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Effect of *Cry*IC toxin from *Bacillus thuringiensis* on larval feeding behavior of *Spodoptera exigua*

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Abstract

The lack of data on the effect of *Bacillus thuringiensis* (B.t.) toxins on larval feeding behavior of the pest *Spodoptera exigua* (Hübner) (Noctuidae: Amphypyrini) prompted us to investigate the effect of three delivery systems of *Cry*IC, a commercial formulation, inclusion bodies, and the activated *Cry*IC toxin. The commercial formulation was the least and *Cry*IC toxin the most lethal form to neonates of susceptible colonies. All but two of the treatments in choice tests with neonates and third instars showed significant avoidance of B.t. treated diet, with greater proportion of larvae from susceptible (UCR-S and AUBURN-S) and resistant (AUBURN-R) colonies on untreated diet than on diet treated with any of the *Cry*IC forms and concentrations tested. Furthermore, third instars consumed significantly more control than treated diet for all *Cry*IC forms, colonies and concentrations. The avoidance of *Cry*IC toxin by neonates and third instars strongly suggests that *Cry*IC, which also is present in the commercial formulation and in the inclusion bodies, is responsible for eliciting avoidance behavior by *S. exigua* larvae. Behavioral observations of third instars in a no-choice test on either treated or control diet indicated that questing behavior in susceptible larvae appears to be positively related with presence of *Cry*IC toxin in the diet. Furthermore, resistant third instars were on the whole more active than susceptible thirds on both treated and control diet. Resistant thirds raised on *Cry*IC treated diet (AUBURN-RC) spent more time eating treated diet than resistant larvae raised on control diet (AUBURN-R), suggesting that diet conditioning plays an important role on feeding behavior of *S. exigua*. The implications of these results are discussed.

Introduction

Increasing environmental awareness, legislative and regulatory actions for the limitation of chemical pesticide use, and occurrence of pesticide resistance are promoting the development of alternative pest control strategies such as use of microbial insecticides (Trumble, 1990). Feitelson et al. (1992) reported that *Bacillus thuringiensis* (B.t.) products account for 90–95 percent of the total biopesticide market. The increasing importance of B.t. as a biopesticide has sparked research on new B.t. proteins, modes of action, genetics, application, and toxicity spectrum (Entwistle et al., 1993). Reports of resistant insect populations in the field during the late 1980s stimulated research on physiological and biochemical resistance mechanisms to B.t. (Van Rie et al., 1990; Marrone & McIntosh, 1993; Tabashnik, 1994). An important difference between B.t. and most synthetic pesticides is that B.t. must be ingested, and studies indicate that B.t. toxins influence larval feeding (Herbert & Harper, 1987; Gharib & Wyman, 1991; Gould et al., 1991); therefore, changes in feeding behaviors may play an important role in development of resistance to B.t.

Nevertheless, there has been limited research on the effect of entomophathogens on larval behavior (Gould, 1988). Furthermore, this limited literature contains conflicting results. Therefore, developing an understanding of how toxins influence behavior is needed for managing potential resistance deveopment. This need has been exacerbated by the refinement of gene transfer techniques during the late 1980s that increased the

potential for the development of new B.t. delivery systems and the engineering of new plants and compounds with one or more complementary B.t. proteins. Hence, research on the effect of B.t. on larval behavior is needed for both a basic understanding of insect-pathogen interactions and for practical reasons involving the efficient and continued use of pathogens as biopesticides (Gould, 1988).

Spodoptera exigua (Hübner) (Lepidoptera: Noctuidae), a major pest of vegetable crops around the world, is a suitable candidate for studying such insect-pathogen interactions. This insect has been examined for toxicity of selected B.t. toxins (Moar et al., 1986; Moar & Trumble, 1987), including the highly effective *Cry*IC toxin (Visser et al., 1988; Masson et al., 1992; Moar & Breed, 1994; Moar et al., 1995). In addition, *S. exigua* has been targeted for suppression by B.t. in several IPM programs (Trumble, 1990; Trumble et al., 1994); suggesting that these data would have practical application in commercial agriculture. Considerable information is also available on interactions between the plant hosts, B.t., and *S. exigua* (Trumble et al., 1991; Meade & Hare, 1994). Finally, several behavioral responses to food sources have been described for this insect (Berdegué & Trumble, 1996). Briefly, these behaviors are: eating, defined as chewing with the mouthparts in contact with the diet surface; questing, when larvae walk or lift their thoraxes, and move from side to side in a searching motion; swallowing, occurs after eating when larvae repeatedly contract esophageal muscles and move their mandibles as if chewing, but mandibles are not in contact with the diet surface; and lastly, resting, when no apparent activity is observed.

However, no data are currently available concerning the effect of B.t. on behavior of *S. exigua* larvae. Therefore the objectives of our study were threefold: firstly, to determine if *S. exigua* larvae detected and avoided *Cry*IC toxin present in three main delivery systems for B.t. toxins, these included commercially formulated material, *Cry*IC inclusion bodies (protoxin), and the activated *Cry*IC toxin that might be encountered by the larvae while foraging. Secondly, if avoidance of *Cry*IC occurred, we were interested in documenting the effect of *Cry*IC toxin on feeding behavior of susceptible and resistant *S. exigua* larvae. Particularly, if feeding by the larvae was negatively affected by the toxin, we expected a reduction in number of occurrences and time spent eating and swallowing as well as an increase in number of occurrences and time spent questing and resting. Thirdly, we intended to document the effect of diet conditioning on feeding behavior by *S. exigua* larvae.

Materials and methods

Insects. Unless otherwise specified, all tests were conducted in incubators at 28 ± 2 °C and L14:D10 photoperiod. Two susceptible (UCR-S and AUBURN-S) and two *Cry*IC resistant (AUBURN-R and AUBURN-RC) colonies were used. Insects from the UCR-S colony were maintained on artificial diet (Patana, 1969) inside an incubator. The colony was originally collected from Orange Co., CA, and had new genetic material added from the same area 6 months prior to the study. Insects for the AUBURN-S, AUBURN-R and AUBURN-RC colonies were obtained from a laboratory colony maintained by WJM at Auburn University. These colonies were established with insects collected from cotton in 1991. The colonies were reared on artificial diet (Chaflant, 1975) at 28 ± 1 °C with L16:D8 photoperiod. The resistant colony (AUBURN-R) was reared on a 320 μg *Cry*IC toxin/g artificial diet (Chaflant, 1975) for >25 generations prior to the experiment. Although we were not able to perform LC_{50} estimates for AUBURN-R larvae, Moar et al. (1995) reported that only 8% mortality was observed with this colony at 320 μg/g while the LC_{50} for AUBURN-S was 4.3 μg *Cry*IC toxin/g diet. Therefore, AUBURN-R larvae typically exhibited at least 100-fold resistance compared to the AUBURN-S colony. Third instars from the AUBURN-R colony were raised from eggs on control diet. Therefore, to discern if larval behavior could be conditioned by the type of diet larvae were raised on, a second resistant colony (AUBURN-RC) was obtained by rearing AUBURN-R eggs to third instars on *Cry*IC treated diet (320 μg/g).

Toxins. A *Cry*IC commercial formulation (XenTari), *Cry*IC inclusion bodies, and the trypsin digested *Cry*IC toxin from B.t. subsp. *entomocidus* were examined for their effect on larval behavior. XenTari, a B.t. subsp. *aizawai*-based product containing *Cry*IA(a), *Cry*IA(b), *Cry*IC, *Cry*ID and *Cry*IIB (Abbott Laboratories, 1992. B.t. Products manual, Abbott Laboratories, North Chicago, IL), is over seven-fold more toxic against *S. exigua* than *B. thuringiensis* subsp. *Kurstaki*-type products (e.g. Dipel 2X) (Moar & Breed, 1994). *Cry*IC inclusion bodies (containing the 130kD protoxin) and trypsinized *Cry*IC toxin (subsequently referred to as *Cry*IC toxin) from the subsp. *entomocidus* strain 60.5

was expressed and purified as described by Moar et al. (1995).

Protein concentrations for *Cry*IC (41.3 mg/ml) inclusion bodies were determined by the Micro BCA Protein Assay (Pierce Chemical Co., Rockford, IL). Purity of protoxins was measured using SDS-PAGE. For toxin purification, *Cry*IC inclusion bodies were digested without previous solubilization using 50 mM CAPs buffer (pH 10.5) containing 1 mg/ml bovine pancreas trypsin (Sigma Chemical Co., St. Louis, Missouri). Trypsin digested toxins (incubated for a maximum of 30 min) were purified and isolated using HPLC (Pusztai-Carey et al., 1994).

Four to five concentrations of XenTari were tested ranging from 5–100 μg of XenTari per g of diet for neonates and 25–200 μg/g for third and fourth instars. Each concentration was obtained according to the methodology followed by Moar & Trumble (1990). The materials were added to artificial diet (Pantana, 1969) and into either 1.5 ml microcentrifuge tubes for choice tests or into disposable petri dishes (150 × 25 mm) for behavior tests. The *Cry*IC inclusion bodies were dissolved in HPLC grade water, each concentration was added to artificial diet, then mixed and poured as before. Four concentrations of *Cry*IC inclusion bodies were tested ranging from 15–200 μg/g for neonates and 25–200 μg/g for third and fourth instars. *Cry*IC toxin also was dissolved using HPLC grade water. Two to four concentrations were tested (1–25 μg/g) and each concentration was mixed and poured as before.

Insect toxicity assays. Bioassays were conducted to determine the relative sensitivity (LC_{50}) of both susceptible colonies to the commercially formulated product, *Cry*IC inclusion bodies, and *Cry*IC toxin. Five to seven concentrations were assayed against susceptible neonates. Toxin was added to 20 g of artificial diet and poured into a 24-well bioassay tray (C–D International Inc., Pitman, N.J.). One neonate was added per well, and the trays were placed in an incubator. Mortality was recorded at seven days. Control mortality was <10%. Assays were repeated three to four times. The LC_{50} for the resistant colony (AUBURN-R) was not obtained because of the limited availability of *Cry*IC toxin and the large concentrations required to establish LC_{50} with this population (Moar et al., 1995).

Choice tests with neonates. Five susceptible or resistant *S. exigua* neonates were placed inside arenas adapted from the methodology of Gould et al. (1991). The arenas were constructed from 30 ml plastic cups with 4% agar (w/v) in the bottom, with 2 holes at opposite sides of the cup where two 1.5 ml micro centrifuge tubes were placed. One of the tubes contained diet alone (control diet) and the second contained treated diet. Choice tests with the commercial formulation and *Cry*IC inclusion bodies were conducted using the UCR-S colony while choice tests with *Cry*IC toxin were conducted using UCR-S, AUBURN-S and AUBURN-R colonies. The concentrations for the choice tests were 5, 15, 25, 50, and 100 μg/g for the commercial formulation; 15, 50, 100, and 200 μg/g for tests with *Cry*IC inclusion bodies; and 1, 5, 10, and 25 μg/g for neonates from the UCR-S colony and 1, 10, and 25 μg/g for neonates from AUBURN-S and AUBURN-R colonies for tests with *Cry*IC toxin. Control diet for choice tests with the commercial formulation was obtained by mixing 2 ml of 0.1% Tween solution (Fisher Scientific, Pittsburgh, PA) in 50 g diet; control diet for the other *Cry*IC forms was obtained by mixing 2 ml of distilled water in 50 g diet. For all choice experiments, *S. exigua* eggs were placed in plastic petri dieshes, with 4% agar in the bottom, inside an incubator. The neonates were placed inside the arenas when more than 50% of the eggs had hatched. All the concentrations per *Cry*IC form were evaluated concurrently and the arenas were placed inside an incubator. The position of the neonates was recorded twice/day (at 8:00 and 17:00 h) for 4 days. Each arena was treated as a replicate and the experiment had a total of 25 replicates per concentration.

Choice tests with third instars. Two susceptible or resistant newly molted third instars were placed inside arenas constructed with 150 ml plastic cups with 4% agar in the bottom and with 4 holes in a cross arrangement in which four 1.5 ml micro centrifuge tubes were placed. Opposing tubes contained the same type of diet (control or treated diet). As previously described, choice tests with the commercial formulation and *Cry*IC inclusion bodies were conducted using the UCR-S colony. Choice tests with *Cry*IC toxin were conducted using UCR-S and AUBURN-R colonies. The concentrations for the choice tests with the commercial formulation were 25, 50, 100, and 200 μg/g; the concentrations for tests with *Cry*IC inclusion bodies were 25, 50, 100, and 200 μg/g; and the concentrations for choice tests with *Cry*IC toxin were 1, 5, 10, and 25 μg/g for larvae from the UCR-S colony and 10 and 25 μg/g for larvae from the AUBURN-R colony. The tests for all the concentrations were run concurrently and the arenas were placed inside an incubator,

the position of the larvae was recorded as before. Consumption was estimated by obtaining the difference between initial and final weights of the microcentrifuge tubes. Water loss for each treatment and concentration was controlled for by estimating weight differences of the microcentrifuge tubes in two arenas without larvae (control arenas) held under the same conditions. Each arena was treated as a replicate and each concentration test per *Cry*IC form had a total of 25 replicates plus two control arenas.

Behavior tests. The behavior of either resistant (AUBURN-R) or susceptible (AUBURN-S) starved (12–18 h), one-day-old third instars was studied by observing the larvae individually on treated (10 μg of *Cry*IC toxin/g diet) or control diet during a nine minute period. As mentioned before, four distinct feeding behaviors were recorded: eating, questing, swallowing and resting (Berdegué & Trumble, 1996). A conservative nine minute period was used because preliminary observations indicated that larvae would go through a cycle exhibiting all four behaviors every 3–4 min. The observations were recorded using a computer-assisted monitoring device (Eigenbrode et al., 1989). Time spent and number of events associated with specific behaviors were recorded by observing the larvae at 10 × magnification with a dissecting microscope.

For these studies, 1.1 cm^3 control or treated diet cylinders were used. Each cylinder was placed in the center of a disposable petri dish with agar (4%) in the bottom (150 × 25 mm). A single larva was placed on the cylinder and the observations began as soon as the larvae showed any activity. Larvae from the AUBURN-R and AUBURN-S colonies were raised from eggs on untreated artificial diet until the experiment took place. Newly molted and starved (12–18 h) third instars were used. The experiment was repeated 25 times for each resistant colony-diet combination (AUBURN-R × control diet, AUBURN-R × *Cry*IC toxin treated diet) and 55 times for each susceptible colony-diet combination (AUBURN-S × control diet, AUBURN-S × *Cry*IC toxin treated diet). To discern if larval behavior could be conditioned by the type of diet on which larvae were raised, we recorded behavioral observations on larvae from the AUBURN-RC colony. This experiment was repeated 30 times for each diet combination (AUBURN-RC × control diet, AUBURN-RC × *Cry*IC toxin treated diet).

Data analysis. Mortality data from toxicity assays were analyzed using probit analysis (Finney, 1971). The assumption of normality for all data sets was tested with the UNIVARIATE procedure (SAS, 1990). Data from choice tests (proportion of larvae on each diet) were transformed by the arcsine square root function. Preference data for neonates that satisfied the assumption of normality were analyzed using the REPEATED statement of the GLM procedure; if the data were not normally distributed they were analyzed using a Chi-square test (χ^2) with the NPAR1WAY procedure (SAS, 1990). Because the preference data from choice tests with third instars were not normally distributed, they were analyzed using a Chi-square test.

The existence of a possible interaction between OBSERVATION × TREATMENT was examined using the CATMOD procedure for non-normal data and using the REPEATED statement of the GLM procedure for normal data (SAS, 1990). However, the OBSERVATION × TREATMENT interaction was not significant for any of the treatments and colonies tested ($P > 0.05$) and will not be considered further.

Normally distributed data for pairwise comparisons between consumption on the *Cry*IC treated diet and the control diet, time spent in a particular behavior, and number of occurrences of a particular behavior were analyzed using TTEST and MEANS procedures (SAS, 1990). Non-normally distributed data were analyzed using a Chi-square test (χ^2) with NPAR1WAY procedure (SAS, 1990). Probabilities of a type II error (β), i.e., accepting the null hypothesis when it is false, for one-tailed tests were obtained using the Z statistic in the NPAR1WAY procedure (Ott, 1988; SAS, 1990).

Results

Insect toxicity assays. Toxicity values for each *Cry*IC form are presented in Table 1. The commercial formulation was the least toxic *Cry*IC form while the *Cry*IC toxin was the most lethal to susceptible colonies. No-choice tests conducted with both susceptible populations (Table 1) showed no significant differences between the LC_{50}s (95% fiducial limits overlap) for UCR-S and the values for AUBURN-S reported by Moar et al. (1995).

Choice tests with neonates. Tests with the four highest concentrations, 15 μg/g ($F = 14.93$; df = 1.46; $P < 0.0003$), .25 μg/g ($F = 18.78$; df = 1.44; $P < 0.0001$), 50 μg/g ($F = 150.49$; df = 1.42; $P < 0.0001$) and 100 μg/g ($F = 21.89$; df = 1.40; $P < 0.0001$), of the commercial formulation showed a

Table 1. Toxicity of *Cry*IC formulations on different colonies of *S. exigua*

Colony	*Cry*IC form	N[a]	LC_{50}^{b} (95% FL)		Slope
UCR-S	XenTari	432	51.60	(40.09–61.71)	3.60 ± 0.71
UCR-S	Inclusion bodies	504	17.12	(5.27–27.38)	1.88 ± 0.46
UCR-S	*Cry*IC toxin	360	4.04	(2.45–5.51)	2.60 ± 0.49
AUBURN-S	*Cry*IC toxin	360	2.34	(1.78–2.91)	4.11 ± 0.81
AUBURN-S[c]	*Cry*IC toxin	515	4.30	(3.80–4.90)	2.36 ± 0.18
AUBURN-R[c]	*Cry*IC toxin	69	8% mortality @ 320		– –

[a] Total number of insects tested; 3–4 replicates using 5–7 concentrations;
[b] LC_{50} values in μg of the *Cry*IC form per gram of diet or % mortality at the highest concentration tested;
[c] From Moar et al. (1995).

significantly greater proportion of neonates from the UCR-S colony on the control diets (Figure 1). The test with the lowest concentration (5 μg commercial formulation/g diet), with approximately a concentration 10-fold lower than the LC_{50}, showed no statistical difference in proportion of neonates on control and treated diets throughout the experiment ($F = 0.01$; df = 1.46; $P > 0.05$) (Figure 1A). For inclusion bodies, all concentrations except one resulted in higher proportions of neonates on control diets, 15 μg/g ($F = 8.77$; df = 1.40; $P < 0.005$), 50 μg/g ($F = 48.12$; df = 1.32; $P < 0.0001$), 100 μg/g ($F = 17.24$; df = 1.24; $P < 0.0005$) (Figure 2). Interestingly, there was a greater proportion of neonates on the diet with the highest concentration of inclusion bodies (200 μg/g; $F = 30.75$; df = 1.26; $P < 0.0001$) (Figure 2D). A significantly higher proportion of neonates was found on the control diets in tests for all concentrations of *Cry*IC toxin using insects from AUBURN-S, 1 μg/g ($F = 111.23$; df = 1.48; $P < 0.0001$), 10 μg/g ($F = 286.47$; df = 1.46; $P < 0.0001$) and 25 μg/g ($F = 792.22$; df = 1.46; $P < 0.0001$), and AUBURN-R colonies, 1 μg/g ($F = 34.31$; df = 1.48; $P < 0.0001$), 10 μg/g ($F = 105.96$; df = 1.48; $P < 0.0001$) and 25 μg/g ($F = 202.27$; df = 1.48; $P < 0.0001$) (Figure 3). Similarly, tests with *Cry*IC toxin using the UCR-S colony indicated a greater proportion of neonates on the control than on the treated diet for all concentrations ($P < 0.0001$) (data not shown).

Choice tests with third instars. Tests with different concentrations of the commercial formulation showed a significantly greater average proportion of larvae from the UCR-S colony on the control, 25 μg/g ($\chi^2 = 34.81$; df = 1.48; $P < 0.0001$), 50 μg/g ($\chi^2 = 35.47$; df = 1.48; $P < 0.0001$), 100 μg/g ($\chi^2 = 35.46$; df = 1.48; $P < 0.0001$) and 200 μg/g ($\chi^2 = 35.52$; df = 1.48; $P < 0.0001$). Tests with *Cry*IC inclusion bodies using UCR-S larvae indicated a greater average proportion of larvae on the control diet for all the concentrations tested, 25 μg/g ($\chi^2 = 15.13$; df = 1.48; $P < 0.0001$), 50 μg/g ($\chi^2 = 35.03$; df = 1.48; $P < 0.0001$), 100 μg/g ($\chi^2 = 24.61$; df = 1.34; $P < 0.0001$) and 200 μg/g ($\chi^2 = 17.05$; df = 1.24; $P < 0.0001$). Tests with *Cry*IC toxin using UCR-S larvae also indicated a greater average on the control diet for all the concentrations tested, 1 μg/g ($\chi^2 = 28.75$; df = 1.48; $P < 0.0001$), 5 μg/g ($\chi^2 = 30.45$; df = 1.48; $P < 0.0001$), 10 μg/g ($\chi^2 = 34.91$; df = 1.48; $P < 0.0001$) and 25 μg/g ($\chi^2 = 25.31$; df = 1.46; $P < 0.0001$). A significantly higher average of larvae was also found on the control diet in tests for all concentrations of *Cry*IC toxin using insects from the AUBURN-RC colony, 10 μg/g ($\chi^2 = 18.20$; df = 1.48; $P < 0.0001$) and 25 μg/g ($\chi^2 = 6.06$; df = 1.50; $P < 0.05$). Larvae consumed significantly more control diet than treated diet, for all *Cry*IC forms, colonies, and concentrations ($P < 0.05$) (Table 2).

Behavior tests. The results from choice tests and consumption data prompted us to perform one-tail *t*-test or chi-square tests for behavioral comparisons of larvae of the same colony on two types of diet (Figure 4). AUBURN-R larvae raised on control diet spent significantly more time eating (One-tailed test; $t = 2.53$; df = 1.48; $P = 0.01$) and less time resting (One-tailed test; $\chi^2 = 3.03$; df = 1.50; $P < 0.05$) and swallowing (One-tailed test; $\chi^2 = 4.82$; df = 1.50; $P = 0.01$) on control than on treated diet. Furthermore these larvae showed a greater number of swallowing occurrences on the treated than on control diet (One-tailed test; $\chi^2 = 4.03$; df = 1.50; $P < 0.05$). There were no statistical differences for time spent questing ($\beta = 0.41$) and

Table 2. Consumption of third-fourth instar *S. exigua*

*Cry*IC form	Concentration[a]	Colony	Consumption (SE)[b]	Statistic	P-value
XenTari	0	UCR-S	0.8123 ± 0.05		
				$\chi^2 = 35.26$	0.0001
XenTari	25	UCR-S	0.1641 ± 0.02		
XenTari	0	UCR-S	0.7556 ± 0.06		
				$\chi^2 = 35.37$	0.0001
XenTari	50	UCR-S	0.0632 ± 0.01		
XenTari	0	UCR-S	0.9100 ± 0.06		
				$\chi^2 = 35.32$	0.0001
XenTari	100	UCR-S	0.1504 ± 0.02		
XenTari	0	UCR-S	0.6625 ± 0.04		
				$\chi^2 = 35.34$	0.0001
XenTari	200	UCR-S	0.0854 ± 0.01		
Inclusion Bodies	0	UCR-S	0.2132 ± 0.03		
				$T = -3.47$	0.001
Inclusion Bodies	25	UCR-S	0.0954 ± 0.02		
Inclusion Bodies	0	UCR-S	0.2391 ± 0.03		
				$\chi^2 = 20.44$	0.0001
Inclusion Bodies	50	UCR-S	0.0475 ± 0.02		
Inclusion Bodies	0	UCR-S	0.1403 ± 0.03		
				$T = -3.63$	0.0008
Inclusion Bodies	100	UCR-S	0.0452 ± 0.01		
Inclusion Bodies	0	UCR-S	0.0634 ± 0.02		
				$\chi^2 = 5.61$	0.0179
Inclusion Bodies	200	UCR-S	0.0083 ± 0.003		
*Cry*IC toxin	0	UCR-S	0.1176 ± 0.01		
				$T = -3.08$	0.0034
*Cry*IC toxin	1	UCR-S	0.0728 ± 0.01		
*Cry*IC toxin	0	UCR-S	0.0851 ± 0.01		
				$\chi^2 = 19.84$	0.0001
*Cry*IC toxin	5	UCR-S	0.0231 ± 0.01		
*Cry*IC toxin	0	UCR-S	0.0732 ± 0.02		
				$\chi^2 = 16.04$	0.0001
*Cry*IC toxin	10	UCR-S	0.0056 ± 0.01		
*Cry*IC toxin	0	UCR-S	0.1013 ± 0.03		
				$\chi^2 = 14.88$	0.0001
*Cry*IC toxin	25	UCR-S	0.0072 ± 0.01		
*Cry*IC toxin	0	AUBURN-R	0.3772 ± 0.04		
				$T = -3.55$	0.0009
*Cry*IC toxin	10	AUBURN-R	0.1994 ± 0.04		
*Cry*IC toxin	0	AUBURN-R	0.4754 ± 0.06		
				$T = -5.47$	0.0001
*Cry*IC toxin	25	AUBURN-R	0.1754 ± 0.03		

[a] μg of *Cry*IC form per g diet;
[b] g of diet consumed ± standard errors.

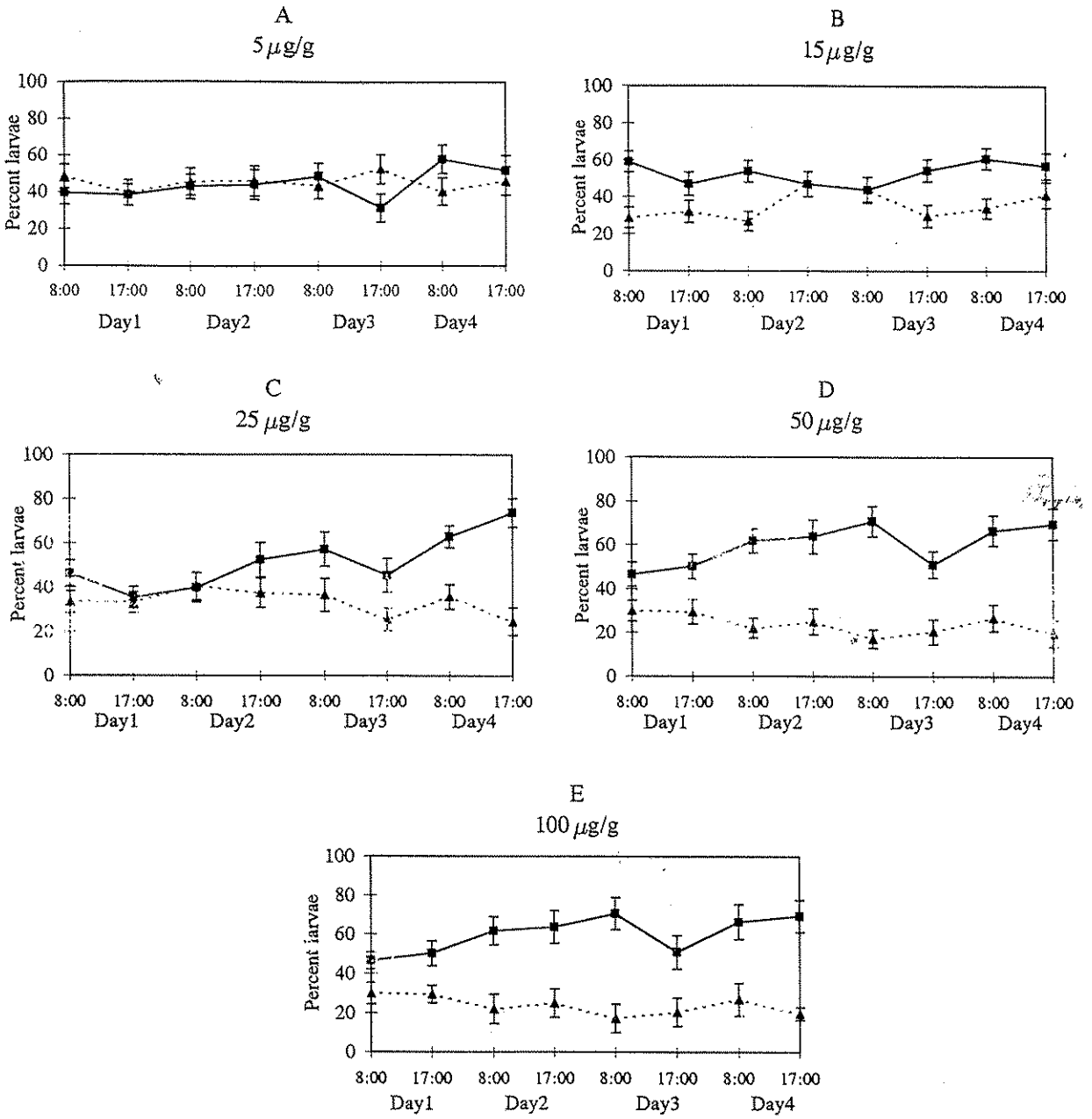

Figure 1. Results of choice tests using UCR-S neonates between diet containing XenTari or diet containing 0.1% Tween solution (Control diet). The concentrations tested were 5 μg/g (A), 15 μg/g (B), 25 μg/g (C), 50 μg/g (D), and 100 μg/g (E). Points indicate the percent neonates on treated diet (solid triangles) and on control diet (solid squares). Lines above each point indicate standard errors.

number of eating ($\beta = 0.48$), questing ($\beta = 0.44$) and resting ($\beta = 0.13$) occurences (One-tailed test; $P > 0.05$).

AUBURN-RC larvae raised on *Cry*IC treated diet showed no differences in time spent eating ($\beta = 0.03$), questing ($\beta = 0.42$), resting ($\beta = 0.32$) and swallowing ($\beta = 0.31$). Also there were no differences in num-

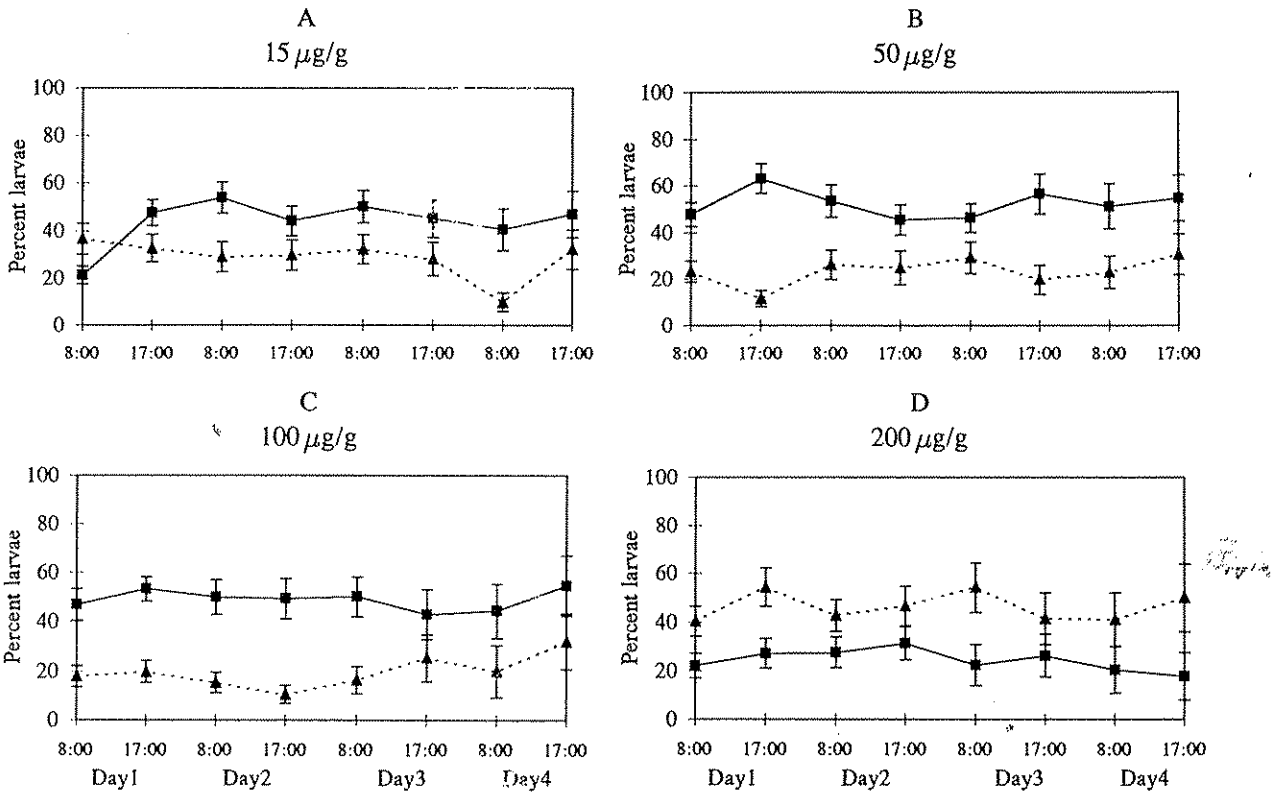

Figure 2. Results of choice test using UCR-S neonates between diet containing inclusion bodies (protoxin) or control diet. The concentrations tested were 15 μg/g (A), 50 μg/g (B), 100 μg/g (C), and 200 μg/g (D). Points indicate the percent neonates on treated diet (solid triangles) and on control diet (solid squares). Lines above each point indicate standard errors.

ber of eating ($\beta = 0.28$), questing ($\beta = 0.17$), resting ($\beta = 0.45$) and swallowing ($\beta = 0.32$) occurrences between larvae on treated and control diet ($P > 0.05$).

AUBURN-S larvae reared on control diet spent significantly less times questing (One-tailed test; $\chi^2 = 3.32$; df = 1.110; $P < 0.05$) and more time swallowing (One-tailed test; $\chi^2 = 9.46$; df = 1.110; $P = 0.001$) on the control than on the treated diet. Susceptible larvae also had fewer questing (One-tailed test; $\chi^2 = 2.76$; df = 1.110; $P < 0.05$) and more swallowing occurrences on control diet than on treated diet (One-tailed test; $\chi^2 = 8.54$; df = 1.110; $P = 0.001$). There were no signficant differences in time spent eating ($\beta = 0.18$) and resting ($\beta = 0.43$) and number of eating ($\beta = 0.43$) and resting ($\beta = 0.19$) occurrences ($P > 0.05$).

Because resistant larvae avoided treated diet, behavioral comparisons between larvae of different colonies on the same diet were conducted using two-tailed tests. Larvae from the AUBURN-R colony had a greater number of eating ($\chi^2 = 16.50$; df = 1.80; $P < 0.0001$), questing ($\chi^2 = 15.64$; df = 1.80; $P < 0.0001$) and swallowing occurrences ($\chi^2 = 8.38$; df = 1.80; $P < 0.005$) and spent significantly more time eating (t =-3.00; df = 1.78; $P < 0.05$) on the treated diet than the larvae from the AUBURN-S colony (Figures 4A,B). When both colonies (AUBURN-R and AUBURN-S) were given control diet the susceptible larvae had a significantly lower number of eating ($\chi^2 = 24.41$; df = 1.80; $P < 0.0001$) and questing ($\chi^2 = 27.51$; df = 1.80; $P < 0.0001$) occurrences, spent significantly more time resting ($\chi^2 = 12.07$; df = 1.80; $P < 0.0005$) and swallowing ($\chi^2 = 20.74$; df = 1.80; $P < 0.0001$), and spent significantly less time eating ($t = -4.07$; df = 1.78; $P < 0.0001$) than resistant larvae (Figures 4C,D).

Resistant larvae raised on control diet had a greater number of questing ($\chi^2 = 3.89$; df = 1.55; $P < 0.05$), resting ($\chi^2 = 3.84$; df = 1.55; $P = 0.05$) and swallowing

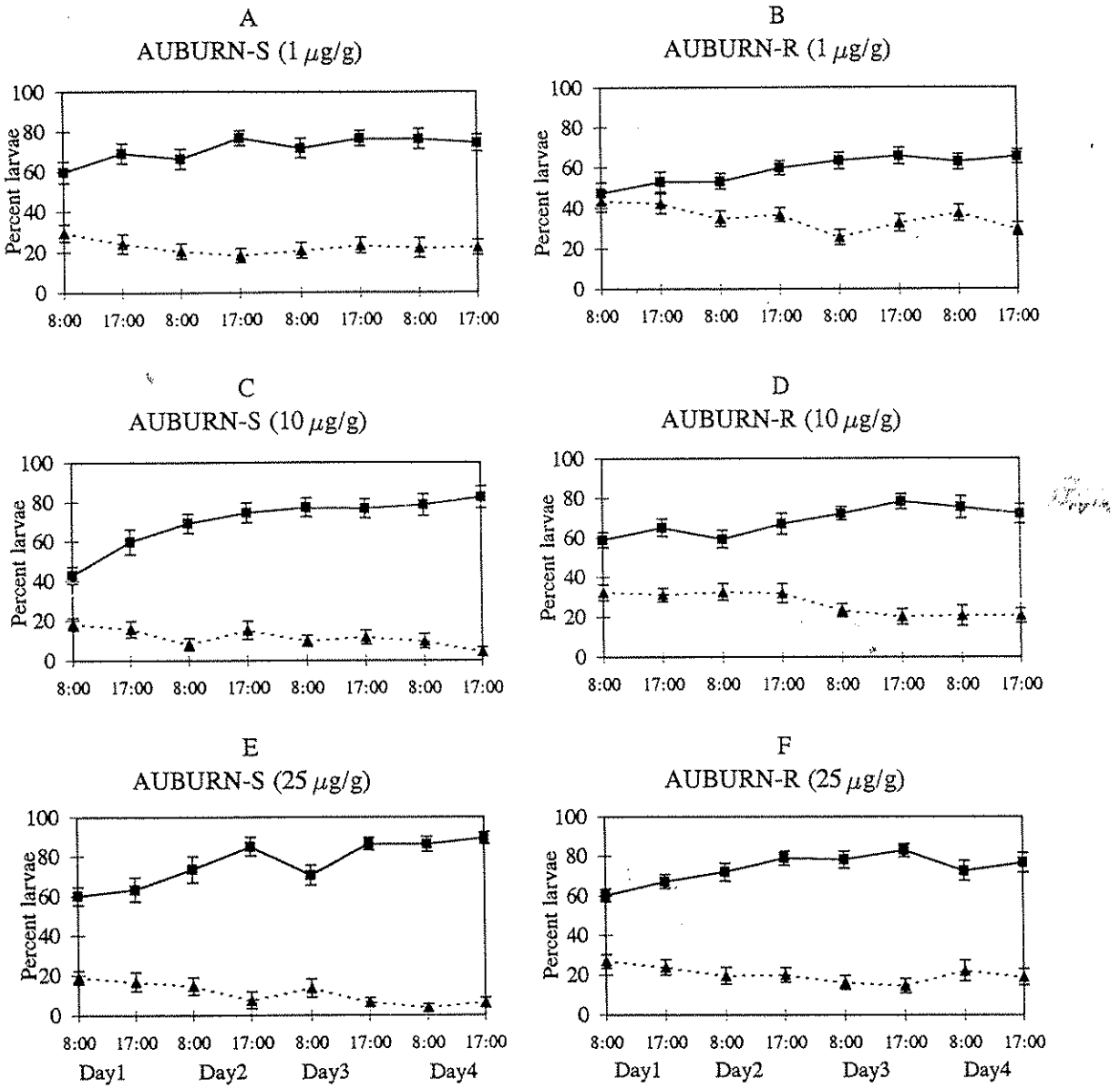

Figure 3. Results of choice test using AUBURN-S (A, C, and E) and AUBURN-R (B, D, and F) neonates beween diet containing *Cry*IC toxin and control diet. The concentrations tested were 1 μg/g (A and B), 10 μg/g (C and D), and 25 μg/g (E and F). Points indicate the percent neonates on treated diet (solid triangles) and on control diet (solid squares). Lines above each point indicate standard errors.

($\chi^2 = 21.10$; df = 1.55; $P < 0.0001$) occurrences, spent significantly less time eating ($t = -2.39$; df = 1.53; $P < 0.05$) and spent significantly more time swallowing ($\chi^2 = 20.34$; df = 1.55; $P < 0.0001$) treated diet than resistant larvae raised on treated diet (AUBURN-RC) (Figures 4A,B). Resistant larvae raised on control diet had a greater number of eating ($t = 2.73$; df = 1.53; $P < 0.01$), questing ($t = 3.35$; df = 1.53; $P = 0.001$) and swallowing ($\chi^2 = 16.66$; df = 1.55; $P < 0.0001$) occurrences and spent significantly more time swallowing ($\chi^2 = 14.07$; df = 1.55; $P < 0.0005$) control

diet than resistant larvae raised on treated diet (Figures 4C,D).

Discussion

Choice tests with neonates and third instars indicated that *S. exigua* larvae have the capacity to detect and avoid diet treated with any of the *Cry*IC forms tested. The fact that *Cry*IC toxin elicits avoidance by neonates and third and fourth instars strongly suggests that *Cry*IC, which also is present in the commercial formulation and in the inclusion bodies, is responsible for eliciting avoidance behavior in all *Cry*IC forms tested. Furthermore, this avoidance was present for all the colony, concentration, and *Cry*IC form combinations tested with the exception of the lowest concentration for the commercial formulation and the highest concentration for the inclusion bodies (Figures 1–3). The observed high neonate mortality in choice tests on diet with 200 μg of inclusion bodies per g of diet (M.B., unpubl.) (around 10 times the LC_{50} value) suggests that the greater number of neonates present on the treated diet was probably due to a rapid paralysis of the insects due to toxicity (Figure 2D). Thus, less lethal concentrations of these *Cry*IC forms, with the exception of the 5 μg/g commercial formulation (Figure 1A), elicited a largely concentration-independent avoidance behavior. This result differs from earlier reports, where concentration-dependent avoidance caused by B.t. has been suggested as a behavioral form of resistance for a few lepidopterous species. Ramachandran et al. (1993) showed that second instar spruce budworm (*Choristoneura fumiferana* (Clemens)) avoid diet treated with *Cry*IA(a) endotoxin at concentrations 1.3 and 6.67 times over the LC_{50} (6 μg/ml); however, this avoidance behavior may be stage dependent because Retnakaran et al. (1983) did not find avoidance of diet with B.t. HD-1 endotoxin by sixth instars. Fourth and fifth instar *Heliothis virescens* showed concentration-dependent avoidance of B.t. endotoxins, whereas second instars showed avoidance at all concentrations (Gould et al., 1991). Finally, a commercial formulation of B.t. (Foray 48B) also caused a concentration-dependent avoidance by gypsy moth larvae (Farrar & Ridgway, 1995).

Gould & Anderson (1991) found that resistant strains avoided only the highest B.t. and dipel concentrations. However, similar to our results, these authors found that susceptible *H. virescens* larvae exhibited concentration-independent avoidance of diets with Dipel and B.t. endotoxins. Finally, some authors have reported the lack of a repellent effect of transgenic plants to economically important herbivores (Wilson et al., 1992; Ebora et al., 1994).

Results from no-choice behavioral observations of *S. exigua* larvae on control and treated diet indicate that *Cry*IC toxin affects feeding behavior of *S. exigua* larvae (Figure 4). The number of occurrences and time spent questing by susceptible *S. exigua* larvae appears to be positively affected by the presence of *Cry*IC toxin in the diet. However, there was no effect of *Cry*IC toxin on eating and resting behaviors for this colony.

Comparisons among colonies showed that resistant larvae were on the whole more active than susceptible larvae on both treated and control diet. Resistant larvae spent significantly more time eating and had a higher number of eating occurrences on *Cry*IC treated and untreated diet than susceptible larvae (Figure 4). This suggests that resistant larvae inherently eat more, possibly because of the metabolic costs involved with resistance. Thus, the level of resistance to *Cry*IC in *S. exigua* affects specific feeding behaviors such as eating, questing, resting, and swallowing (Figure 4).

When offered control diet, AUBURN-R larvae raised on control diet had a greater number of short swallowing, eating, and questing occurrences than AUBURN-RC. However, although both cohorts had similar overall behaviors, AUBURN-RC larvae spent more time eating treated diet than did the larvae raised on control diet (Figure 4A). This indicates that larval conditioning may play an important role on eating behavior of *S. exigua*.

Although the mechanism through which *Cry*IC toxin affects feeding behavior of the larvae remains unknown, feeding inhibition of larvae after ingestion of B.t. has been well documented (Farrar & Ridgway, 1995; Gill et al., 1992; Honée & Visser, 1993). Lhoste & Martouret (1968) showed that only the δ-endotoxin produces this response. Our results with susceptible larvae indicate a concentration-dependent decrease in overall diet consumption for the protoxin and *Cry*IC toxin forms (Table 2). Therefore, we believe that *Cry*IC toxin negatively affected diet consumption by *S. exigua*.

Fast & Videnova (1974) reported that labeled protein fragments were present in the hemolymph 1 min after ^{3}H-labeled B.t. crystals were fed to the spruce budworm, *C. fumiferana*. Retnakaran et al. (1983) proposed that these materials could be directly or indirectly affecting behavior by acting upon the central nervous system. They speculated that at low levels the toxin molecules could pass through the damaged

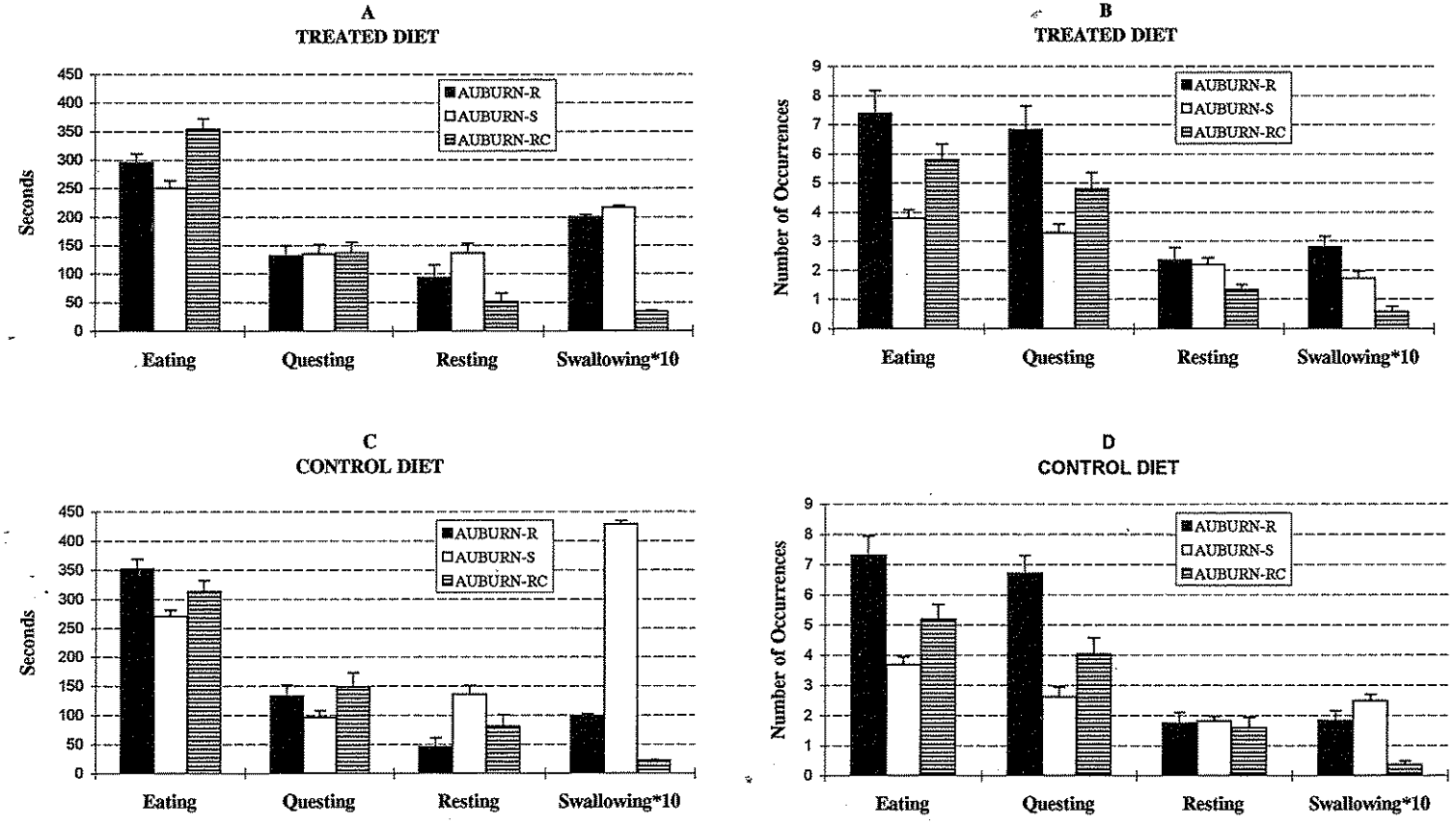

Figure 4. Comparisons between larvae of different cohorts of results of nine minute observations of the behavior of third and fourth instars when confronted with either control or treated (10 μg of *Cry*IC toxin/g diet) diet. Tests were performed with larvae from AUBURN-R, AUBURN-S and AUBURN-RC colonies. Bars above each individual behavior indicate total time spent (sec.) (A and C) and number of occurrences (B and D) of that particular behavior. Lines above each bar indicate standard errors.

midgut cells into the hemocoel and inhibit feeding. The cessation of feeding could then permit the larva to degrade the toxin and repair the midgut by replacing the damaged cells from the regenerative nidi. If the recovered larva was not exposed to the toxin again after the feeding resumed, recovery would be permanent. Ramachandran et al. (1993) showed that the spruce budworm could recover repeatedly from effects of the *Cry*1A(a) toxin, reinfecting themselves if the toxin was still available, or recovering to grow normally in its absence.

Our results indicated concentration dependent effect on diet consumption by two *Cry*IC forms (Table 2). The differences in consumption between the control and the *Cry*IC toxin treated diets for the UCR-S colony were <2X, ¼X, >10X, and >10X for 1, 5, 10, and 25 μg/g, respectively, while the difference in consumption for the AUBURN-R colony were 2X and 3X for 10 and 25 μg/g. This indicates that although there is an effect on consumption by sublethal concentrations (1 μg/g for UCR-S and 10–25 μg/g for AUBURN-R) this effect increases with an increase of toxicity. Therefore, we would also expect a concentration dependent decrease of consumption on AUBURN-R larvae with sufficiently high concentrations of the toxin (i.e., 1–2 mg). Nevertheless, feeding inhibition by intoxication may not be solely responsible for the observed behaviors because susceptible larvae were on the whole more active (questing criterion only) after being fed the toxin than after being fed control diet. Furthermore, avoidance of the treated diet was observed by susceptible larvae when confronted with sublethal concentrations of the toxin (1 μg/g *Cry*IC/g diet). This effect was also observed on resistant larvae, which should have suffered no more than a minor toxic effect from the tested concentrations. Therefore, we suggest that for those concentrations where initial exposure of the larvae to the treated diet does not result in paralysis, *Cry*IC toxin is acting as a feeding deterrent rather than an inhibitor (at least within the first 9 min of exposure). A possible explanation for the non-toxic effect of *Cry*IC on the behavior of resistant *S. exigua* larvae is that *Cry*IC or a behaviorally active component of this protein may flow to the hemocoel from the midgut and affect the CNS as suggested by Retnakaran et al. (1983).

The fact that *S. exigua* can detect and avoid *Cry*IC toxin could have important implications on established and new pest management strategies such as the use of transgenic plants which express B.t. toxins in either the 'protoxin' or *Cry*IC toxin. For example in a field with a transgenic variety with different levels of toxin expression within a plant, or a field with alternative weed hosts or alternative susceptible plants of the same species, *S. exigua* larvae, which are highly mobile (Smits et al., 1987; Berdegué & Trumble, 1996), could potentially detect and avoid those plant parts or whole plants that express the toxin and feed on non-toxic plant material only. Therefore, higher-than-expected pest populations could result from the use of transgenic plants if larval behavioral response is not taken into consideration. Ultimately, understanding the mechanism by which larval behavior is affected by *Cry*IC and other toxins may enable a more efficient use of existing B.t. compounds and improve the effective application of new technologies. One such technology could actually rely on differential toxin expression within a plant so that feeding preferentially occurs on non-marketable parts and on plant parts or plant growth stages where herbivory has no negative effect on yield due to regrowth/compensation mechanisms (Trumble et al., 1993).

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