 3) AR has been selected with Cry1Ac toxin for 26 generations on selection diet containing 20% more water and therefore 20% less nutrients; AR have adapted to these conditions, as would be expected for a highly polyphagous insect (Woods 1999); and 4) exposure to Cry1Ac toxin affects the physiology of the insects such that they obtain higher fitness values from the increased nutrition of Bt (Sayyed et al. 2003); or other factors. In the confused flour beetle, *Tribolium confusum* Jacquelin du Val reduced fitness was observed in a selection-free population compared with population with more intense selection (Lomnicki and Jasienski 2000).

AR required 27 d for adult eclosion on regular diet, compared with 25 d for SC. This resulted in developmental asynchrony (Liu et al. 1999, Bird and Akhurst 2004, 2005) as has been observed in other insects, and may lead to assortative mating (Liu et al. 1999) thereby accelerating the rate of resistance evolution. This should not be relative to *H. zea*, because peak mating occurs on the fourth night after emergence (K.J.A., unpublished data; Shorey et al. 1968). Assortative mating fitness differences will favor restoration of susceptibility in the absence of insecticide treatments (Groeters et al. 1993). Caprio (2001), using a spatially descriptive model, found that nonrandom mating along with nonrandom oviposition could significantly delay resistance evolution.

Long-term rearing of insects in the laboratory results in reduced fitness mainly because of the founder effect and/or inbreeding (Roush and Daly 1990). Therefore, AR was crossed to SC1 to ascertain whether observed reduction in fitness was linked to resistance (Bird and Akhurst 2004) and to save AR from extinction. Even after one generation of crossing with SC1, AR1 had increased fitness costs while feeding on Cry1Ac-treated and untreated diet. These observations strongly suggest that they may be linked to Cry1Ac-resistance as reported in Cry1Ac-resistant *H. armigera* after four crosses with a susceptible strain (Bird and Akhurst 2004). Although AR1 seems similar to AR in terms of survivorship at 500 μg Cry1Ac toxin/g diet, the RR for AR1 is lower than for AR because of increased tolerance of SC1 (LC_{50} = 31.25 μg/g

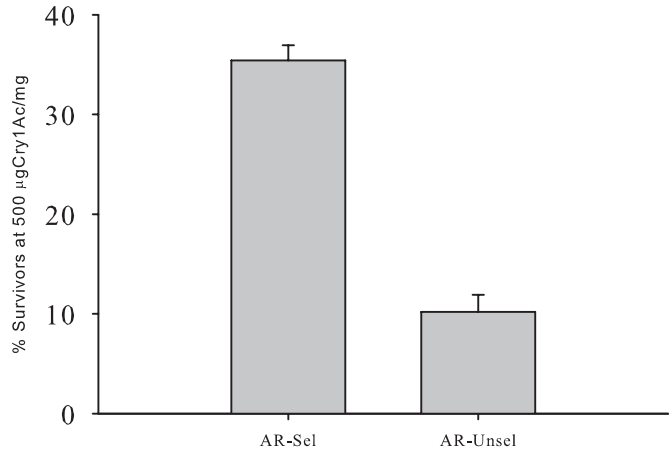

Fig. 4. Percentage of survivors of Cry1Ac-resistant *H. zea* at 500 μg/g Cry1Ac toxin when selected continuously at 500 μg/g Cry1Ac toxin (AR-Sel) compared with when removed from selection for one generation (AR-Unsel).

diet) compared with SC (LC_{50} = 9–15 μg/g diet, Anilkumar et al. 2008). This increased tolerance to Cry1Ac in SC1 may also come with fitness costs that were reflected in larval weight, larval and pupal period not differing between AR1 and SC1; these fitness costs may not be linked to Cry1Ac-resistance.

Resistance in AR was not stable; after one generation of rearing on regular diet AR lost a significant amount of resistance. Similar unstable resistance (from >500-fold to >74-fold) also was reported in Cry1C resistant *Spodoptera littoralis* (Boisduval) (Muller-Cohn et al. 1996). However, Bt resistance was stable in *Spodoptera exigua* (Hübner) (Moar et al. 1995) and *P. interpunctella* (343-R) (McGaughey and Beeman 1988). Both stable and unstable Bt resistance was observed in *P. xylostella* (Ferre and Van Rie 2002). Unstable Cry1Ac resistance in AR may help in understanding observed reductions in the LC_{50} values of field-collected populations, which had elevated LC_{50} values in F_1, but declined rapidly during laboratory colonization (R. Luttrell, personal communication). The reduction in resistance may be linked to fitness costs and/or accumulation of deleterious mutations (Lynch et al. 1999).

Contrary to the initial expectations of rapid evolution of *H. zea* resistance to Bt cotton (Harris 1991, Roush 1997), there are no reports of field control failure(s) after more than a decade of Bollgard and Bt corn use (Ali et al. 2006, Moar and Anilkumar 2007). This lack of observed field-evolved resistance occurred despite widespread use of Bollgard and Bt corn during this period. There are several mitigating factors that might have contributed to the delay of this pest developing resistance to Bollgard: 1) the "high dose plus structured refuge," 2) use of pyrethroid insecticide(s) to control bollworms during high infestations Bt cotton (Anilkumar et al. 2008), 3) substantial temporal and spatial bollworm production from noncotton crop hosts (Gustafson et al. 2006), and 4) fitness costs associated with elevated Cry1Ac resistance or tolerance as shown in these studies and others. The latter has likely played the most important role in delaying resistance development in bollworms. Indeed, Gustafson et al. (2006) incorporated assumed values (none, low, and moderate) of fitness costs associated with either recessive or additive inheritance for resistance in modeling the effect of non-Bt crops as effective refuges for insect resistance management. For the Mississippi region, this model predicted a delay in resistance for 6–10, 7–14, and >30 yr with none, low, and moderate fitness costs, respectively. We believe that this model has been validated and may indicate even greater delays in resistance development if results from this study (moderate to high fitness costs) are incorporated in their model, assuming laboratory generated results are applicable to the field. Recently, Tabashnik et al. (2008) reported field-evolved Cry1Ac-resistance in *H. zea* based on laboratory assays of different strains collected from the field before (Luttrell et al. 1999) and after (Ali et al. 2006) commercial cultivation of Bt cotton. However, the conclusions of Tabashnik et al. (2008) are directly contradicted by the lack of observed changes in Bt cotton efficacy against *H. zea* and the lack of confirmed Bt resistant *H. zea* populations in the Environmental Protection Agency-mandated Bt resistance monitoring program. We believe that the data presented in this present manuscript help to explain why field-evolved resistance has not yet occurred in this pest.

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