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Effects of 5-aza-2′-deoxycytidine on biological parameters of *Achroia grisella* F. (Lepidoptera: Pyralidae)

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Abstract: The non-target effects of 5-aza-2′-deoxycytidine (5-aza-dC), an epigenetically effective agent, were assessed on different life-history traits of two successive generations of *Achroia grisella* F. (Lepidoptera: Pyralidae) by trophic exposure. The results did not reveal any prominent effect of 5-Aza-dC on emergence times and morphological disorders of offspring of both sexes, and dry weight of F_1 females (except for 0.1 mg/ml) and males according to controls. However, 5-Aza-dC caused a considerable decrease in wet weight of F_1 females at >0.1 mg/mL and in F_1 males only at 0.5 mg/mL. The mean longevity of F_1 and F_2 females was almost unchanged after exposure to 5-Aza-dC treatment. However, the longevity was considerably shorter, by 16% at a dose of 0.75 mg/mL for F_1 males and 28% longer at 1.0 mg/mL for F_2 males with respect to the controls. When the two generations were compared with each other in terms of adult longevity, the differences were not significant for the longevity of females, whereas F_2 males lived significantly longer than F_1 males in all groups except for the control and 0.5 mg/mL groups. 5-Aza-dC also markedly decreased the total number of both offspring but no dose-related alterations were observed. Analysis of the data for the number of viable and damaged eggs laid per F_1 females revealed that 5-Aza-dC adversely affected the reproductive potential of *A. grisella* based on daily and three-day observations. The most striking effect was a decline in fecundity of females by 57% at 1.0 mg/mL. These observations suggest that 5-Aza-dC has a negative effect on developing moth progeny across trophic levels.

Key words: 5-aza-2′-deoxycytidine; developmental time; longevity; reproductive potential

INTRODUCTION

Each chemical used for different purposes has its own physical, chemical and biochemical properties. Even newer compounds with less-threatening toxicological features can have harmful effects on human health and other non-target species. Though initially chemotherapeutic agents, 5-Aza-2′-deoxycytidine (5-Aza-dC) and 5-azacytidine (5-AzaC) have also shown to be mutagenic or cytotoxic and can induce cancer [1-4]. Moreover, the adverse effects of the drugs could be transferred to subsequent generations that were not exposed to the chemicals, showing transgenerational epigenetic inheritance [5]. Thus, the effect of the drugs on the population density of organisms has been noted, and chemicals such as 5-Aza-dC could be toxic from an environmental point of view.

Research into the toxic effects of 5-Aza-dC has mostly been undertaken in vertebrates rather than invertebrates. Besides, very little has been published on the effects of 5-Aza-dC on insect species [6-11]. The lesser wax moth, *Achroia grisella* F. (Lepidoptera: Pyralidae), is one of the economically important pests of wax and is frequently used as a model organism to evaluate the effects of toxic substances on both target and nontarget species [7,12]. There are four developmental stages of *A. grisella*, including egg, larva, pupa and adult. Being the most harmful developmental stage, the newly hatched larva immediately begins to eat. Caterpillars of this insect damage beehives by leaving silk-lined tunnels or galleries in the combs and by feeding on wax, honeycombs and pollen. On the other hand, this moth has an important role in the ecosystem and is often preferred in biological control studies as they are the natural hosts of some beneficial insects [13,14]. In the present study, *A. grisella* was selected as a model organism to investigate the possible adverse effects of 5-Aza-dC on insects. The effects of

5-Aza-dC on egg-to-adult developmental time of *A. grisella* and on some biological parameters of larval endoparasitoid *Apanteles galleriae* Wilkinson (Hymenoptera: Braconidae) have been determined before [7]. Here, I have developed my investigations in detail to show the possible deleterious effect of the cytosine analog 5-Aza-dC on both F_1 and F_2 individuals of *A. grisella*. Studies investigating the effect of toxicants such as 5-Aza-dC on different generations could help us to acquire a better understanding about the toxic effects of this and other similar chemicals on insects and may be of value for similar studies with other invertebrate species.

MATERIALS AND METHODS

Insects and bioassay

A. grisella cultures were established from adults that were collected from the honeycombs maintained in several beehives located in the vicinity of Rize, Turkey. The wax moth cultures and the experimental groups were held in two different rearing rooms at 25±1°C, 60±5% relative humidity and a photoperiod of 12 h. Uçkan and Gülel [13] and Uçkan and Ergin [14] were followed for cultivating *A. grisella*. The effects of 5-Aza-dC (EEC no.219-089-4, Sigma-Aldrich, St. Louis, MO) on the biological parameters of F_1 and F_2 individuals of *A. grisella* were investigated according to the method designed by Uçkan et al. [7]. Briefly, 5-Aza-dC solutions (0.1, 0.5, 0.75 and 1.0 mg/ml) were prepared in distilled water and then added to the insect diet [15,16] as the water source. *A. grisella* cultures reared on a chemical-free diet were used as controls. Different doses of 5-Aza-dC and controls were added to each of 1-L jars containing 30 g of the diet. Newly emerged females and males (1- to 3-days-old) were removed from the stock culture and placed in jars to provide a mating and oviposition.

F_1 offspring

Three newly emerged parent females and males were held in 1-L jars containing 5-Aza-dC-treated diet or distilled water (control group) for five days. All jars were observed daily until F_1 adult female and male emergence. The time required for completion of development from egg deposition of parent females to adult eclosion was recorded as the egg-to-adult developmental time for F_1 offspring. After the first adult eclosion, insect cultures were controlled for ten days on a daily basis to determine the total number of F_1 female and male adults. Each experimental group was also examined for morphological disorders (reduced body length, curved wings, etc.). For examining the effects of 5-Aza-dC on the weight and adult longevity of *A. grisella*, newly emerged F_1 female and male adults were collected from the jars during a period of ten days. Twenty pairs of fresh-weighed adults were placed in 80-mL cups in four replicates and observed daily until death. Then the adults were weighed again and the values were recorded as the dry weight. In a parallel set of experiments, the longevity of newly emerged F_1 female and male adults was assessed by placing 12 individual mating pairs in another 80-mL cup. They were observed daily, and the longevity of each individual was recorded.

To determine the adult fecundity and fertility of F_1 females after 5-Aza-dC exposure, individual mating pairs of *A. grisella* adults was kept in 80-mL cups that were covered with gauze, which allows the circulation of air. Females laid eggs on the surface of the paper through the gauze. The papers placed on the gauze were changed daily for three days and the number of eggs on the gauze was counted for viable and damaged ones. Then the three pieces of paper with the eggs were transferred into another 80-mL cup containing 1.5 g of natural blackened comb. Hatching larvae migrated from the papers to the comb. The hatchability of eggs was calculated at the end of the seventh day after being placed in natural blackened comb jars. In addition, three types of unhatched eggs were distinguished according to the microscopic observation: (i) dark-colored eggs in which dead larvae were observed, (ii) global and transparent-looking unfertilized eggs in which no development was observed, and (iii) wrinkled, dried and rigid-shaped eggs that were described as damaged eggs. Fecundity was expressed as the total number of eggs per female based on daily and three-day counts of all tested groups. The percent fecundity, percent fertility and corrected percent sterility [37] of F_1 females were also calculated.

F_2 offspring

Hatched larvae of F_1 females that were put into the jars with natural blackened comb were observed daily until the F_2 adult emergence to determine the egg-to-adult developmental time for F_2 offspring, which was recorded as the time elapsed from the day the second pieces of paper were placed into the jars to the first adult emergence. After the first adult eclosion, the jars were controlled every day to determine the total number of F_2 female and male adults for about two months until the adult emergence was completed; these individuals in the experimental and control groups were also examined for morphological disorders. To examine the effects of 5-Aza-dC on the adult longevity of F_2 offspring, newly emerged and mating pairs of adults were collected from the jars and placed in another 80-mL cup. All of the cups were observed at 24-h intervals until the death of individuals, and the times between adult emergence and death were recorded as female and male longevity.

The experiments were repeated four times with specimens chosen randomly from different populations at different intervals in terms of egg-to-adult developmental time, the total number of progeny, morphological disorders, adult weight (five pairs in each replicate), and adult longevity (three pairs in each replicate) of F_1 offspring. Nine randomly selected pairs of F_1 adults were also used to evaluate the effects of 5-Aza-dC on the fecundity and fertility of F_1 females and on the biological parameters of F_2 offspring for each dose and control group in the three replicates. When the jars with natural blackened comb were examined at the end of the seventh day, it was observed that all of the eggs of F_1 females had hatched. However, no adults were obtained from some of the jars except for the control and 0.1 mg/mL groups. The jars with no adult emergence were recorded as zero for the number of F_2 offspring and were not evaluated in terms of egg-to-adult developmental time, adult longevity and morphological disorders of F_2 offspring. Twelve pairs from F_2 offspring were examined in terms of adult longevity for the control, 0.1 mg/mL, and 0.75 mg/mL groups, while only four pairs in the 0.5 mg/mL and three pairs in the 1.0 mg/mL groups because of the inability to obtain F_2 female and male individuals on the same day.

Statistical analysis

The effect of 5-Aza-dC on the biological parameters of F_1 and F_2 offspring of *A. grisella* was tested with one-way analysis of variance (ANOVA). Tukey's honestly significant *post hoc* test (HSD) was used to compare the means according to the homogeneity of variances. An arcsine square-root transformation was conducted on the percentage values before analyses. The values of F_1 and F_2 generations were compared with each other in terms of adult longevity using the independent-samples t-test. An SPSS software program (SPSS 10.0 for windows) was used for data analysis. Results were considered statistically significant when $P<0.05$.

RESULTS

Effects of 5-Aza-dC on F_1 offspring

The effects of 5-Aza-dC on the egg-to-adult developmental time and adult weight of F_1 offspring are presented in Table 1. Female ($F=0.364$; df=4, 15; $P=0.830$) and male ($F=0.378$; df=4, 15; $P=0.821$) emergence

Table 1. 5-Aza-dC-related changes in the egg-to-adult developmental time and adult weight of F_1 *A. grisella*.

	Developmental time (d) [a]		Female weight (mg) [a]		Male weight (mg) [a]	
5-Aza-dC	Female	Male	Wet	Dry	Wet	Dry
(mg/mL)	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]
Control	46.50±1.32a	42.50±0.87a	21.40±0.97a	5.73±0.54a	10.24±0.41a	3.43±0.28ab
0.1	46.75±0.85a	42.75±0.48a	21.37±0.43a	7.92±0.65b	10.40±0.33a	3.27±0.17ab
0.5	45.75±1.18a	43.00±0.82a	16.94±0.33b	5.09±0.38a	8.45±0.20b	2.91±0.12b
0.75	45.00±1.23a	42.00±0.82a	18.20±0.78b	5.90±0.42a	9.38±0.42ab	3.18±0.16b
1.0	45.75±1.11a	42.00±0.58a	17.91±0.34b	6.45±0.35ab	10.06±0.16a	3.91±0.11a

[a]Means in each column followed by the same letter are not significantly different ($P>0.05$; d – day).
[b]Data are average of four replicates.

times were almost constant for all of the experimental groups. Female wet weight (F=11.036; df=4, 95; P=0.000) significantly decreased at all doses compared to the control and 0.1 mg/mL groups, while the dry weight (F=4.890; df=4, 95; P=0.001) considerably increased only at the dose of 0.1 mg/mL as compared to the untreated group. There was a significant decrease in the wet weight of males only at 0.5 mg/mL with respect to control group (F=6.143; df=4, 95; P=0.000), but the differences were not important for the dry weight relative to the control. However, the dry weight of males at 1.0 mg/mL was significantly higher than at doses of 0.5 and 0.75 mg/mL (F=4.395; df=4, 95; P=0.003) (Table 1).

The total number of progeny produced by three F_1 females during the period of ten days was 230±26 in the control group (Table 2). However, fecundity was noticeably lowered at all doses, except for 0.5 mg/mL (F=2.238; df=4, 15; P=0.114). The total number of females (F=2.329; df=4, 15; P=0.103) at 0.5 mg/mL was markedly higher than for all tested groups but the differences were not significant. The 5-Aza-dC treatment also caused an insignificant increment in female sex ratios (F=1.895; df=4, 15; P=0.164) at all doses (especially for 0.5 mg/mL), and a decrease in the number of males (F=1.995; df=4, 15; P=0.147). The sex ratio of adults was male-biased in all tested groups. The mean number of 62 F_1 females showed 3% morphological disorders in the control group while this ratio was 5-8% in the experimental groups. However, the differences in the morphological disorders were not significant for both female (F=0.721; df=4, 15; P=0.591) and male (F=1.565; df=4, 15; P=0.235) insects.

Table 2 also shows the effects of 5-Aza-dC on adult longevity of F_1 offspring. The mean longevity of females (F=0.394; df=4, 55; P=0.812) was almost unchanged after exposure to the 5-Aza-dC treatment, but the male adults lived shorter than the control group at all tested doses. However, the reduction in longevity was significant only at 0.75 mg/mL relative to the control (F=3.858; df=4, 55; P=0.008).

Effects of 5-Aza-dC on the fecundity of F_1 females

The total number of viable eggs based on three daily readings was noticeably decreased on the first day (F=1.544; df=4, 40; P=0.208), the second day (except for 0.1 mg/mL) (F=1.380; df=4, 40; P=0.258) and the third day (F=3.115; df=4, 40; P=0.025). However, the decline was significant only at 1.0 mg/mL when compared to the control based on the third-day observation. In addition, the reduced number of damaged eggs was important only at 1.0 mg/mL on the first day relative to the control group (day 1: F=3.239; df=4, 40; P=0.022; day 2: F=1.324; df=4, 40; P=0.278; day 3: F=2.412; df=4, 40; P=0.065). The three-day results showed that the total number of viable (F=3.851; df= 4, 40; P=0.010) and damaged (F=3.317; df=4, 40; P=0.019) eggs had decreased after exposure to 5-Aza-dC. However, the decline was significant only at 1.0 mg/mL for viable eggs, and at 0.75 and 1.0 mg/mL for damaged eggs relative to the untreated groups (Table 3). Moreover, 5-Aza-dC also caused prominent changes in the number of unhatched eggs when compared to the control (the ratio of damaged eggs: F=1.536; df=4, 40; P=0.210; unfertilized eggs: total number: F=1.028; df=4, 40; P=0.405, ratio: F=0.415; df=4, 40; P=0.797). However, significant changes were obtained only from the dead larvae, with a reduction observed for 0.5 and 1.0 mg/mL compared to the control group when the ratios of unhatched eggs were evaluated (dead larvae: total number: F=3.125; df=4, 40; P=0.025, ratio: F=2.977; df=4, 40; P=0.030) (Table 4).

5-Aza-dC-related changes in the reproductive potential of F_1 *A. grisella* females based on the three daily results are shown in Table 5. The fecundity of a single female fed on a chemical-free diet was 101.56±14.43. The highest egg value of 91.78±12.99 for 0.1 mg/mL and the lowest egg value of 43.67±12.93 for 1.0 mg/mL were obtained after the 5-Aza-dC treatment of the parent insects. However, the decreases in fecundity (F=3.952; df=4, 40; P=0.009) of F_1 females was statistically significant only at 1.0 mg/mL. The relative fecundity percentage (F=5.258; df=4, 40; P=0.002) of the experimental groups exhibited a tendency to decline when the percentage of fecundity of the control group was assumed to be 100%. The decline was significant only at doses of 0.5 and 1.0 mg/mL, and at 1.0 mg/mL relative to 0.1 mg/mL. The mean number of eggs hatched (F=2.944; df=4, 40; P=0.032) decreased significantly only at 1.0 mg/mL with respect to the control and the 0.1 mg/ml groups, whereas 5-Aza-dC caused an insignificant increase in the percentage of fertility (F=0.537; df=4, 40; P=0.709) relative to the control group. The corrected percent of sterility of F_1

Table 2. 5-Aza-dC-related changes in the number of offspring, sex ratio, adult longevity and morphological disorders in F_1 *A. grisella*.

5-Aza-dC (mg/mL)	No. of offspring and sex ratio[a]				Adult longevity (d) [a]		Morphological disorders (%)[a]	
	Female	Male	Total no. of progeny	Female sex ratio (%)	Female	Male	Female	Male
	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]
Control	62.00±18.87a	167.75±23.47a	229.75±26.23a	26.70±6.21a	9.42±1.15a	17.50±0.57a	2.68±1.01a	5.30±2.07a
0.1	54.00±3.49a	110.00±12.13a	164.00±15.30a	33.20±1.25a	9.50±0.82a	16.83±0.52ab	7.71±4.31a	6.97±1.23a
0.5	97.00±5.10a	157.75±4.29a	254.75±9.31a	38.01±0.68a	9.00±0.28a	14.83±0.99ab	6.27±2.63a	6.83±0.75a
0.75	66.25±11.35a	141.25±32.66a	207.50±43.44a	33.54±2.83a	9.00±0.65a	14.67±0.57b	4.88±1.38a	7.67±1.06a
1.0	62.75±8.29a	105.25±13.32a	171.25±20.17a	36.52±1.22a	10.17±0.65a	17.00±0.59ab	7.73±1.75a	3.85±0.26a

[a]Means in each column followed by the same letter are not significantly different (P>0.05; d – day).
[b]Data are average of four replicates.

Table 3. 5-Aza-dC-related changes in the total number of eggs laid by F_1 females of *A. grisella* based on three daily results.

5-Aza-dC (mg/mL)	Time posttreatment (day) [a]							
	Day 1		Day 2		Day 3		Total no. of eggs/female[a]	
	Viable eggs	Damaged eggs	Viable eggs	Damaged eggs	Viable eggs	Damaged eggs	Viable eggs	Damaged eggs
	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]
Control	30.33±6.35a	6.11±1.75a	26.56±8.43a	4.00±1.19a	31.00±6.32a	3.56±0.99a	87.89±13.08a	13.67±2.71a
0.1	26.33±7.45a	1.89±0.68ab	36.00±9.27a	4.22±1.61a	22.11±7.00ab	1.22±0.36a	84.44±11.75ab	7.33±1.85ab
0.5	16.00±4.24a	2.56±1.00ab	16.33±4.67a	1.56±0.77a	12.56±5.10ab	2.11±0.82a	44.89±10.69ab	6.22±1.76ab
0.75	10.11±3.94a	1.78±0.85ab	20.78±6.51a	1.33±0.47a	22.44±6.67ab	2.00±0.65a	53.33±11.30ab	5.11±1.17b
1.0	16.67±9.53a	1.33±0.75b	16.33±5.31a	3.67±1.60a	4.89±1.81b	0.78±0.36a	37.89±11.75b	5.78±1.72b

[a]Means in each column followed by the same letter are not significantly different (P>0.05).
[b]Average of eggs laid by nine F_1 females per treatment.

Table 4. 5-Aza-dC-related changes on the fertilization rate of unhatched eggs laid by F_1 females of *A. grisella*.

5-Aza-dC (mg/mL)	Total no. of unhatched eggs/female[a]					
	Dead larvae		Unfertilized eggs		Damaged eggs	
	Total no.	Ratio (%)	Total no.	Ratio (%)	Total no.	Ratio (%)
	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]
Control	8.78±2.73a	10.01±3.14a	2.44±0.90a	3.13±1.04a	13.67±2.71a	13.81±2.07a
0.1	8.00±2.91a	8.37±2.26a	2.67±0.82a	3.36±1.56a	7.33±1.85ab	8.02±1.30a
0.5	1.11±0.59b	2.29±1.20b	2.00±0.69a	3.59±1.30a	6.22±1.76ab	13.34±2.53a
0.75	7.56±3.14a	10.16±4.26a	3.56±1.85a	4.10±1.76a	5.11±1.17b	11.46±2.27a
1.0	0.44±0.34b	0.50±0.33b	0.67±0.29a	1.77±0.92a	5.78±1.72b	20.03±6.71a

[a]Means in each column followed by the same letter are not significantly different (P>0.05).
[b]Average of unhatched eggs laid by nine F_1 females per treatment.

Table 5. 5-Aza-dC-related changes in the reproductive potential of F_1 *A. grisella* females based on three daily results.

5-Aza-dC (mg/mL)	Total no. of eggs/ female[a]	% Fecundity[a]	No. of eggs hatched/ female[a]	% Fertility[a]	Corrected % sterility[a]
	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]	($\bar{x}$ ± SE) [b]
Control	101.56 ± 14.43a	100.00 ± 0.00a	76.67±13.73a	73.05 ± 4.13a	---
0.1	91.78 ± 12.99ab	90.37 ± 12.79ab	73.67±10.96a	80.13 ± 4.04a	-9.69 ± 5.52a
0.5	51.11 ± 12.07ab	50.33 ± 11.88bc	41.89±10.33ab	81.02 ± 2.98a	-10.92 ± 4.07a
0.75	58.44 ± 12.21ab	57.55 ± 12.02abc	42.22±9.07ab	74.29 ± 5.35a	-1.69 ± 7.33a
1.0	43.67 ± 12.93b	43.00 ± 12.73c	36.78±11.48b	77.70 ± 6.60a	-6.36 ± 9.04a

[a]Means in each column followed by the same letter are not significantly different (P>0.05).
[b]Average of nine individuals per treatment.

females (F=0.373; df=3, 32; P=0.773) was higher at 0.75 and 1.0 mg/mL, but insignificant when compared to the others (Table 5).

Effects of 5-Aza-dC on F_2 offspring

An insignificant reduction in the number of females, males and total progeny was observed in the F_2 offspring from the F_1 *A. grisella* that was supplemented with 5-Aza-dC and also in the adult ratio of hatched F_1 eggs at all doses except for 0.1 mg/mL. When the experimental groups were compared with each other, there was a significant decrease only at 1.0 mg/mL relative to 0.1 mg/mL in terms of the total number of females (F=3.146; df=4, 40; P=0.024), males (F=3.179; df=4, 40; P=0.023), progeny (F=3.460; df=4, 40; P=0.016), and the adult ratio (F=3.099; df=4, 40; P=0.026) of F_2 offspring. Similarly, the 5-Aza-dC treatment also caused a significant decrease in the female sex ratio (F=3.322; df=4, 40; P=0.019) of F_2 offspring at the 1.0 mg/mL dose relative to 0.1 and 0.5 mg/mL, but the differences were not statistically important with respect to the control (Table 6).

The effects of 5-Aza-dC on egg-to-adult developmental time, adult longevity and the morphological disorders of F_2 offspring are presented in Table 7. The developmental time (F=0.560; df=4, 31; P=0.693) and female longevity (F=0.368; df=4, 38; P=0.830) were almost constant in all tested groups; however, the male

Table 6. 5-Aza-dC-related changes in the number of F_2 offspring and the sex ratio of *A. grisella*.

5-Aza-dC	No. of females[a]	No. of males[a]	Total no. of progeny[a]	Adult ratio (%)[a]	Female sex ratio (%)[a]
(mg/mL)	($\bar{x}$ ± SE)[b]	($\bar{x}$ ± SE)[b]	($\bar{x}$ ± SE)[b]	($\bar{x}$ ± SE)[b]	($\bar{x}$ ± SE)[b]
Control	5.67±1.61ab	5.22±1.41ab	10.89±2.88ab	13.59±2.75ab	44.19±6.92ab
0.1	6.67±1.55a	7.67±2.26a	14.33±3.47b	19.26±3.07b	48.15±3.65a
0.5	3.11±1.42ab	2.22±1.16ab	5.33±2.57ab	10.08±3.18ab	52.89±11.88a
0.75	3.22±1.21ab	2.67±0.94ab	5.89±2.14ab	9.86±3.04ab	35.79±9.11ab
1.0	0.78±0.36b	1.67±0.76b	2.44±1.09a	5.45±2.51a	15.28±6.66b

[a]Means in each column followed by the same letter are not significantly different (P>0.05).
[b]Average of F_2 adults obtained from nine F_1 females per treatment.

Table 7. 5-Aza-dC-related changes in egg-to-adult developmental time, adult longevity and morphological disorders of F_2 *A. grisella*.

	Developmental	Adult longevity (d)[a]		Morphological disorders (%)[a]	
5-Aza-dC	time (d)[a]	Female	Male	Female	Male
(mg/mL)	($\bar{x}$ ± SE)[b]	($\bar{x}$ ± SE)[b]	($\bar{x}$ ± SE)[b]	($\bar{x}$ ± SE)[b]	($\bar{x}$ ± SE)[b]
Control	59.11±4.82a	7.50±0.50a	16.92±0.75a	8.04±6.25a	3.67±1.91a
0.1	53.22±4.22a	8.08±0.42a	19.92±0.61ab	9.00±5.47a	7.86±3.92a
0.5	56.38±5.19a	8.00±1.08a	18.00±1.58ab	0.89±0.89a	1.82±1.82a
0.75	56.33±4.42a	7.50±0.50a	19.75±0.81ab	5.56±5.56a	2.38±2.38a
1.0	48.50±2.47a	7.00±1.53a	21.67±2.19b	8.33±8.33a	11.25±6.58a

[a]Means in each column followed by the same letter are not significantly different (P>0.05; d – day).
[b]Data are average of three replicates.

Table 8. 5-Aza-dC-related changes in adult longevity (day) between F_1 and F_2 individuals of *A. grisella*.

	Female					Male				
5-Aza-dC	F_1	F_2	Statistics (*t*-test)			F_1	F_2	Statistics (*t*-test)		
(mg/mL)	($\bar{x}$ ± SE)[a-b]	($\bar{x}$ ± SE)[a-b]	*t*	df	P	($\bar{x}$ ± SE)[a-b]	($\bar{x}$ ± SE)[a-b]	*t*	df	P
Control	9.42±1.15x	7.50±0.50x	1.535	22	0.139	17.50±0.57x	16.92±0.75x	0.617	22	0.543
0.1	9.50±0.82x	8.08±0.42x	1.539	22	0.138	16.83±0.52x	19.92±0.61y	-3.852	22	0.001
0.5	9.00±0.28x	8.00±1.08x	1.323	14	0.207	14.83±0.99x	18.00±1.58x	-1.624	14	0.127
0.75	9.00±0.65x	7.50±0.50x	1.827	22	0.081	14.67±0.57x	19.75±0.81y	-5.144	22	0.000
1.0	10.17±0.65x	7.00±1.53x	2.119	13	0.054	17.00±0.59x	21.67±2.19y	-3.017	13	0.010

[a]Means in the same horizontal row (x-y) followed by the same letter are not significantly different (P>0.05; *t*-test).
[b]Data are average of four and three replicates for F_1 and F_2, respectively.

adults lived longer than the control group at all examined doses. The increase in male longevity was significant only at 1.0 mg/mL (F=3.331; df=4, 38; P=0.020) relative to the untreated group. The percentages of morphological disorders of F_2 females (F=0.419; df=4, 30; P=0.793) and males (F=1.107; df=4, 28; P=0.373) fluctuated among the applied doses but the differences were not significant (Table 7).

When the two generations were compared with each other in terms of adult longevity, the differences were not significant for female longevity (P>0.05). On the other hand, the F_2 males lived longer than the F_1 males and the differences were also significant in all examined groups (P<0.05), except for the control and 0.5 mg/mL groups (Table 8).

DISCUSSION

5-Aza-dC exerts potentially dual effects on an organism; it is a more effective drug at low doses and it can be cytotoxic at higher doses, as is the case with the majority of chemicals [3,4,17,18]. The anticancer properties or the mutagenic potential of the cytosine analog 5-Aza-dC have always been more attractive properties for researchers rather than its possible toxic effects on insects [7-9]. Thus, the present study was carried out to investigate in detail the potential deleterious effects of 5-Aza-dC on different biological parameters of F_1 and F_2 individuals of *A. grisella*. 5-Aza-dC application did not cause significant changes in the egg-to-adult developmental times for either generation when compared to the control. These results are in agreement with those of Uçkan et al. [7] who reported that 5-Aza-dC application slightly increased the immature development of *A. grisella*. In the current study, the development of F_2 offspring took slightly longer than that of F_1 individuals in all tested groups. Because of the same elongation time in the immature development of control group, 5-Aza-dC is not likely to cause such differences between two generations. In addition, Uçkan et al. [7] also observed that 5-Aza-dC application rarely caused morphological disorders such as reduced body length, half- or curved wings that were not linked to sex in *A. grisella*. A detailed examination of the morphological disorders here in two generations showed that these deformities probably stemmed from the effects of the chemical on females (as it produced a prominent increase in F_1 progeny) rather than on males. Nevertheless, the effects of 5-Aza-dC on this parameter seem temporary since the prominent increase in F_1 females disappeared in F_2 females. Hence, it is clear that 5-Aza-dC does not have a toxic effect on the emergence time and morphological disorders of *A. grisella* across trophic levels according to the obtained results and according to Uçkan et al. [7]. However, 5-Aza-dC caused an increase in adult emergence time and a decrease in adult body size of *A. galleriae* reared on 5-Aza-dC-contaminated host species, *A. grisella* [7]. This situation proved once more that parasitoids were often more sensitive to toxicants than their hosts [12,16,19,20]. Furthermore, the prolonged immature developmental period of *A. galleriae* after exposure to chemicals may impair the survival of this parasitoid species due to the possibility of emergence in an unfavorable environment [7].

F_1 adults tended to lose their wet weight in all 5-Aza-dC-treated groups except for 0.1 mg/mL. However, the results did not reveal any considerable effect of 5-Aza-dC on the dry weight of F_1 females (except for 0.1 mg/mL) and males when compared to the control groups. Studies on *Galleria mellonella* L. (Lepidoptera: Pyralidae) [21] and *Lymantria dispar* L. (Lepidoptera: Lymantriidae) [22] larvae feeding on heavy-metal-contaminated food showed that larvae lose weight especially at high doses of exposure. The dose-wise decline in pupal weight in cypermethrin-treated groups at >5 ppm has also been observed in *G. mellonella* after a 7-day exposure of larvae to a diet containing cypermethrin [20]. In another study, weight loses in *Hermetia illucens* (L.) (Diptera: Stratiomyidae) were also shown after larval exposure to cyromazine and pyriproxyfen [23]. The wet weight loss in *A. grisella* may be attributed to the insufficient food supply because 5-Aza-dC-induced a decline in diet quality [7]. Also, the decrease in adult weight in response to 5-Aza-dC indicates that the size during early developmental stages of *A. grisella* was also affected, which also negatively affects the beneficial species that develop on this insect. Thus, the increased adult emergence time and decreased adult size, longevity and fecundity of *A. galleriae* reared on *A. grisella* larvae exposed to different doses of 5-Aza-dC [7] support this assumption. The developmental biology of biological control agents is considerably influenced by several

factors that depend on the host itself [12,20,24-27]. For instance, there is a positive relationship between host size and parasitoid size, and host size is also an effective factor influencing the sex ratio, longevity and fecundity of parasitoids [24,28-30].

Examining the effects of 5-Aza-dC on the longevity of F_1 and F_2 adults revealed that the chemical treatment significantly affected the longevity of *A. grisella*. Surprisingly, the longevity of F_1 males exposed to 5-Aza-dC tended to decrease and of F_2 males tended to increase more drastically compared to F_1 and F_2 females that displayed no significant difference in longevity at all of the doses when compared with the controls. The differences were considerably shorter by 16% at 0.75 mg/mL for F_1 males, and 28% longer at 1.0 mg/mL for F_2 males with respect to the controls. It seems that the differences in male longevity resulted from the chemical treatment rather than mating activity, because the females lived almost as long as the control at every tested dose. We have also found that 5-Aza-dC caused a significant decrease in adult longevity of *A. galleriae* reared on *A. grisella* larvae exposed to different doses of 5-Aza-dC [7]. A comparison of the longevity of *A. galleriae* [7] and *A. grisella* at different doses of 5-Aza-dC showed that the toxic effect of the chemical on longevity was higher at 0.75 and 1.0 mg/mL. It is very possible that the longevity of *A. grisella* and *A. galleriae* are affected by the increasing doses of 5-Aza-dC in diet. Although female longevity of *A. grisella* did not change after the chemical treatment, the 5-Aza-dC-induced effect on the longevity of both F_1 and F_2 males showed that males were more sensitive than females. We also concluded before that *A. galleriae* males have a slightly shorter life at the higher 5-Aza-dC doses [7] and were more susceptible than females. Sexual difference in susceptibility to chemicals has also been noted for other insect species, with males being generally more sensitive than females [31] or vice versa [12,20]. The differences may be attributed partly to the differences between sexes in terms of size and physiology. When two generations were compared to each other, the differences were not significant for the longevity of *A. grisella* females at any dose. On the other hand, F_2 males lived significantly longer than F_1 males, especially at higher doses as compared to the controls. 5-Aza-dC-induced stress seems to produce this adverse effect on the longevity of *A. grisella*. The affected longevity of *A. grisella* may cause unexpected consequences to the population of this moth in next generations by affecting the number of eggs, a changed mating time and activity. Moreover, the insignificant reduction in the total number of F_1 offspring essentially stemmed from the decline in the number of male progeny because of the 5-Aza-dC treatment. Similarly, 5-Aza-dC also decreased the total number of F_2 offspring (especially at the 1.0 mg/mL dose) despite of the decline in the number of both male and female progeny. Although the eggs of F_1 females in the entire natural blackened comb jars hatched, 11, 33, and 56% of the jars did not reach the adult stage at 0.5, 0.75, and 1 mg/mL doses, respectively. The negative effects of 5-Aza-dC on male longevity (especially significant at 0.75 mg/mL for F_1 and at 1.0 mg/mL for F_2 progeny) and on the total number of offspring of both sexes (more striking at 1.0 mg/mL for the F_2 progeny) indicates that the toxicity could be transferred to subsequent generations that were not exposed to the chemicals. Alternatively, the effects of 5-Aza-dC on longevity and the number of offspring could probably decrease the population rate of beneficial species, such as parasitoids dependent on host sources. In line with this, Uçkan et al. [7] showed that the most striking decline was in the number of *A. galleriae* emerging from the 5-Aza-dC-treated host larvae. The negative influence of other chemicals on parasitoids indirectly through host physiology was also reported in several studies [12,16,20,27,32-34].

Fecundity is an important parameter of an insect's life cycle and host fecundity has a vital role in parasitoid life [30,35]. *A. grisella* females that ingested 5-Aza-dC during the larval stage displayed the most striking response in the number of eggs laid per female and on the percentage fecundity. The lowering effect of the chemical on egg numbers (viable and damaged ones) was especially important at higher doses (0.75 and 1.0 mg/mL) based on three-day observations. Moreover, some females laid no eggs in all experimental groups, although the total number of eggs per F_1 female was minimally 41 and maximally 169 in the controls. In addition, 5-Aza-dC elicited a sharp decrease in percentage fecundity at >0.1 mg/ml doses as compared to the control; however, the effects on percentage fertility was to a lesser extent. Therefore, according to the current results it is obvious that 5-Aza-dC had an adverse activity on the reproductive potential of *A. grisella*. The insignificant but high

value of corrected percentage sterility at 0.75 and 1.0 mg/mL doses supports this inference. The decrease in egg fecundity of *A. grisella* can be attributed to the toxicity of the diet because of the increasing amount of 5-Aza-dC, which caused a decline in diet quality [7,20]. The adverse activity of chemicals on the reproductive potential of insects has been mostly attributed to chemical-based interference with the neurosecretory system [36,37]. The significant decrease in the percentage fecundity and the insignificant changes in the percentage of fertility, corrected by the percentage of sterility, also suggest that 5-Aza-dC could not cause the extinction of *A. grisella*. However, decreased host fecundity will eventually cause a decrease in the population rate of parasitoids dependent on the host sources. Previous data showing important decreases in the fecundity of the endoparasitoid *A. galleriae* obtained from 5-Aza-dC-treated host larvae [7] are consistent with this assumption. A reduction in the fecundity of parasitoids caused by chemicals in host species was also reported in other studies [12,27]. Therefore, a hidden damage that would further affect population density might have occurred when insects were exposed to chemicals by feeding.

Chemicals can affect biological parameters such as developmental time, weight and the total number of progeny [12,16,20,23,38], although insects can continue their development normally after the elimination of the inhibitory effects of toxicants. Feeding parent *A. grisella* with a diet containing 5-Aza-dC resulted in some adverse effects, especially on adult longevity, weight and egg fecundity, as well as on some biological parameters of the endoparasitoid *A. galleriae* [7]. Apart for our previous results [7], I could not find any other report showing the detrimental effects of 5-Aza-dC on the life parameters of insects. However, Amarasinghe et al. [8] reported that altered methylation by 5-Aza-dC caused an increased aggression and induced the development of ovaries in *Bombus terrestris* workers. It is a well-known fact that animals require high energy under stress conditions to use in repair mechanisms. Thereby, the decreases in metabolites to compensate the stress factors [16] could adversely affect the biological parameters of insects [12,16,20,38]. In line with these data, the stress-induced, trophic interaction of 5-Aza-dC seems to produce the abovementioned adverse effects on some biological parameters of *A. grisella* and its endoparasitoid, *A. galleriae* [7]. Chemicals disrupt the ecological balance among all living organisms in some way, even when they are produced for good purposes. This, in turn, may present a threat to the continuity of species in nature from an evolutionary perspective.

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