RESPONSES OF TOBACCO BUDWORM[1] LARVAE TO CYPERMETHRIN: EXAMINATION OF OBSERVATION TIMES AND DEATH CRITERIA[2]

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ABSTRACT

Tobacco budworm larvae from four strains killed by topically applied cypermethrin died slowly regardless of larval weight and at all tested doses. In one set of experiments, third instars (15 - 25 mg) were treated with a range of doses (0.004 - 1060.0 ug per larva, depending on strain) and classified as dead, moribund, or alive, 24, 48, 72, and 96 h after treatment. Because the ultimate response of many larvae could not be determined even after 96 h, each larva was again observed 192 - 288 h (8 - 12 days) after treatment when all larvae were either clearly dead or alive. Whether moribund larvae were included with dead or live larvae significantly affected LD_{50} estimates. For example, using the 96 h mortality observation, estimated LD_{50} was 167 ug per larvae when moribund larvae were considered dead and 1,682 ug per larva when they were considered alive. The effect was largest for earlier observation times and more tolerant strains. Of larvae classified as moribund, about one-third ultimately recovered and two-thirds died. In another set of experiments, 173 third instars (9.4 - 36.1 mg) were treated with 1.0 ug cypermethrin. Although survival after 24, 48, and 72 h was 100, 97.7, and 88.4 % respectively, all died from cypermethrin treatment. Even at high doses (e.g., LD_{90}), the ultimate fate of most larvae could not be predicted accurately until at least 4 - 5 days after treatment. For larvae (9.0 - 175.4 mg, n = 241) treated with 0.1 ug cypermethrin (about LD_{50}), observations 120 h after treatment which specified larval activity provided the best predictor of ultimate fate. Thus, when larval toxicity studies are used to estimate long-term population response, mortality estimates should be made at least 72 h after treatment.

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[1]Lepidoptera: Noctuidae.

[2]This paper reports the results of research only. Mention of a proprietary product or pesticide does not constitute an endorsement or a recommendation for its use by USDA nor does it imply registration under FIFRA as amended.

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INTRODUCTION

Efforts to manage development of resistance to cypermethrin in tobacco budworm, *Heliothis virescens* (F.), depend on accurate assessments of both short- and long-term effects of insecticide applications. Estimates of short-term response of a population to cypermethrin are most common and valuable because they demonstrate immediate benefits of application. Typically, larvae are classified as dead or alive 24 or 48 h after treatment. Often, however, many insects classified as dead are actually still alive (Payne et al. 1988, Roush and Luttrell 1989). When tobacco budworm larvae are treated with cypermethrin, some insects that are initially knocked down may recover, reach adult stage and reproduce; others that appear only mildly affected 24 or 48 h after treatment die from the insecticide (Firko and Hayes 1990). Thus, with cypermethrin it is difficult to obtain accurate estimates of mortality after only 24 or 48 h. Significant error may result if these observations are used as indicators of long-term population response (i.e., indicators of how the population will be genetically different in subsequent generations).

With each insecticide application the genetic structure of the pest population changes and genetic changes in tolerance and life history traits may profoundly affect ability of the population to increase, as well as tolerance levels in subsequent generations. To predict accurately development of resistance it is necessary to estimate the proportion of the population that can survive to the reproductive stage. In two separate sets of experiments we examined specific observation times and death criteria to determine the most accurate type of observation as a means of predicting actual mortality of tobacco budworm larvae treated with cypermethrin.

MATERIALS AND METHODS

Probit Analysis and Moribund Larvae. Three strains of tobacco budworm with known tolerance levels were treated topically with cypermethrin as third instars. Our resistant strain (R) was started with eggs obtained from ICI Americas, Inc. (PEG-87 strain). We maintained R with selection pressure for resistance to cypermethrin (topical application of 490 - 530 ug per third instar) for two generations before toxicity tests. Our intermediate tolerance strain (RBC) was produced by crossing susceptible tobacco budworm sterile backcross (TBW-BC, from USDA-ARS, Stoneville, Miss.) females with R males, and then backcrossing female F_1 with male R. TBW-BC was originally produced by crossing *H. virescens* males with *H. subflexa* females (Laster 1972), but has been maintained for the past 20 years as a pure *H. virescens* colony. Our lowest tolerance strain (RS) was produced by reciprocally-crossing normal, susceptible tobacco budworm (USDA-ARS, Stoneville, Miss.) with R. Larvae were reared individually in plastic cups (22.5 ml) with about 15 ml of artificial diet (King and Hartley 1985), with mold inhibitor (Powell and Hartley 1987) at 25 $\pm$ 2°C, 60 $\pm$ 10 % RH, and a photoperiod of 14:10 (L:D).

Third instars (15.0 - 25.0 mg) were treated topically with acetone (1.0 ul, analytic-grade) containing technical-grade cypermethrin (FMC Corp., Princeton, New Jersey). Larvae were originally observed 24, 48, 72, and 96 h after treatment and

scored as dead, alive (able to stand and crawl after being turned onto back), or moribund (alive but clearly adversely affected by the insecticide, unable to stand and crawl). Moribund larvae either died from exposure to cypermethrin or recovered completely and it was not possible to predict which moribund larvae would recover. Because many larvae were classified as moribund even after 96 h, we made a final observation 8 - 12 days (192 - 288 h) after treatment to determine ultimate response to cypermethrin; most survivors had pupated by this time. Observations at 24 and 48 h were subsequently dropped because most larvae were classified as moribund and these observations were poor indicators of response. Probit analyses and comparisons were based on 72 and 96 h and final observations. We performed 5 separate probit analyses for each strain: two each based on the 72 and 96 h mortality observations with moribund larvae included with either live or dead larvae, and one based on the final observation when larvae were clearly either dead or alive.

Quantified Response To Cypermethrin. Larvae (strain MS) used in this experiment originated from eggs collected in 1987 from a 380 ha cotton field near Leland, Washington Co., Mississippi, USA. Larvae were reared as described above. The colony was maintained free of insecticides until production of the first experimental generation in May 1988 (3 - 5 overlapping generations). Larvae were weighed just before topical treatment with cypermethrin (described above). Three separate groups of larvae were treated. Small larvae (9.4 - 36.1 mg, n = 173) from the first experimental generation, were treated with 1.0 ug cypermethrin. Another group (9.0 - 175.4 mg, n = 241) was treated with 0.1 ug. Larvae that survived 0.1 ug were reared to the adult stage and mated in mass colony (two containers) to produce a second experimental generation whose parents had been selected for tolerance to cypermethrin. Small larvae (10.3 - 35.5 mg, n = 345) from this second generation also were treated with 0.1 ug cypermethrin.

Beginning 0.5 h after treatment, we observed larval response by agitating gently with a blunt probe; upright larvae were rolled onto their backs. Responsiveness, activity level, type of activity, and ability to roll-over constituted scoring criteria (Table 1). Condition of each larva was scored at each observation. Because some larvae were alive but still debilitated 120 h after treatment, we made a final observation 240 - 288 h (10 - 12 days) after treatment to determine final condition; by this time, all test specimens had reached fifth instar or pupated or were dead. Larvae treated with 1.0 ug cypermethrin were observed and scored 0.5, 1, 2, 4, 8, 24, 48, 72, 120, and 240 h after treatment. Those treated with 0.1 ug also were observed 96 h after treatment.

We examined optimal observation time and response criteria for classifying treated individuals as dead or alive; final condition was compared with mortality classifications (dead vs. alive) based on various death criteria at the 24, 48, 72, 96, and 120 h observations. Death criteria were based on the response criteria in Table 1. For example, a death criterion of 4/5 (dead/alive) meant that larvae with scores of 4 or less were classified as dead and larvae with scores of 5 or above were classified as alive. There were two possible types of errors in classifying larvae as dead or alive at the 24, 48, 72, 96 and 120 h observations: a larva was incorrectly classi-

TABLE 1.[a] Response Criteria For Experiments In Which Larval Response to Cypermethrin Was Quantified.

Score	Ability to right	Activity and capabilities
0	no	None
1	no	Minor activity after persistent probing
2	no	Minor activity, immediate response
3	no	Slight independent activity, responsive
4	no	Active, slow writhing, no control
5	no	Active, vigorous writhing, no attempts to right
6	no	Active, attempts to right
7	yes	Active, rights with difficulty
8	yes	Active, rights easily, but adverse effects
9	yes	Active, rights easily, minor effects only
10	yes	Active, no visible effects

[a]Reprinted from Firko and Hayes (1990) with permission.

fied as dead if it subsequently recovered and survived, or incorrectly classified as alive if it was dead at the final observation with little or no evidence of further growth or development. SAS (SAS Institute 1988a,b) was used for data handling and statistical analyses. Percentages were compared statistically with G-tests (Sokal and Rohlf 1981).

RESULTS AND DISCUSSION

Probit Analysis and Moribund Larvae. Final observations (8 - 12 days after treatment) were unambiguous assessments of mortality because all larvae were clearly either dead with no visual evidence of weight gain, or completely recovered. We assumed the final observation provided the most accurate assessment of response and used it to represent ultimate response. For each strain, LD_{50} estimates based on 72 and 96 h observations with moribund larvae considered dead were not significantly different from LD_{50}s based on final mortality. However, the 96 h observation consistently gave results most similar to probit analyses based on ultimate condition (Table 2). Observations 24 and 48 h after treatment provided the least accurate assessment of population tolerance regardless of how moribund larvae were considered because at most doses, nearly all larvae were classified as moribund; they dominated the category in which they were included. When moribund larvae were included with live larvae at the 72 and 96 h observations, LD_{50} estimates were closer than with the 24 and 48 h observations but significantly different from LD_{50} estimates based on final mortality. Classifying moribund larvae as dead was most accurate because roughly one third ultimately recovered while two thirds died. Probit analyses based on 72 or 96 h mortality with moribund larvae classified as dead most accurately reflected final mortality (Table 2).

Differences in estimated LD_{50} resulting from classifying moribund larvae as either dead or alive were associated with tolerance level and were largest for the most tolerant (R) strain: a greater proportion of moribund R larvae recovered and survived. Our R strain was derived from PEG-87 which

possesses a variety of physiologically distinct tolerance mechanisms (Dowd and Sparks 1987, Lee et al. 1989, Little et al. 1989a,b, McCaffery et al. 1989). Moribund R larvae may be better able to recover because of the many tolerance mechanisms they possess.

TABLE 2. Variation in Results of Probit Analyses After Classifying Moribund Larvae as Either Dead or Alive.

Strain	Obs. time	Moribund larvae	n[a]	LD_{50}[b] (95 % limits)	Slope ± sd
RS	72 h	dead	517	0.24 (0.17-0.35)	1.7 ± 0.2
	72 h	alive	517	0.78 (0.38-1.58)	1.4 ± 0.3
	96 h	dead	517	0.25 (0.18-0.36)	1.5 ± 0.2
	96 h	alive	517	0.48[c]	1.8 ± 1.4
	8-12 d	N/A[d]	517	0.29 (0.23-0.38)	1.5 ± 0.1
RBC	72 h	dead	849	13.0 (9.5-17.4)	0.8 ± 0.1
	72 h	alive	849	397.7 (263.6-652.9)	0.6 ± 0.1
	96 h	dead	994	14.3 (9.5-21.1)	0.7 ± 0.1
	96 h	alive	994	40.3 (29.4-55.8)	0.6 ± 0.1
	8-12 d	N/A	994	14.4 (9.5-21.5)	0.7 ± 0.1
R	72 h	dead	346	115 (80-180)	0.9 ± 0.1
	72 h	alive	346	137,080 (6305-4X10^{12})	0.4 ± 0.2
	96 h	dead	671	167 (128-222)	0.9 ± 0.1
	96 h	alive	671	1,682 (905-4503)	0.7 ± 0.1
	8-12 d	N/A	671	234 (177-325)	0.9 ± 0.1

[a]Some 72 h observations were not made.
[b]LD_{50} in ug per larva.
[c]SAS did not estimate confidence intervals in this analysis.
[d]There were no moribund larvae 8 - 12 days after treatment.

Quantified Response To Cypermethrin. Cypermethrin killed larvae slowly. Although all MS larvae treated with 1.0 ug cypermethrin died, survival after 24, 48, and 72 h was 100, 98, and 88 % respectively. Of larvae treated with 0.1 ug, few had died or completely recovered after 24 h and most had intermediate response scores (Fig. 1). After 120 h, most were either dead or completely recovered. Of larvae debilitated but still alive after 120 h, the proportion that recovered increased with score, but on average, one-third recovered and two-thirds died. The various death criteria varied in their ability to predict ultimate response to treatment with cypermethrin (Table 3). The number of larvae incorrectly classified as alive (because they subsequently died) was greatest for the strictest death criterion (0/1). Conversely, few larvae were incorrectly classified as alive with the least strict death criterion (9/10). The number of larvae incorrectly classified as dead showed the opposite pattern; the strictest death criterion resulted in the fewest number of larvae incorrectly classified as dead and the least strict death criterion resulted in the greatest number of larvae incorrectly classified as dead. Intermediate death criteria were most accurate at all observation times. Table 3 also

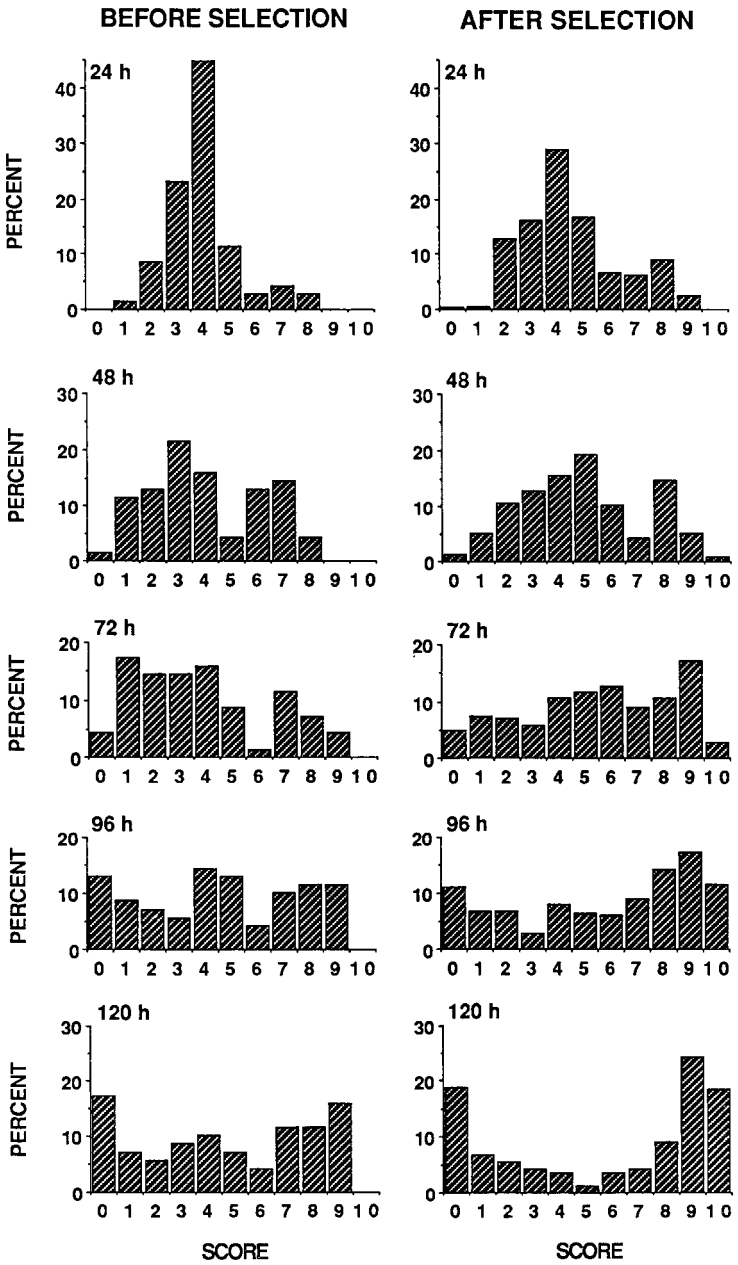

FIG. 1. Frequency histograms (percent) of tolerance scores of MS larvae treated with 0.1 ug cypermethrin and observed 24, 48, 72, 96, and 120 h after treatment.

TABLE 3. Number of MS Larvae Incorrectly Classified as Alive or Dead, Total Percent Error (Total Incorrect / Total), and Correlation Coefficients Between Mortality Classifications (Observation Time and Death Criterion) and Final Condition.

		Before Selection (n=241)				After Selection (n=345[a])			
		number of incorrect				number of incorrect			
Obs (h)	Death crit[b]	live	dead	percent error[c]	corr. coef.[d]	live	dead	percent error	corr. coef.
24	0/1	66	0	27.4 a	0.10	125	1	36.5 ab	0.04ns
	1/2	54	3	23.6 ab	0.32	123	1	36.0 ab	0.06ns
	2/3	44	7	21.2 ab	0.41	95	17	32.4 ab	0.23
	3/4	29	18	19.5 bc	0.49	62	40	29.6 a	0.34
	4/5	21	31	21.6 ab	0.49	29	107	39.4 bc	0.28
	5/6	19	42	25.3 ab	0.44	9	145	44.6 cd	0.30
	6/7	11	57	28.3 a	0.46	5	164	48.9 de	0.27
	7/8	6	93	41.1 d	0.35	3	183	53.9 ef	0.22
	8/9	0	142	58.9 e	0.24	0	211	61.2 fg	0.12
	9/10	0	171	71.0 f	0.07ns	0	220	63.8 g	0.00
48	0/1	62	0	25.7 a	0.23	121	0	35.1 ab	0.14
	1/2	45	1	19.1 ab	0.49	105	2	31.0 a	0.30
	2/3	37	7	18.3 b	0.51	74	7	23.4 c	0.48
	3/4	29	11	16.6 b	0.56	48	25	21.1 c	0.53
	4/5	20	19	16.2 b	0.60	20	51	20.6 c	0.59
	5/6	14	31	18.7 ab	0.58	6	104	31.8 a	0.48
	6/7	12	37	20.4 ab	0.56	4	137	40.9 bd	0.38
	7/8	5	57	25.8 a	0.54	3	151	44.7 d	0.34
	8/9	2	86	36.5 c	0.44	1	200	58.3 e	0.17
	9/10	0	167	69.3 d	0.11ns	0	217	62.9 e	0.07ns
72	0/1	55	0	22.8 ab	0.37	110	2	32.5 ab	0.25
	1/2	44	0	18.3 ac	0.52	84	2	24.9 cd	0.46
	2/3	39	2	17.0 acd	0.55	63	5	19.7 ce	0.58
	3/4	31	2	13.7 cde	0.64	46	8	15.6 e	0.66
	4/5	19	7	10.8 e	0.72	26	25	14.7 e	0.68
	5/6	14	14	11.6 de	0.71	12	51	18.3 e	0.65
	6/7	11	23	14.1 cde	0.67	5	88	26.9 ad	0.55
	7/8	7	36	17.8 acd	0.63	3	117	34.8 b	0.46
	8/9	6	64	29.1 b	0.49	3	154	45.5 f	0.33
	9/10	0	164	68.0 g	0.13	1	211	61.5 g	0.09ns
96	0/1	49	0	26.3 ab	0.46	89	2	26.4 a	0.43
	1/2	42	0	17.4 ab	0.55	67	3	20.3 ab	0.57
	2/3	34	0	14.1 acd	0.64	48	7	15.9 bc	0.65
	3/4	24	1	10.4 cd	0.74	40	9	14.2 c	0.69
	4/5	16	6	9.1 c	0.77	23	20	12.5 c	0.73
	5/6	13	11	10.0 cd	0.75	10	29	11.3 c	0.77
	6/7	11	15	10.8 cd	0.74	7	47	15.6 bc	0.70
	7/8	9	28	15.3 ad	0.66	4	75	22.9 a	0.61
	8/9	6	53	24.5 be	0.54	3	123	36.6 d	0.44
	9/10	0	160	66.4 f	0.15	2	182	53.4 e	0.24

TABLE 3 (Continued).

Obs (h)	Death crit[b]	Before Selection (n=241) number of incorrect live	dead	percent error[c]	corr. coef.[d]	After Selection (n=345[a]) number of incorrect live	dead	percent error	corr. coef.
120	0/1	40	1	21.0 a	0.56	46	0	18.1 ab	0.63
	1/2	32	1	13.7 ab	0.65	30	1	12.2 ac	0.75
	2/3	23	3	10.7 bc	0.72	18	3	8.3 cd	0.82
	3/4	21	4	10.4 bc	0.73	10	6	6.3 d	0.86
	4/5	14	4	7.5 c	0.81	4	9	5.1 d	0.89
	5/6	11	9	8.3 bc	0.79	4	12	6.3 d	0.87
	6/7	10	11	8.7 bc	0.78	2	19	8.3 cd	0.84
	7/8	7	22	12.0 abc	0.73	1	29	11.8 ac	0.78
	8/9	7	34	17.0 a	0.64	1	52	20.9 b	0.65
	9/10	1	86	36.1 d	0.46	0	113	44.5 e	0.37

[a]For the 120 h observation, n = 254.
[b]Maximum/minimum scores for larvae classified as dead/alive.
[c]Percent errors within columns and death criteria followed by the same letter are not different (p = 0.05, G-test).
[d]All correlations significant (p = 0.05) except "ns".

shows correlations between actual (final) mortality and mortality classifications made at the 24, 48, 72, 96 and 120 h observation times using various death criteria.

The ability to classify larvae accurately as dead or alive increased with time regardless of death criterion (Table 4). Percent error in classifying larvae was smaller with later observation times; in each case, the 120 h observation had the smallest error and was significantly better than either the 24 or 48 h observation. In most cases, the 120 h observation was significantly better than the 72 h observation. These results suggest that mortality classifications of tobacco budworm larvae treated with cypermethrin should be made no less than 72 - 96 h after treatment; observations made before 72 h could result in significant error in mortality and subsequent errors in estimates of population tolerance.

In some cases, estimates of knockdown made shortly after field applications of cypermethrin may provide adequate estimates of field efficacy because many larvae that are knocked down probably die. Although some larvae that are only knocked-down may recover, they may succumb to other sources of mortality (e.g., predators, excessive heat) because of their weakened condition and sublethal effects (Haynes 1988). However, these data show that the ability to withstand the debilitating effects of cypermethrin for a sustained period of time (e.g., 4 days) and still recover is an important aspect of tolerance in tobacco budworm. We demonstrated that the ability to survive for a longer time is an inherited trait by successfully selecting for this trait. These results suggest that the most precise method for estimating long-term population consequences of cypermethrin applications are different from the most precise methods for estimating the short-term response and benefits of cypermethrin applications.

TABLE 4. Percent Error Of Mortality Classifications Observed 24, 48, 72, 96, and 120 h After Treatment.

Death Criterion (dead/alive)	observation time (h)	Percent error[a] before[b] selection	Percent error[a] after selection
3/4	24	19.5 a	29.6 a
	48	16.6 a	21.1 b
	72	13.7 a	15.6 bc
	96	10.4 b	14.2 c
	120	10.4 b	6.3 d
4/5	24	21.6 a	39.4 a
	48	16.2 ab	20.6 b
	72	10.8 bc	14.7 c
	96	9.1 c	12.5 c
	120	7.5 c	5.1 d
5/6	24	25.3 a	44.6 a
	48	18.7 a	31.8 b
	72	11.6 b	18.3 c
	96	10.0 b	11.3 d
	120	8.3 b	6.3 e
6/7	24	28.3 a	48.9 a
	48	20.4 b	40.9 b
	72	14.1 bc	26.9 c
	96	10.8 c	15.6 d
	120	8.7 c	8.3 e

[a]Values within columns and death criterion followed by the same letter not significantly different ($p = 0.05$, G-test).
[b]Larvae weighed 10 - 36 mg; before selection, $n = 69$; after selection, $n = 345$.

If estimates of population tolerance (e.g., LD_{50}) are based on early observation times (e.g., 72 h after treatment with moribund larvae classified as alive), significant error in mortality classifications may result regardless of death criterion. In these toxicity tests, greatest error resulted when death criteria were extreme (i.e., when either only clearly dead larvae or all but completely recovered larvae were classified as dead). If results based on early observation times are used to predict the long-term effects of applications of cypermethrin (e.g., predict changes in tolerance within populations), they will have little predictive power. Short of observing treated larvae until they reach the pupal stage, mortality classifications based on intermediate death criteria, made at least 96 h after treatment, should most accurately predict ultimate fate of treated larvae, and would be most useful for predictions of long-term effects of field applications of cypermethrin intended to control populations of tobacco budworm.

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