2003 BELTWIDE COTTON CONFERENCES, NASHVILLE, TN – JANUARY 6-10

B. T. RESISTANCE EVOLUTION IN THE *H. ZEA* POPULATION IN EASTERN NORTH CAROLINA

R.E. Jackson, J.R. Bradley, Jr., F. Gould, J.W. Van Duyn, and A.D. Burd
North Carolina State University
Raleigh, NC

Abstract

Adult female bollworm moths were collected from various light trap locations in eastern North Carolina from August-October during 2000-2002. Female moths were allowed to oviposit, and upon hatch, 24 neonates from each female (F_1 families) were screened for growth rate on each of three diets: non-*B. t.*, Cry1Ac, and Cry2Ab; *B. t.* diets contained 5.0 µg toxin per milliliter of diet. One individual out of 1834 screened was found that appeared to carry a major dominant gene for resistance to Cry1Ac. Assuming four genomes per individual, then the gene frequency is 1/7336 or 0.000136. Another individual was also found out of 1897 screened that appeared to carry a major gene for resistance to Cry2A. The gene frequency for Cry2A resistance would be estimated at 1/7588 or 0.000132. Adults from F_1 families with growth scores in the highest (R) and lowest (S) quartiles were mated in 4 combinations, RxR, SxR, RxS, and SxS. Differences in growth rates of larvae from these crosses demonstrated that there is substantial quantitative genetic variation for resistance to both CryIAc and Cry2Ab. Along with results suggesting partially dominant inheritance of resistance to Cry1Ac and Cry2A, these estimates become critically important when determining appropriate resistance management strategies for the sustainability of transgenic cottons.

Introduction

Bollgard cottons have been widely planted in recent years, comprising approximately 70% of the cotton acreage in North Carolina over the last few years (Bacheler per. comm.). Although these transgenic cottons provide absolute control of the tobacco budworm, *Heliothis virescens* (F.), recent laboratory and field studies have demonstrated that Bollgard cottons do not adequately control bollworm and that *B. t.* resistance genes can be found in North Carolina bollworm populations (Burd et al. 1999, 2001; Jackson et al. 2000, 2001; Lambert et al. 1996, 1997; Mahaffey et al. 1994, 1995). Due to these findings, resistance management issues associated with the deployment and sustainability of *B. t.* cottons have become a concern (Tabashnik 1994).

In order for resistance evolution to be controlled, *B. t.* cottons must express a high dose of toxin, inheritance of resistance must be incompletely or completely recessive, and frequency of resistance genes in the population must be low (Union of Concerned Scientists 1998); however, recent studies have suggested that none of these assumptions are true for bollworm (Burd et al. 1999). Current resistance management strategies and modeling efforts are dependent upon these assumptions; therefore, these unknowns must be resolved in order for appropriate resistance management strategies to be implemented.

The objectives of the study were 1) to estimate the frequency of major resistance genes for Cry1Ac and Cry2A in the general North Carolina bollworm population, 2) to determine if there has been a substantial shift in the frequency of resistance genes in the bollworm population from 2000-2002, and 3) to determine if individuals that responded moderately well to discriminating doses of Cry1Ac and Cry2Ab toxins carried minor resistance genes that could decrease the efficacy of Bollgard cottons.

Materials and Methods

From August-October during 2000-2002, adult female bollworm moths were collected from light traps at various locations in eastern North Carolina. A total of 583, 561, and 690 female moths (lines) were collected in 2000, 2001, and 2002, respectively, and were used for the bioassays. Adult females were placed individually into 10-oz. clear plastic cups that were covered with cheesecloth to provide a substrate for oviposition. Moths were retained in rearing facilities at North Carolina State University at 27-30°C, 55-60% relative humidity, and a 14:10 light:dark photoperiod. Cheesecloths were checked daily for the presence of eggs.

Upon hatch, 24 neonates from each female line were placed onto each of three diets: non-*B. t.* (NBT), Cry1Ac-containing, and Cry2A-containing diets. Both *B. t.* diets contained 5.0 µg toxin per milliliter of diet. Cry1Ac was obtained from Mycogen, Corp. in a formulation of MVP®. Cry2Aa was purified from the NRD-12 isolate of *Bacillus thuringiensis* ssp. *kurstaki* and was expressed in a recombinant *Escherichia coli* strain (Moar et al. 1994) and was used in the 2000 study. Cry2Ab was acquired by producing lyophilized corn tissue containing the Cry2Ab toxin and grinding the tissue into powder; Monsanto Agric. Co. determined the concentration of Cry2Ab within the corn powder. Cry2Ab was utilized in the studies in 2001-2002. Diet was poured into 24-well plastic bioassay plates and refrigerated before use. A single neonate was placed into each well using a fine camel hair paintbrush. Mylar film was heat-sealed onto bioassay plates to prevent escape of larvae. Two holes were punched through the film over each well with No. 2 insect pins to allow air exchange. All plates were observed after

seven days when larvae were scored based on instar size. Instar size was determined based on head capsule and body size (Neunzig 1969). Instar sizes were converted to an ordinal ranking system as shown in Table 1. Data were analyzed using PROC UNIVARIATE (SAS Institute 1990).

In 2000, survivors from the original bioassays were used for subsequent selection experiments. Any lines that performed just as well on either *B. t.* diet as they did on NBT diet were saved and screened again. These lines were mass mated among themselves and selected on the appropriate *B. t.* diet the next generation. Several lines that had no survivors on either *B. t.* diet but performed well on NBT diet were saved as well. These were used as control lines for subsequent experiments. Reciprocal crosses were made between these control lines and the survivors from selected lines to determine possible inheritance of resistance. Larvae from these crosses and also from the selected and control lines were placed on the appropriate *B. t.* diet and weighed after 10 days.

For those lines whose performance ranked in the upper or lower quartile for either of the *B. t.* diets in 2001, larvae from the non-*B. t.* diet were reared to the adult stage. Reciprocal crosses were made between adults from lines that ranked in the highest (R) and lowest (S) quartiles for each diet. RxR and SxS crosses were also conducted. Neonates from successful crosses were placed on the appropriate *B. t.* diet and weighed after 10 days.

Results

Distribution of average ratings for each female line on NBT diet during each year of the study is shown in Figure 1. The average ratings were approximately 9.0, 9.4, and 6.7 for 2000, 2001, and 2002, respectively. These ratings corresponded to a mid 4th instar in 2000, a mid 4^{th} to late 4^{th} instar in 2001, and a late 3rd instar in 2002. The 2002 distribution of families on NBT diet was shifted to the left as compared to 2000 and 2001 distributions. This may have been due to a change in the corn-soy blend used in preparing the artificial diet or to a general lower vigor of the moths in 2002. The age class distributions for 2002 larvae on Cry1Ac and Cry2Ab diets were also shifted in a similar manner. Therefore, relationships of performance distributions on *B. t.* diets to those on NBT diet should be similar among years.

The distribution of average ratings for female lines on Cry1Ac-containing diet during each year of the study is shown in Figure 2. The average ratings for all larvae on Cry1Ac-containing diet were 4.0, 4.4, and 2.6 for 2000, 2001, and 2002, respectively. Ratings corresponded to a late 2nd instar in 2000, a late 2^{nd} to early 3^{rd} instar in 2001, and an early 2nd to mid 2nd instar in 2002. Relationships of distributions on Cry1Ac-containing diet to that of the NBT diet were similar across years; the average performance ratings of families on Cry1Ac-containing diet were 44, 47, and 39% of the average ratings of families on NBT diet in 2000, 2001, and 2002, respectively. Bioassays on Cry1Ac in 2000 revealed one family with most individuals as large as control larvae. The average rating of this family was 7.4 which falls between a late 3rd and early 4th instar. Ten out of twenty-four larvae survived on the *B. t.* diet with survivors ranging from late 3rd to early 4th instar. The controls (NBT) for this family ranged from late 3rd to mid 4th instar with a mean rating of 8.25.

Figure 3 illustrates the distribution of average ratings for female lines on Cry2A-containing diet during each year of the study. The average ratings for all larvae on Cry2A-containing diet were 3.0, 4.9, and 2.6 for 2000, 2001, and 2002, respectively. These ratings corresponded to a mid 2nd instar in 2000, an early 3^{rd} instar in 2001, and an early 2nd to mid 2nd instar in 2002. As with Cry1Ac, relationships of distributions on Cry2A-containing diet to that of the NBT diet were similar across years; average performance ratings of families on Cry2A-containing diet were 33, 52, and 39% of the average ratings of families on NBT diet in 2000, 2001, and 2002, respectively. In 2000, bioassays from Cry2Aa lines also revealed one family with most individuals as large as controls. The average rating for this family was 6.5 which is between a mid 3rd and late 3rd instar. Nineteen out of twenty-four larvae from this family survived on the Cry2Aa diet with growth stages ranging from early 2nd to mid 4th instars. The controls (NBT) for this family ranged from late 3rd to mid 4th instar with a mean rating of 8.55.

Figure 4 illustrates weights from the selected family that performed best on Cry1Ac in the original 2000 bioassay. These weights were taken from the second generation of larvae (the original field collected females would be their grandmothers). Figure 4 also shows weights for second-generation control larvae (individuals from a particular family line that died on both toxin diets but not on NBT diet). Weights of reciprocal crosses between this selected line and the control line are also shown. As seen in figure 4, the average larval weight for the selected line was 28 milligrams. The average weights for the selected female x control male and the control female x selected male crosses were 16 mg and 14 mg, respectively. All control larvae once again died on the 5.0 µg/ml Cry1Ac diet.

In 2001, seven and eight successful crosses were obtained for Cry1Ac and Cry2Ab, respectively, for use in the reciprocal cross study. Successful crosses for Cry1Ac screening were as follows: 2 resistant crosses (RxR), 1 resistant female x control male cross (RxS), 2 control female x resistant male crosses (SxR), and 2 control crosses (SxS). Cry2Ab crosses that were successful were: 2 resistant crosses, 2 resistant female x control male crosses, 3 control female x resistant male crosses, and 1 control cross.

Since no major *B. t.* resistance genes were detected in 2001, tests were conducted to determine if there was genetic variation among families whose average ratings were in the upper and lower quartiles. To determine if single female lines with growth in the highest 25% of all 561 single female lines on Cry1Ac were genetically different from lines with growth in the lowest 25%, lines within these ranges were used (that portion of the line on NBT diet) to perform reciprocal crosses. The upper quartile rating for growth on Cry1Ac-containing diet was 5.0 (early 3rd instar), while the lower quartile rating was 4.0 (late 2nd instar). Mean ratings for single female lines used to perform reciprocal crosses were 5.4 for upper quartile lines and undefined for lower quartile lines since there were no survivors on Cry1Ac-containing diet.

As with Cry1Ac, no major *B. t.* resistance genes were detected in 2001, so experiments were conducted to determine whether genetic variation existed between upper and lower quartile families. As with those single female lines on Cry1Ac, upper and lower quartile lines on Cry2Ab-containing diet were saved to perform reciprocal crosses. The upper quartile growth rating for lines on Cry2Ab was 6.0 compared to 4.0 for lower quartile lines. Mean growth ratings for lines within these ranges used for reciprocal crosses were 6.0 for the upper quartile lines and 2.9 for the lower quartile lines.

Average weights of larvae from RxR, RxS, SxR, and SxS crosses after rearing on Cry1Ac-containing diet for 10 days are shown in Figure 5. The average (SE) larval weight for the RxR lines was 19.4 (0.99) mg compared to 6.3 (0.86) mg for the SxS lines. Average larval weights for the R female x S male and S female x R male crosses were 15.2 (1.51) and 12.7 (0.97) mg, respectively. Average larval weights from crosses of RxR, RxS, SxR, and SxS crosses after rearing on Cry2Ab-containing diet are shown in Figure 6. The average (SE) larval weight for the RxR, RxS, SxR, and SxS crosses were 14.6 (1.38), 14.6 (1.07), 13.9 (0.78), and 7.95 (1.19) mg, respectively.

No major resistance genes to Cry1Ac or Cry2A were detected in the 2002 study, and reciprocal cross experiments were not conducted among survivors from families due to minimal variation in average ratings among families.

Discussion

Based on our lab studies from 1999 (Burd et al. 2000), we concluded that inheritance for resistance to Cry1Ac toxin was dominant or incompletely dominant. Therefore, any individuals carrying genes for resistance were likely to survive when screened on a discriminating dose of this toxin in artificial diet. This type of inheritance enables heterozygotes to survive when selected on *B.t.* diet. Statistically, the most probable carrier for this resistance gene would be a heterozygote in current field populations. Also the most probable mating would occur between heterozygote individuals and homozygote susceptible individuals. With this in mind, the likely offspring form this cross would be ½ heterozygote and ½ homozygote susceptible. From this, we assumed that if a screened line had 50% of the individuals that were the same size as their NBT counterparts then this would be considered a resistant line.

Screening 1834 total females on Cry1Ac diet allowed us to characterize 7336 genomes because each mated female carries 2 of her own alleles and 2 from her male counterpart. With this in mind, our estimated gene frequency for resistance to Cry1Ac toxin would be 1/7336 or 0.000136. It should be noted that this estimate is conservative. As seen in figure 1, many female lines had higher than average growth on Cry1Ac diet (i.e. the entire family reached 3rd instar but was not quite as large as the controls). This is likely due to the presence of minor genes for Cry1Ac resistance as indicated by the 2001 reciprocal cross results. In either case this would serve to increase our estimate of the initial gene frequency for Cry1Ac resistance. The one family which survived on the Cry1Ac had a total of 10 out of 24 individuals survive. This is consistent with the 50% that we expected. Also, all survivors were consistent in size with their NBT counterparts.

Since 1897 female lines were screened on Cry2A diet, then the number of genomes we actually tested was 7588. Therefore, our estimated gene frequency for resistance to Cry2Aa toxin would be 1/7588 or 0.000132. Again, this is a conservative estimate based on the fact that other female lines performed above average and were likely carrying minor resistance genes. This would also increase our estimate of initial gene frequency for Cry2A resistance. The one family that survived from the Cry2A bioassays had 19 out of 24 survivors. This is greater than the 50% that we expected; however, there were 6 of these individuals that were mid 2nd instar and below. Therefore, only 13 out of 24 individuals were as large as the control counterparts.

Because Bollgard cottons do not produce a "high dose" for bollworm, larvae with minor resistance genes would be selected for and could, over time, decrease the efficacy of Bollgard cottons. The 2001 study demonstrated that single female lines with growth in the highest 25% of all 561 single female lines on *B. t.* toxins were genetically different from lines whose growth was in the lowest 25%. The fact that larvae from the S female X R male crosses performed better than the larvae of the SxS crosses proves that the difference was not due to a maternal effect. Given that there is heritable variation for minor genes, monitoring programs for bollworm and other pests exposed to moderate doses of *B. t.* toxins should not only search for major genes.

Results from selection experiments and reciprocal cross experiments reported in this paper are consistent with those reported by Burd et al. (2000). These studies indicated that resistance to Cry1Ac and Cry2A is inherited as a dominant or incom-

pletely dominant trait. Comparisons of frequency distributions of average ratings of families on each *B. t.* toxin and the relationships of distributions on *B. t.* diet and NBT diet among the three years of the study demonstrate that there has been no substantial change in the shape of the growth distributions for single female lines on either Cry1Ac or Cry2A. This indicates that if there has been an increase in the frequency of major or minor resistance genes it has been too small to detect, even with data on over 500 females in each season. Along with the results from our current gene frequency estimates, this may allow us to quantify certain parameters that are typically assumed when modeling evolution of *B. t.* resistance in field populations of bollworms. If the initial gene frequencies for resistance are indeed this high coupled with partially dominant inheritance, then properly structured refuge systems become critically important for transgenic *B. t.* cotton technology to be sustainable.

Acknowledgments

The authors express appreciation to Cotton, Inc. for providing a graduate research assistantship for the senior author and to Monsanto Agric. Co. for providing partial project funding. Special thanks to Rogan Jackson, Margery Ambrose, Ryan Kurtz, and Carlton Purvis for technical assistance.

Literature Cited

Bacheler, J. S. 2002. Personal communication.

Burd, A. D., J. R. Bradley, Jr., and J. W. Van Duyn. 1999. Performance of selected *Bt* cotton genotypes against bollworm in North Carolina. Proc. Beltwide Cotton Conf. 931-934.

Jackson, R. E., J. R. Bradley, Jr., A. D. Burd, and J. W. Van Duyn. 2000. Field and greenhouse performance of bollworm on Bollgard II cotton genotypes. Proc. Beltwide Cotton Conf. 1048-1052.

Jackson, R. E., J. R. Bradley, Jr., J. W. Van Duyn, and A. D. Burd. 2001. Efficacy of Bollgard and Bollgard II cottons against bollworm, *Helicoverpa zea* (Boddie), in field and greenhouse studies. Proc. Beltwide Cotton Conf. 815-818.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1996. Effects of natural enemy conservation and planting date on the susceptibility of *Bt* cotton to *Helicoverpa zea* in North Carolina. Proc. Beltwide Cotton Conf. 931-935.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1997. Interactions of *Helicoverpa zea* and *Bt* cotton in North Carolina. Proc. Beltwide Cotton Conf. 870-873.

Mahaffey, J. S., J. S. Bacheler, J. R. Bradley, Jr., and J. W. Van Duyn. 1994. Performance of Monsanto's transgenic *B.t.* cotton against high populations of lepidopterous pests in North Carolina. Proc. Beltwide Cotton Conf. 1061-1063.

Mahaffey, J. S., J. R. Bradley, Jr., and J. W. Van Duyn. 1995. *B. t.* cotton: field performance in North Carolina under conditions of unusually high bollworm populations. Proc. Beltwide Cotton Conf. 795-798.

Neunzig, H. H. 1969. The biology of the tobacco budworm and the corn earworm in North Carolina with particular reference to tobacco as a host. North Carolina Agricultural Experiment Station. Tech. Bul. No. 196. 63 pp.

SAS Institute. 1990. SAS/STAT User's Guide, Vol. 2. SAS Institute, Cary, NC, 795 pp.

Tabashnik, B. E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Ann. Rev. Entomol. 39: 47-79.

Union of Concerned Scientists. 1998. Now or never: serious new plans to save a natural pest control. M. Mellon and J. Rissler, eds. pp. 149.

Table 1. Rating scale used to convert Helicoverpa zea instar size to appropriate ordinal ranking after seven days on artificial diet.

Larval Size	Ordinal Ranking
1st instar	1
Early 2nd instar	2
Mid 2nd instar	3
Late 2nd instar	4
Early 3rd instar	5
Mid 3rd instar	6
Late 3rd instar	7
Early 4th instar	8
Mid 4th instar	9
Late 4th instar	10
Early 5th instar	11

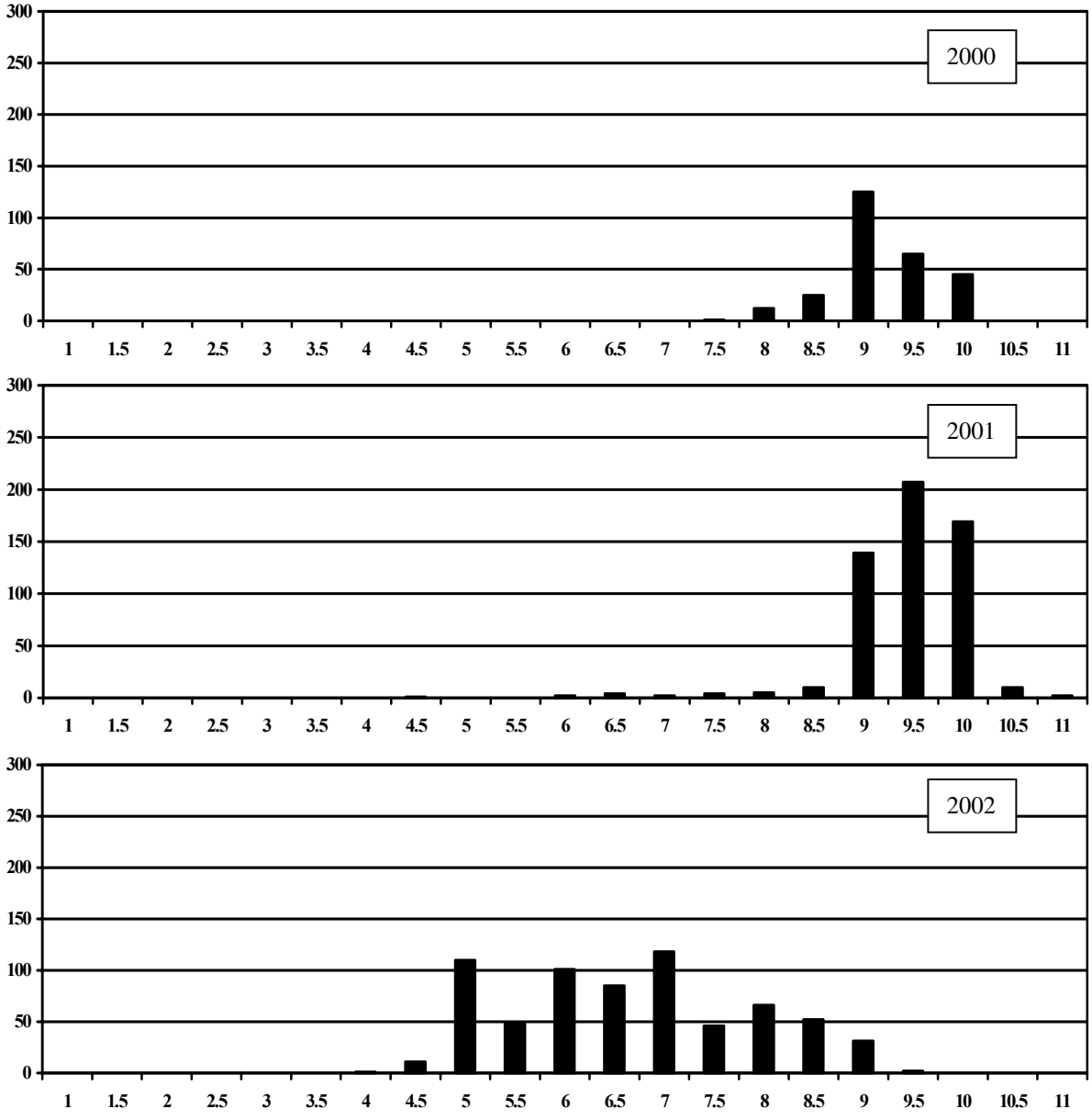

Figure 1. Distributions of average ratings for *Helicoverpa zea* female lines on NBT diet during August-October in 2000-2002.

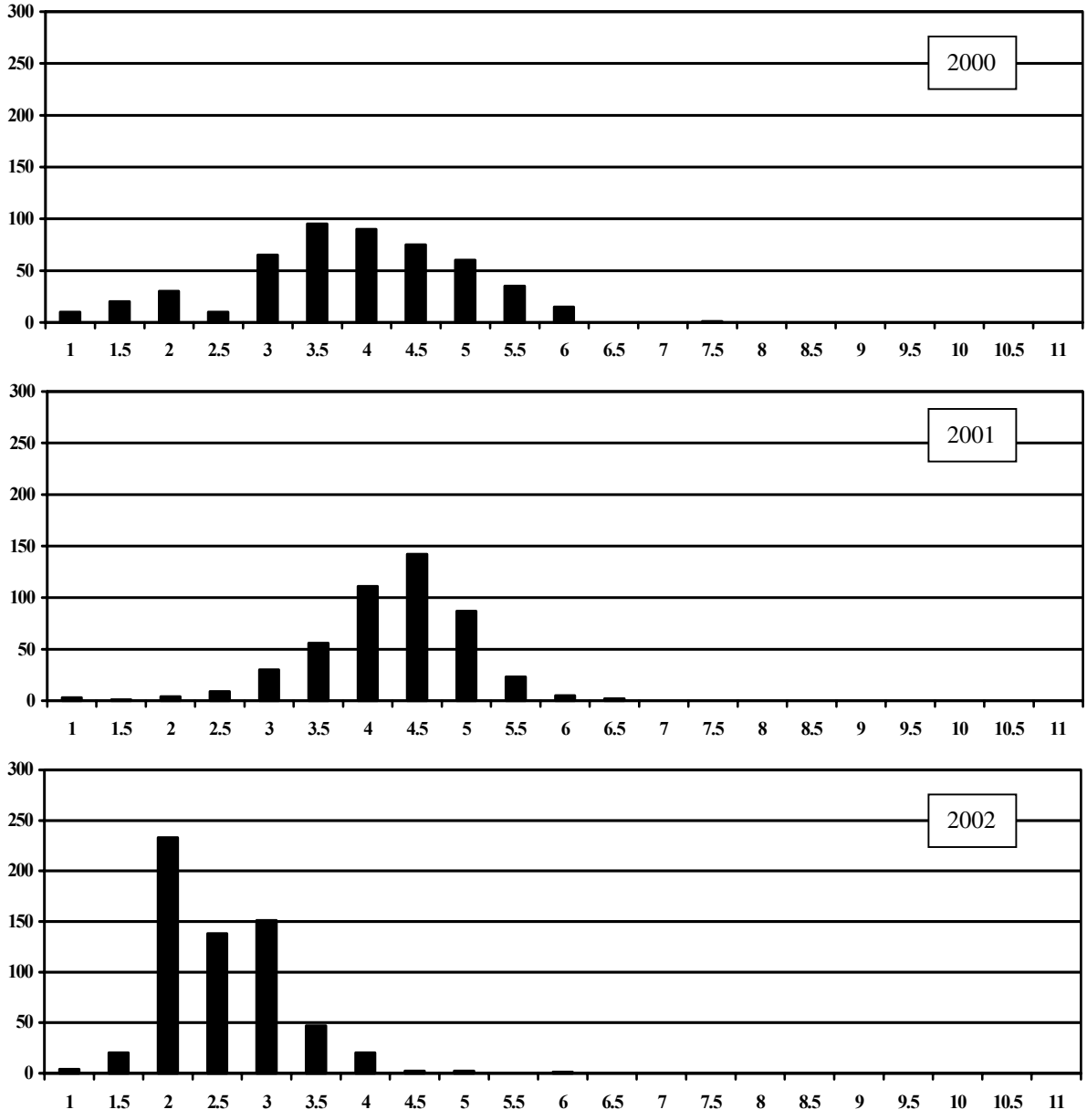

Figure 2. Distributions of average ratings for *Helicoverpa zea* female lines on Cry1Ac-containing diet during August-October in 2000-2002.

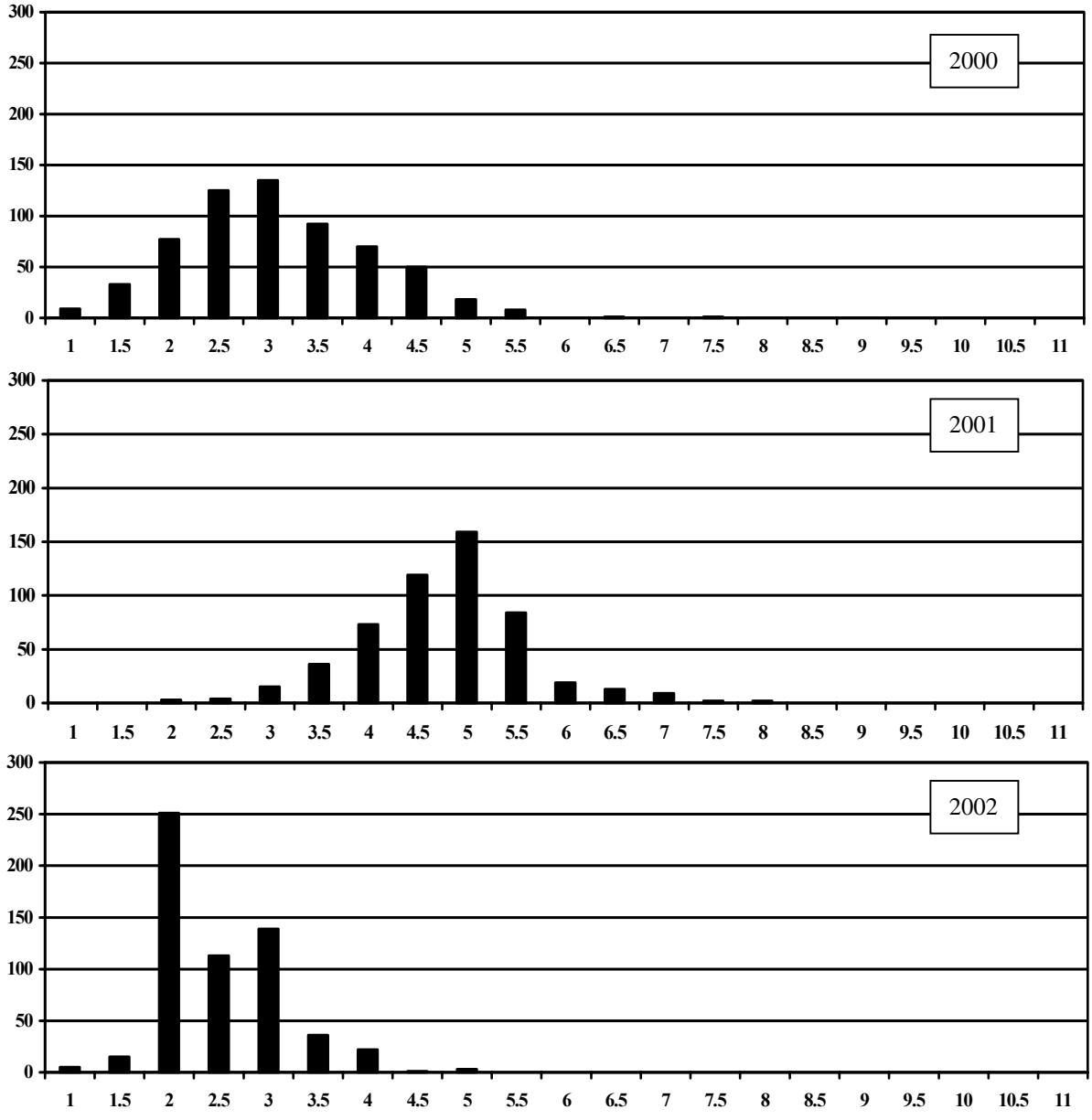

Figure 3. Distributions of average ratings for *Helicoverpa zea* female lines on Cry2A-containing diet during August-October in 2000-2002.

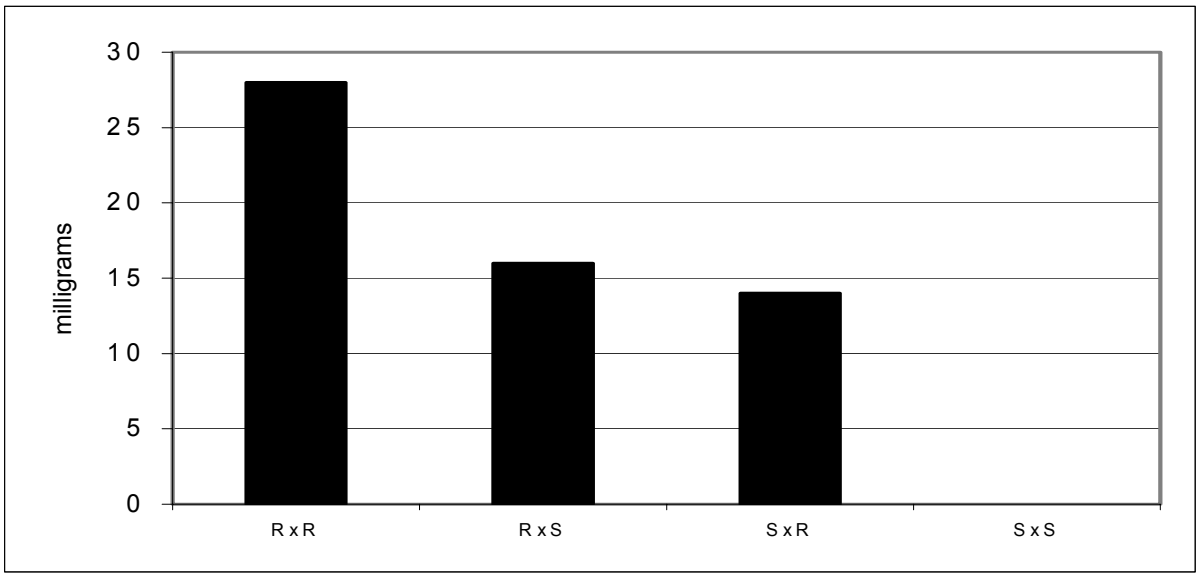

Figure 4. Weight in grams for *Helicoverpa zea* resistant line, control line and reciprocal crosses between these two lines taken after ten days on 5.0 *ug*/ml of CryIA(c) diet.

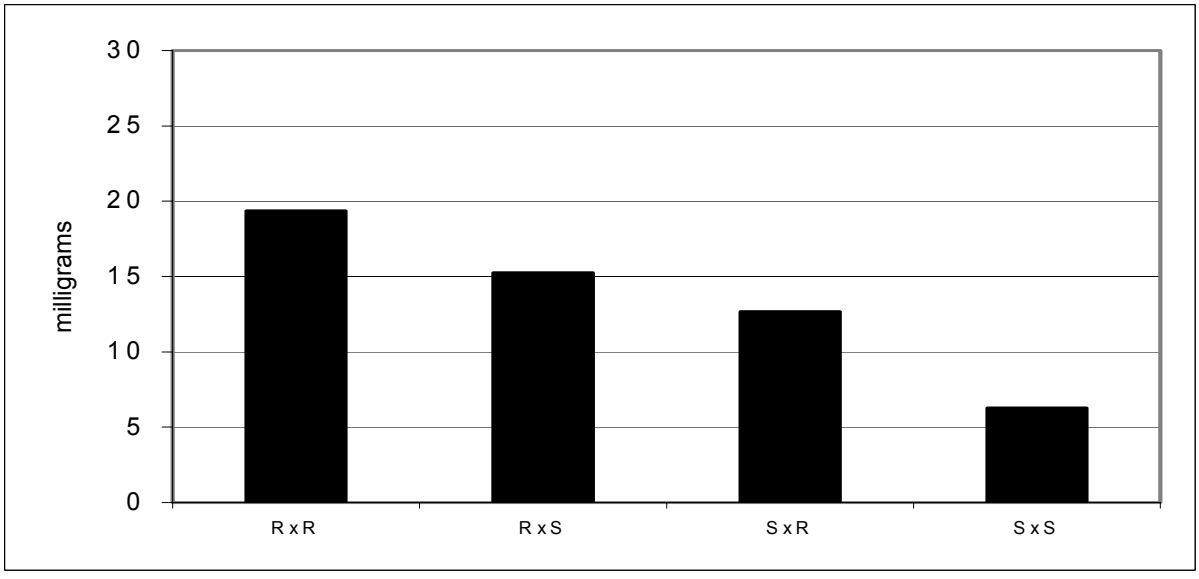

Figure 5. Mean larval weight in milligrams for *Helicoverpa zea* resistant line (RxR), control line (SxS), and reciprocal crosses between these lines (RxS and SxR) taken after ten days on 5.0 µg/ml of Cry1Ac-containing diet.

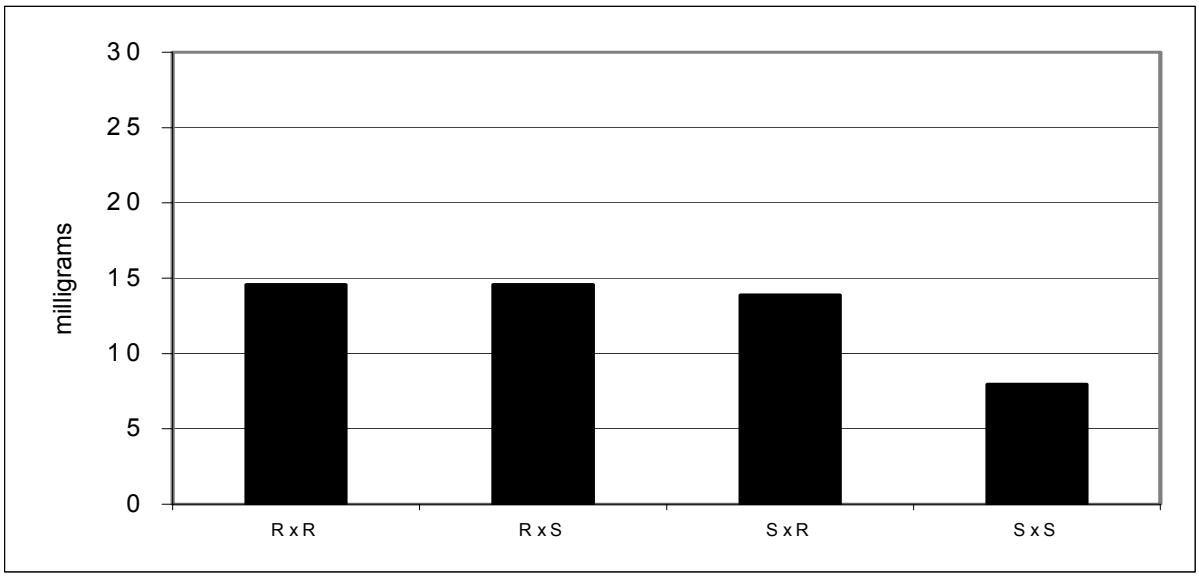

Figure 6. Mean larval weight in milligrams for *Helicoverpa zea* resistant line (RxR), control line (SxS), and reciprocal crosses between these lines (RxS and SxR) taken after ten days on 5.0 µg/ml of Cry2Ab-containing diet.