Arch Biol Sci. 2020;72(2):181-192 https://doi.org/10.2298/ABS200107012C

Effects of kinetin on biological parameters and hemocytes of *Achroia grisella* (Lepidoptera: Pyralidae)

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Received: January 7, 2020; **Revised**: March 20, 2020; **Accepted**: March 27, 2020; **Published online**: April 8, 2020

Abstract: The effects of the cytokinin hormone kinetin on the life history traits and hemocytes of the smaller wax moth *Achroia grisella* F. (Lepidoptera: Pyralidae) were examined in order to better understand the physiological impacts of plant growth regulators on insects. Based on the obtained results, it was found that kinetin did not lead to significant changes in larval mortality, development time, morphological disorders and egg fertility. Female and male longevity were almost unchanged when early instars were fed with a kinetin-added diet, and it tended to be higher for males when kinetin was applied at the egg stage, especially at 5 mg/L. The weight of females decreased significantly with 25 and 3000 mg/L of kinetin and that of males with 5 mg/L. The most striking effect was a considerable decline in the number of progeny, particularly at 400 and 3000 mg/L, as compared to the control. The injection of kinetin caused noticeable decreases in the number of hemocytes in the circulation at 3 and 24 h. This work presents the first evidence that kinetin adversely affects the development and the hemocyte counts of an economically-important host species, *A. grisella*.

Keywords: *Achroia grisella*; cytokinin; life history traits; morphological disorder; hemocyte number

INTRODUCTION

Over the years of developing pest control programs, scientists have started exploring eco-friendly methods. One of them is the application of plant growth regulators (PGRs), which were initially used only to contribute to the growth, differentiation and development of plants in limited agricultural areas. However, overuse of these natural and synthetic chemicals can have detrimental effects on environmental health as well as on plants. Hence, the availability of PGRs in nature has led researchers to investigate their neglected roles on other organisms. Kinetin, a member of the cytokinin class of plant hormones, is an amphoteric compound derived from adenine base [1,2]. The name was proposed because of its cell division-promoting activity in tobacco tissues [1,3,4]. After its discovery about 60 years ago [3], kinetin was classified in the category of pesticides by the Environmental Protection Agency (EPA) [5]. Despite the fact that it was purified from DNA during initial studies, kinetin (N^6-furfuryladenine) was assumed to be an unnaturally-occurring synthetic cytokinin [1]. However, kinetin was also detected in natural samples, including plants [6,7], human cell extracts and DNA [8] and human urine [9], with the help of recent technological developments using more sensitive techniques such as high-performance liquid chromatography with an electrochemical detector (HPLC/UV/EC) system, or mass-spectrometric analysis [8,9]. The data concerning the biological properties of kinetin are mostly related to plants. Kinetin induces tRNA synthesis, the cell cycle and the activity of cyclin-dependent kinases, and serves as an anti-stress molecule in plant cells [10,11]. Moreover, studies with both plants and animals showed its antioxidative and free radical scavenger characteristics and anti-aging properties [12-15]. Due to these three features, kinetin as the main component of cosmetic, pharmaceutical and cosmeceutical substances, was thought to be useable for human dermo-cosmetic research [2,10,11,16-18]. Because of the intriguing effects of kinetin, it is still the subject of much research including interactions in biological systems.

PGRs can have adverse effects on all living organisms even at lower toxicological values. Namely, kinetin and

similar cytokinins that are applied to plants in nature, can cause the accumulation in both target and non-target species through direct chemical contact or by feeding, and can also have considerable impacts at third trophic levels, such as fitness changes of natural enemies. Therefore, it has become necessary to investigate the possible deleterious effects of kinetin on both harmful and beneficial insect species as part of integrated pest management (IPM) studies. Kinetin research about insects has shown that the data were mostly related to the orders of Orthoptera [19], Hemiptera [20-23], Lepidoptera [24], and especially Diptera [12,13,25-31]. Yeşilada et al. [25-28] investigated the effects of kinetin, abscisic acid (ABA), gibberellic acid (GA_3) and indole acetic acid (IAA) on some biological features of Meigen and Oregon R strain of *Drosophila melanogaster* (Diptera: Drosophilidae). They found that kinetin added to the standard nutrient medium of *Drosophila*, especially at high doses (10^{-3} and 10^{-4} M), increased egg fecundity and the number of pupae and adults, while it also decreased the percentage viability of this insect [25,26]. When adult morphology was examined, it was observed that 10^{-3} M kinetin caused the highest rate of phenotypic abnormality [27] and decreased the mutant wing spots [28]. However, the detection of an increase in the number of wing spots at 10^{-4} M kinetin may indicate a dose-dependent mutagenic effect of this hormone [28]. In another fruit fly, *Zaprionus paravittiger* (Godbole and Vaidya) (Diptera: Drosophilidae), low doses of kinetin slowed down growth and ageing, improved lifespan, reduced egg fecundity and increased catalase activity, while high doses had toxic effects by shortening the lifespan of insects [12,13]. Kaur and Rup [29,30] treated *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) with four PGRs (kinetin, coumarin, GA_3 and IAA) and reported inhibitory effects on growth, development, adult emergence, longevity and reproductive potential of this fruit fly. Their results also revealed that the percentage of abnormal flies emerging increased [30] and quantitative changes in the biochemical profile (i.e. proteins, carbohydrates, hydrolytic enzymes) occurred [31] under exposure to these hormones. The most striking effect of PGRs on *B. cucurbitae* was with kinetin and coumarin, followed by GA_3 and IAA [29,30]. Evidence from earlier studies [25,26,29,30] strongly supports that kinetin has an inestimable effect on insects. Despite this, the biological effects of kinetin on insects have not yet been sufficiently investigated, so further studies on different types of insects are needed.

The smaller wax moth, *Achroia grisella* F. (Lepidoptera: Pyralidae), is a serious pest in beehives because its caterpillars feed on wax, honeycombs and pollen by leaving silk-lined tunnels or galleries in the combs. This holometabolic insect is frequently used as a model organism to evaluate the effects of toxic substances [32-38]. As summarized above, kinetin has adverse effects on some biological and biochemical properties of insects. However, we lack knowledge about its possible toxic effects on hemocytes, which are important elements of the insect immune system. Insects that encounter xenobiotics/toxic compounds or viruses respond effectively by means of innate immunity, which consists of cellular and humoral components. Hemocytes are more sensitive than other cells to different environmental factors [39-41] since they have multifunctional roles such as phagocytosis, nodulation, cell agglutination, and detoxification [42]. Hence, changes in the free hemocyte numbers in insect hemolymph are routinely used as an indicator when investigating the cytotoxic effects of chemicals such as PGRs [36,43,44]. To further increase our awareness of how insect biology and physiology can be influenced by PGRs, we studied the exposure response to different doses of kinetin in a lepidopteran wax moth, *A. grisella*. According to the literature, this is the first research that shows the kinetin-induced changes in the total hemocyte count (THC) and on some biological parameters (mortality, adult eclosion, total number of progeny, morphological disorders, weight, adult longevity and egg fecundity) of *A. grisella*.

MATERIALS AND METHODS

Insects

Stock cultures of *A. grisella* were obtained from adults reared on blackened honeycomb at 29±1°C and 60±5% relative humidity, with a photoperiod of 12:12h (L:D) in our laboratory (Balikesir University, Turkey). As for the original stocks, the insects were obtained from individuals collected from beehives in apicultural regions near Balıkesir. The details of the cultivation method were presented in articles by Uçkan and Gülel [45] and Uçkan and Ergin [46]. The stock cultures and

experimental groups were maintained on a diet described by Bronskill [47] and modified by Sak et al. [48].

Kinetin

Technical kinetin (CAS no. K3378-16, Sigma-Aldrich, St. Louis, MO) was used for experimental analysis. A stock solution of kinetin was prepared by first dissolving this hormone in 0.1 N NaOH (Sigma) and the final volume was adjusted with phosphate buffer saline (PBS; Sigma, pH=7.4). The other kinetin solutions were obtained by dilution with PBS from this stock solution (3000 mg/L) kept at +4°C in a refrigerator, and were used immediately in the experiments.

Treatment and bioassays

Three series of experiments were carried out to examine the effects of kinetin on this moth species. In the first one (toxicity test), different kinetin doses (5, 25, 50, 100 and 3000 mg/L) were prepared in distilled water and then added to the insect diet as the water source. For this purpose, five randomly selected early instars (1.8±0.6 mg) were removed from the stock culture and placed on a glass Petri dish (60x15 mm) containing 2.5 g of the kinetin-applied diet or a distilled water control (25 larvae for each tested group in the five replicates). All Petri dishes were held in another rearing room under the same conditions as mentioned for the stock cultures. Dead larvae and emerged adults were removed from the Petri dishes, after which larval mortality, larva-to-adult developmental time, adult weight and adult longevity for each dose were observed for 60 days.

The inert larvae that did not respond to a mechanical stimulus were designated as dead. The time required for completion of development from the day when early instars were placed in the Petri dishes to the adult emergence was recorded as the larva-to-adult developmental time. To examine the effects of kinetin on adult weight and longevity of *A. grisella*, newly emerged individuals were collected from the Petri dishes and fresh-weighed. Female and male adults were placed in the same rearing room, but in separate 80-mL cups, and observed daily until their death. The time between adult emergence and death was recorded as the longevity of virgin adults. The dead individuals were kept in an incubator set to 50±1°C for 24 h to determine their dry weights [49].

The 2nd round of experiments

In the 2nd series of experiments, the adverse effects of kinetin on *A. grisella* were investigated in more detail with only three selected doses (5, 400 and 3000 mg/L) because of kinetin-independent deaths in toxicity tests. For this, two newly-emerged females and males (0- to 1-day-old) were removed from the stock cultures and placed in 1-L jars containing 20 g of kinetin-treated diets or 20 g of three different control diets supplemented with distilled water, 0.1 N NaOH or PBS. The jars were maintained in the same rearing room under the same conditions as the Petri dishes. Adults were removed from the jars on the 5th day after mating and laying eggs. When F_1 larvae were observed in the jars, 20 g more of the kinetin-treated diet or NaOH- and PBS-treated control diets were added. Thus, this moth species was exposed to the chemicals by the diet two times after the egg stage. Moreover, 20-40 g of distilled water control diet were also added to all tested groups from time to time to prevent the larvae from starving. All jars were observed daily until the first adult eclosion and then checked for daily 10 days to determine the egg-to-adult developmental time, the total number of progeny, morphological disorders, adult weight, adult longevity and egg fecundity of *A. grisella*. For the egg-to-adult developmental time, the day after two parent adults were placed into the jars was recorded as the 1st day. Then, the time elapsed from this day until the 1st F_1 female or male emergence was determined. After the 1st adult eclosion, the jars were checked for 10 days to determine morphological disorders and the total number of F_1 female and male adults. The parameter of morphological disorder was counted in three ways, as follows: (i) with curved wings, (ii) shortened wings or (iii) as fully disrupted individuals having both curved and shortened wings or reduced body size. Unlike the toxicity tests, the effects of kinetin on adult weight and longevity of *A. grisella* were investigated along with the mating effect. For this purpose, newly-emerged and mating pairs of F_1 adults were collected from the jars, fresh-weighed and then placed in 80-mL cups. All cups, kept in the same room as the Petri dishes, were observed at 24-h intervals until the individuals died. The time between adult emergence and death was marked as female and male longevity. The dry weights of dead adults were measured as in the toxicity tests. In a parallel set of experiments, the fecundity

of newly emerged F_1 females was assessed by placing individual mating pairs in another 80-mL cup covered with gauze. A piece of paper was placed on the gauze and females were allowed to lay eggs on the surface of the paper through the gauze. The papers on the gauze were changed daily until the females died, while the eggs on the papers were counted under a microscope (Olympus CX21, Japan). The total number of eggs obtained from all of the papers was recorded as the egg fecundity of F_1 females.

The 3rd round of experiments

In the 3rd series of experiments, the influence of kinetin on THC, as an indicator of immunity, was studied with 4 different doses (5, 25, 400 and 3000 mg/L). Last instars (30±20 mg), obtained from the stock culture, were injected with 2 µL of kinetin solution on the 2nd hind leg by using a 5-µL Hamilton microsyringe (Hamilton, 75 N). Kinetin-applied larvae were held in plastic Petri dishes (60x15 mm) for 3 h or 24 h under the same laboratory conditions as for the toxicity tests. Controls consisted of untreated larvae, null-injected, 0.1 N NaOH-injected, PBS-injected, and larvae injected with a mixture of 0.1 N NaOH and PBS (NaOH-PBS-injected). THC was determined [50] as follows: briefly, 3 and 24 h post-injection, the hemolymph was taken from the 1st hind leg by a sterile 19-gauge needle. Then, 4 µL of obtained hemolymph were immediately mixed with an anticoagulant buffer to prevent clotting and the THC was detected under an Olympus CX21 (Olympus, Japan) microscope. The experiments were repeated four times for F_1 offspring to determine the egg-to-adult developmental time, the total number of progeny, morphological disorders and egg fecundity (10 pairs in each replicate). Also, the experiments were repeated three times with specimens chosen randomly from different populations at different intervals to analyze adult weight and longevity (10 pairs in each replicate), as well as the THC (10 larvae in each replicate).

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the means of the noted biological parameters (mortality, developmental time, total number of progeny, morphological disorders, weight, longevity, and fecundity) or the THC as the assumptions for the parametric tests were met. Tukey's *post hoc* (HSD) test was used to compare the means according to the homogeneity of variances. Dunnett's procedure was also used to determine if the means of the treatment groups differed from the control group. The THC at 3 and 24 h was compared by using the independent-samples *t*-test. SPSS software program (version 18.0 for Windows, SPSS Science, Chicago, IL) was used for data analysis. The results were considered statistically significant when $P<0.05$.

RESULTS

Toxicity of kinetin

The mortality percentage of *A. grisella* larvae after exposure to different doses of kinetin-supplemented diet is shown in Fig. 1. The mortality reached the highest value at 100 mg/L of kinetin and the lowest value at 3000 mg/L; however, the chemical concentration did not affect mortality according to one-way ANOVA (F=0.703; df=5, 24; P=0.627). In support of ANOVA, Dunnett's test showed that the mortality of the experimental groups did not differ significantly (P>0.05) from the controls. Therefore, the toxicity results showed that the deaths occurred independent of kinetin doses, and probit analysis could not be performed due to insufficient mortality ratios (Fig. 1). Similarly, the kinetin-related changes in some biological properties (larva-to-adult developmental time, adult longevity and adult weight) of *A. grisella* could be determined for the larvae reaching adult stage in

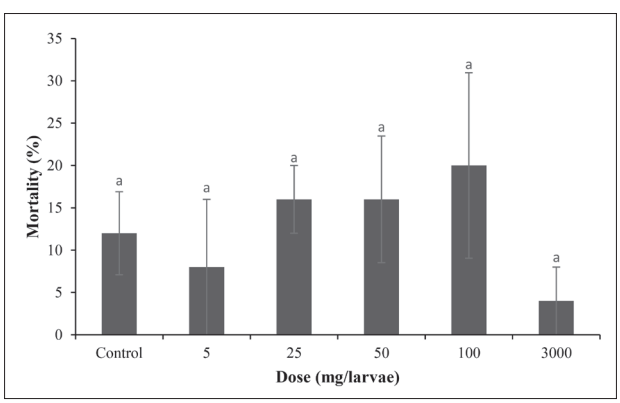

Fig. 1. Mortality of *A. grisella* after exposure to different doses of kinetin (mg/L).

toxicity tests that also showed an insignificant influence of kinetin (Table 1). The developmental time (F=0.424; df=5, 57; P=0.830) and longevity of adult females (F=0.645; df=5, 57; P=0.667) slightly decreased at all doses when compared to the control groups (Dunnett's test; P>0.05), while for the same parameters we found increases for males (developmental time: F=0.272; df=5, 62; P=0.927, longevity: F=0.577, df=5, 62; P=0.718). However, kinetin-dependent changes were not statistically significant (Dunnett's test; P>0.05). Insignificant decreases were also observed in the wet (F=2.712; df=5, 57; P=0.029) (except for 25 mg/L; Dunnett's test; P= 0.004) and dry weights of females (F=1.471; df=5, 57; P=0.214) at all tested doses when compared to the controls, while the wet (F=0.844, df=5, 62, P=0.524) and dry weights of males (F=0.194, df=5, 62, P=0.964) were almost constant (Table 1).

Effects of kinetin on F_1 offspring

Kinetin-related changes in F_1 *A. grisella* based on ten daily observations are shown in Tables 2-4. The developmental times of females (F=0.431; df=5, 18; P=0.821) and males (F=0.523; df=5, 18; P=0.756) were almost unchanged after exposure to kinetin. The longevity of females (F=0.821; df=5, 174; P=0.536) was also constant in all tested groups when compared to the control (Dunnett's test; P>0.05), while the longevity of males (F=2.650; df=5, 174; P=0.025) tended to be higher (significantly higher than the control longevity at 5 mg/L, Dunnett's test; P=0.017). Table 2 also shows that kinetin application decreased slightly the weights of female and male individuals. The reduction in the dry weight of females (F=1.485; df=5, 174; P=0.197) was not statistically significant when compared to the controls (Dunnett's test; P>0.05), however, Dunnett's test showed a significant decrease in wet weight only at 3000 mg/L (P=0.032). When the wet weights of females (F=4.198; df= 5, 174; P=0.001) were compared between different treatments, significantly lower values were observed for the NaOH-treated (Tukey test; P=0.013) and 3000 mg/L groups (Tukey test; P=0.004) than for 400 mg/L. Moreover, considerable decreases in the wet weights of males (F=3.482; df=5, 174; P=0.005) for NaOH-treated (Tukey test; P= 0.024), 5 mg/L (Tukey test; P= 0.025) and 3000 mg/L groups (Tukey test; P= 0.010) were observed, whereas for the dry weight (F=2.595; df=5, 174; P=0.027) a decrease was detected

Table 1. Kinetin-related changes in larva-to-adult developmental time, adult longevity and weight of *A. grisella*.

	Development time (day)*		Adult longevity (day)*		Female weight (mg)*		Male weight (mg)*	
Kinetin (mg/L)	Female	Male	Female	Male	Wet	Dry	Wet	Dry
	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†
Control	29.33±1.56a	24.30±2.57a	8.50±0.58a	15.50±0.50a	23.37±1.25a	4.64±0.28a	8.10±0.53a	1.35±0.09a
5	27.90±1.45a	25.85±1.57a	7.20±0.63a	16.69±0.92a	19.99±1.12a	3.71±0.30a	8.89±0.38a	1.38±0.07a
25	27.00±0.59a	25.40±0.99a	8.27±1.34a	16.70±0.54a	17.37±1.44a	3.55±0.31a	8.88±0.53a	1.34±0.09a
50	29.20±1.03a	26.82±1.38a	7.20±0.68a	17.00±0.74a	20.61±1.55a	3.95±0.35a	7.99±0.45a	1.29±0.09a
100	28.00±0.78a	25.64±1.36a	7.11±0.46a	15.45±1.18a	20.98±1.32a	4.50±0.66a	8.72±0.39a	1.36±0.09a
3000	29.36±2.54a	26.00±0.97a	7.82±0.33a	15.62±1.16a	19.32±1.16a	3.88±0.27a	8.65±0.30a	1.42±0.12a

*Means in each column followed by the same letter are not significantly different (P>0.05; Tukey's HSD test).
†Data are average of 25 individual in five replicates.

Table 2. Kinetin-related changes in the egg-to-adult developmental time, adult longevity and weight of F_1 *A. grisella*.

	Development time (day)*		Adult longevity (day)*		Female weight (mg)*		Male weight (mg)*	
Kinetin (mg/L)	Female	Male	Female	Male	Wet	Dry	Wet	Dry
	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†
Control	38.25±1.65a	36.00±1.08a	4.80±0.20a	11.00±0.42a	19.24±0.59ab	5.56±0.47a	9.59±0.25ab	2.05±0.17ab
NaOH	37.50±0.96a	36.25±0.95a	4.87±0.20a	12.37±0.45ab	17.36±0.55a	4.49±0.43a	8.77±0.28a	1.87±0.20ab
PBS	36.75±1.44a	35.50±1.19a	5.33±0.22a	12.43±0.29ab	17.80±0.49ab	5.18±0.49a	10.25±0.48b	2.11±0.18b
5	37.00±0.41a	34.75±0.25a	4.83±0.21a	12.83±0.41b	17.79±0.34ab	4.02±0.13a	8.77±0.21a	1.43±0.05a
400	37.25±0.63a	36.00±0.71a	4.90±0.22a	11.40±0.43ab	20.06±0.75b	5.21±0.51a	9.29±0.38ab	2.05±0.14ab
3000	36.25±0.48a	35.00±0.41a	4.77±0.32a	12.53±0.60ab	17.04±0.64a	5.19±0.62a	8.64±0.31a	1.78±0.16ab

*Means in each column followed by the same letter are not significantly different (P>0.05; Tukey's HSD test).
†Data are average of three replicates for adult longevity and weight and four replicates for developmental time.

Table 3. Kinetin-related changes in morphological disorders of F_1 *A. grisella*.

Kinetin (mg/L)	Female*				Male*			
	Curved wings	Shortened wings	Fully disrupted	Morpholog. disorders (%)	Curved wings	Shortened wings	Fully disrupted	Morpholog. disorders (%)
	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†
Control	4.25±1.03a	5.50±0.96ab	3.75±1.03ab	12.45±0.85a	2.25±0.25a	1.00±0.41a	1.25±0.63a	2.90±0.32a
NaOH	2.50±0.87a	3.00±0.58a	1.00±1.00ab	10.63±2.03a	1.50±0.29a	0.75±0.48a	0.50±0.29a	2.03±0.37a
PBS	8.50±3.66a	9.50±1.56b	5.25±1.70b	21.98±1.84b	1.50±0.65a	0.25±0.25a	1.25±0.48a	2.19±0.79a
5	2.00±0.00a	3.75±1.55ab	1.00±0.71ab	7.76±0.69a	1.75±0.48a	1.25±0.63a	1.25±0.95a	3.00±0.64a
400	2.00±0.41a	1.25±0.63a	1.00±0.00ab	9.44±1.30a	1.75±0.63a	0.00±0.00a	1.75±0.25a	4.04±1.17a
3000	2.75±1.55a	4.25±2.02ab	0.50±0.29a	12.29±3.93a	3.75±1.03a	0.75±0.25a	1.25±0.48a	5.38±0.99a

*Means in each column followed by the same letter are not significantly different (P>0.05; Tukey's HSD test).
†Data are average of four replicates.
Morpholog. – Morphological

Table 4. Kinetin-related changes in the number of offspring, sex ratio, and egg fecundity of F_1 *A. grisella*.

Kinetin (mg/L)	No. of offspring and sex ratio*				Egg Fecundity*	
	Female	Male	Total no. of progeny	Female sex ratio (%)	Total no. of eggs/ female	% Fecundity
	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†	($\bar{x}$ ± SE)†
Control	107.75±11.67a	155.00±13.05a	262.50±12.82a	41.07±3.98a	134.65±18.26a	100.00±0.00a
NaOH	59.00±11.11ab	136.00±8.54ab	194.75±10.87abc	29.86±4.77a	164.80±17.66a	122.39±13.11a
PBS	102.25±14.50ab	134.75±15.44ab	237.00±28.06ab	42.93±1.98a	181.03±20.89a	134.44±15.51a
5	85.75±17.16ab	144.00±4.55ab	229.75±18.38ab	36.30±4.48a	126.13±15.23a	93.67±11.31a
400	48.75±9.74b	98.75±14.29b	147.50±23.66c	32.59±1.97a	143.38±13.70a	106.48±10.18a
3000	54.00±8.44ab	108.75±4.17ab	162.75±4.33bc	32.84±4.23a	174.98±16.19a	129.95±12.03a

*Means in each column followed by the same letter are not significantly different (P>0.05; Tukey's HSD test).
†Data are average of four replicates.

only for 5 mg/L (Tukey test; P= 0.032) when compared to the PBS-treated group. However, the differences were not statistically significant for the wet and dry weights of males (except for 5 mg/L; Dunnett's test; P=0.025) when compared to the controls (Table 2).

The effects of kinetin on the morphological disorders of F_1 individuals are presented in Table 3. The total number of females with curved wings (F=2.122; df=5, 18; P=0.110), shortened wings and fully disrupted individuals did not significantly change in all tested groups when compared to the control (Dunnett's test; P>0.05). However, the application of PBS considerably increased the number of females with shortened wings (F=4.497; df=5, 18; P=0.008) compared to NaOH-treated (Tukey test; P= 0.028) and 400 mg/L (Tukey test; P=0.004) groups, as well as the number of fully disrupted females (F=4.078; df=5, 18; P=0.012) as compared to the 3000 mg/L group (Tukey test; P=0.026). When the percentages of the total morphological disorders were evaluated, the increase in the PBS-treated group was significant when compared to both control (Dunnett's test; P=0.018) and other test groups (F=5.807; df=5, 18; P=0.002). The total number of males with curved wings (F=1.978; df=5, 18; P=0.131), shortened wings (F=1.418; df=5, 18; P=0.265), fully disrupted individuals (F=0.508; df=5, 18; P=0.767) and the percentage of total morphological disorders (F=2.623; df=5, 18; P=0.060) fluctuated among the test groups, but the differences were not significant when compared to the controls (Dunnett's test; P>0.05) (Table 3).

Kinetin application caused a noticeable decline in the total number of F_1 progeny produced by two female parents during a period of 10 days (F=6.125; df=5, 18; P=0.002) (Table 4). The reduction in F_1 progeny was significant at 400 mg/L (Dunnett's test; P=0.001) and 3000 mg/L (Dunnett's test; P=0.005) when compared to the control. The decrease in the number of progeny at 400 mg/L was also significant when compared to 5 mg/L (Tukey test; P=0.048) and the PBS-treated control (Tukey test; P=0.027). The total numbers of females (F=4.266; df=5, 18; P=0.010) and males (F=3.825; df=5, 18; P=0.015) were prominently

lowered in all test groups. Dunnett's test showed that the reduction in the number of females was significant for the NaOH-treated (P=0.049), 400 mg/L (P=0.015) and 3000 mg/L groups (P=0.028), and for males in the 400 mg/L (P=0.008) and 3000 mg/L groups (P=0.032) when compared to the controls. Kinetin treatment also caused an insignificant decrease in the female sex ratios (F=1.893; df=5, 18; P=0.146) (Dunnett's test; P>0.05). The sex ratio of adults was male-biased in all test groups. When the total number of eggs laid by newly emerged F_1 females was examined until their death, it was found that the fecundity fluctuated between doses (F=1.736; df=5, 234; P=0.127), but these differences were not statistically significantly different from the control group (Dunnett's test; P>0.05). The relative fecundity percentage of the experimental groups was also variable when the percentage fecundity of the control group was assumed to be 100% (F=2.141; df=5, 234; P= 0.061). However, the fluctuations among the tested groups were not significant when compared to the control (Dunnett's test; P>0.05) (Table 4).

Effects of kinetin on the THC

Comparisons of the THC among the control groups (untreated, null-injected, NaOH-injected, PBS-injected, and NaOH-PBS-injected) are shown in Fig. 2A. The THC decreased significantly (F= 10.509; df=4, 145; P=0.000) in all control groups except for the PBS-injected group when compared to the untreated ones (Dunnett's test; P<0.05). As the mixture of NaOH and PBS solutions included the effects of other controls on the THC, the results of kinetin-treated groups at 3 h and 24 h were statistically compared only with the untreated and NaOH-PBS-injected controls (Fig. 2B). Hemolymph samples from the untreated last instars normally displayed 20.64±2.04 and 34.33±1.93 x10^6 cells/mL at 3 h and 24 h, respectively. The minimum count of 12.95±0.93 x10^6 cells/mL was observed at 3 h after the 400-mg/L kinetin injection, whereas a maximum count of 31.02±2.42 x10^6 cells/mL was recorded for the same dose at 24 h. Kinetin application caused prominent decreases in all test groups except for 5 mg/L at 3 h (F=3.752; df=5, 174; P= 0.003), but the differences were only significant for the NaOH-PBS-injected (Dunnett's test; P=0.041) and 400 mg/L groups (Dunnett's test; P= 0.010) when compared to the untreated controls. There was also a significant

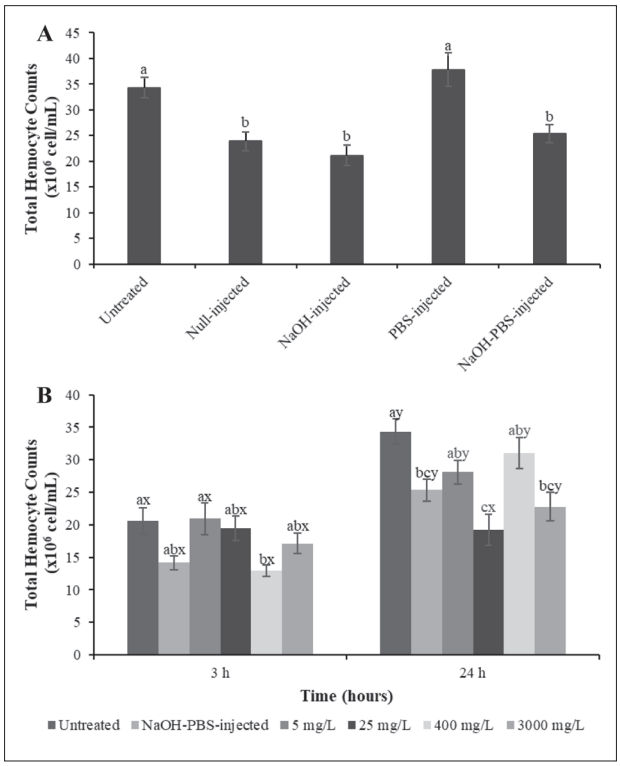

Fig. 2. Total hemocyte counts (x10^6 cell/mL) of *A. grisella* larvae in control groups (**A**) and in kinetin-treated groups (**B**). Each bar represents the mean (±SE) of three replicates with 10 larvae per replicate. Groups (a-c) and time points (x-y) labeled with the same lowercase letter are not significantly different (Tukey's HSD test and *t*-test, P>0.05)

decrease at 400 mg/L compared to 5 mg/L at 3 h (Tukey test; P=0.048) when the THC was compared between treatments. At 24 h post-kinetin injection, the THC declined noticeably in all tested groups when compared to the untreated control (F=6.863; df=5, 174; P<0.0001), but the differences were only significant for the NaOH-PBS-injected (Dunnett's test; P=0.013), 25 mg/L (Dunnett's test; P=0.000) and 3000 mg/L groups (Dunnett's test; P=0.001). When the kinetin-injected groups were compared with each other at 24 h, there was a significant decrease at 25 mg/L compared to the 5 mg/L (Tukey test; P=0.039) and 400 mg/L (Tukey test; P=0.002) groups. THC considerably increased at 24 h compared to 3 h for both controls and kinetin-injected larvae, except for 25 mg/L (untreated: *t*=-4.872; df= 58; P=0.000; NaOH-PBS-injected: *t*=-5.439; df=48.059; P=0.000; 5 mg/L: *t*=-2.347; df=58; P=0.022, 25 mg/L: *t*=0.071; df=58; P=0.943; 400 mg/L: *t*=-6.972; df=37.343; P=0.000; 3000 mg/L: *t*=-2.070; df=58; P=0.043).

DISCUSSION

Pesticides and PGRs accumulate in both living and non-living systems through chemical cycles whereby their harmful effects manifest after an extended time [51,52]. Even the unknown effects of chemicals such as PGRs should be investigated carefully for different insect species. According to the literature, this is the first detailed evaluation of the potential impact of a cytokinin hormone, kinetin, on some biological aspects and the immune system of the wax moth, *A. grisella*. The insects from the early instars were initially fed a kinetin diet and the percentage of larval mortality was determined. Mortality values in the experimental groups showed insignificant decreases (5 and 3000 mg/L) or increases (25, 50 and 100 mg/L) when compared to the controls, but the presence of a similar number of dead larvae in untreated groups clearly revealed that this situation was independent of kinetin dose. In a study using different insect species, kinetin, coumarin and GA_3 were shown to cause 100% mortality in the first instars of *B. cucurbitae* at higher doses (125, 625 and 3125 μg/mL) [30]. Studies have also demonstrated that kinetin can cause unexpected effects by changing metabolic pathways [13,22,23,31], though it has no lethal effect on insects and is considered to be safer, biodegradable and environmentally friendly. Namely, the activity of some detoxification enzymes involved in the metabolism of xenobiotics can be increased (catalase, esterase, NADH dehydrogenase, glutathione peroxidase, superoxide dismutase and acid phosphatase) or decreased (alkaline phosphatase, esterase, ATPase and O-demethylase) with the influence of kinetin in the aphid *Lipaphis erysimi* (Kaltenbach) (Hemiptera: Aphididae) [22,23] and fruit flies *Z. paravittiger* [13] and *B. cucurbitae* [31]. This type of metabolic regulation aimed at compensating for the negative effects of foreign materials may not always have positive effects on living organisms over the long term. For example, Sharma et al. [13] found that the maximum catalase activity at a 100 ppm kinetin dose, reduced the lifespan of *Z. paravittiger*. In the current study, adult longevity was almost unchanged in the toxicity test, while kinetin applied from the egg stage significantly prolonged the longevity of males at 5 mg/L compared to the control. Because of the decreased trend in F_1 progeny and the male-biased sex ratio in all tested groups, in the current study, male larvae were influenced more by kinetin than female larvae. Another explanation for this situation may be differences in the physiology of males and females. The sexes could also respond differently to xenobiotics/toxic compounds and could differ in resistance to oxidative stress, hormone levels, immune traits, etc. Current results about longevity are in agreement with those showing the prolongation of adult life in insects depending on exposure to low kinetin doses [12,13], which was assumed to be related to the anti-aging properties of kinetin. Namely, kinetin delays the onset of several cellular and biochemical characteristics associated with cellular aging by maintaining the efficiency of various repair and defense systems or by reducing the accumulation of metabolic defects and debris that can have harmful effects on cellular physiology and biochemistry [2,12,13]. On the other hand, high doses of kinetin were also shown to cause toxic effects in fruit flies by shortening the life span [12,13,29], which once again reveals the importance of the dose of application.

The developmental time for *A. grisella* was almost unchanged after exposure to different kinetin doses for both the 1st and 2nd series of experiments. Therefore, independent of the developmental stage when the chemical was applied, it can be concluded that kinetin has no adverse effects on this biological parameter. Contrary to the present findings, a prolongation of the developmental time in the larval and pupal stages of *Zaprionus* fruit flies was reported when their diet medium contained low doses of added kinetin (25 ppm and above). The researchers attributed this to the fact that kinetin has certain protective effects, which slowed down the developmental stages and reduced the age-specific death rates throughout the adult lifespan [12,13]. However, lack of correlation or negative correlation between the development time and longevity have also been recorded in holometabolous insects [53]. Reduced larval duration in larvae exposed to kinetin and GA_3 topically for one day, was observed in the silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) [24]. In the current study, the wet and dry weights of males were almost constant in the toxicity test, while kinetin applied from the egg stage significantly reduced the dry weight only at 5 mg/L as compared to the control. On the other hand, kinetin application slightly decreased the weight of females in general. The only exception of this trend was a significant increase in wet weight at 400 mg/L when compared to the NaOH-treated and 3000 mg/L

groups. Due to the reduced number of progeny in the 400-mg/L kinetin group, the amount of nutrients per individual could be increased, which led to weight gain due to the ingestion of more food. Another result linked to female weight was an insignificant decrease in the dry weight in both series of experiments when compared to the control. However, the wet weight of females showed significant decreases, especially at 25 mg/L for the toxicity test, and at 3000 mg/L in the 2nd series of experiments. In contrast to the present study, larval, pupal, cocoon and shell weights in *B. mori* increased significantly after the topical application of kinetin and GA_3 to the 4th and 5th instars [24]. In a study conducted with another PGR, the effects of IAA added to the host diet were examined within the host-parasitoid interactions. Researchers found that neither the host *Galleria mellonella* L. (Lepidoptera: Pyralidae) nor the endoparasitoid *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) displayed significant changes in adult size and weight upon IAA treatment [54]. The reason for the differences between our result and others' findings may be attributed in part to the use of different insect species.

Examining the effects of kinetin on the morphological disorders of F_1 individuals revealed that the total number of female and male moths with curved wings, shortened wings and fully disrupted individuals did not considerably change in the test groups when compared to the control. In a study of *D. melanogaster*, the effects of different concentrations of kinetin and ABA (10^{-3} and 10^{-4} M) on adult morphology were investigated by adding kinetin or ABA to the standard nutrient medium for different developmental stages (egg, larva and pupa) [27]. In contrast to our results, 10^{-3} M kinetin caused the highest rate of phenotypic abnormalities such as wings or thorax disorders, blackened body color and black-pigmented eye [27]. Because a significant effect of the mutation was not shown, these phenotypic abnormalities were attributed to the teratogenic effect of the PGRs. The fact that our data on morphological disorders do not overlap with those of *D. melanogaster* could be attributed to the differences in the duration of kinetin exposure and the insect species used. At the same time, analysis of the data for the percentage of total morphological disorders in the present study revealed that PBS treatment caused significant increases in females unlike males when compared to both control and other tested groups. These PBS-induced disorders in females apparently disappeared after kinetin treatment. In a previous study of the antimutagenic effect of PGRs in *D. melanogaster* [28], it was found that 10^{-3} and 10^{-4} M GA_3 reduced all types of ethyl methane sulfonate (EMS)-induced mutant wing spots, whereas 10^{-3} M kinetin reduced only twin spots, and 10^{-4} M IAA decreased only large single spots. These results [28] and our data indicate that PGRs may have healing power against stress factors. However, in the same article it was also found that kinetin and IAA caused mutagenic effects in *D. melanogaster* since they increased the number of EMS-induced mutant wing spots at 10^{-4} and 10^{-3} M, respectively. Thus, the effects of a chemical on an organism vary greatly depending on the type and dose of the substance.

A. grisella administered with kinetin from the egg stage displayed the most striking response in the total number of F_1 progeny. The reducing effect of the chemical on the progeny was especially significant at higher doses (400 and 3000 mg/L) based on 10 daily observations. The reduced number of F_1 offspring stemmed from the decline in the number of both female and male progeny because of the kinetin treatment. In a study conducted with another PGR, the reducing effect of IAA on the total number of both F_1 and F_2 progeny of *Apanteles galleriae* Wilkinson (Hymenoptera: Braconidae) was also reported at higher doses (>200 ppm) [35]. The adverse effects of PGRs on the reproductive potential of insects were attributed to their interference with the neurosecretory system [29]. There are also other investigations showing the adverse effect of PGRs on beneficial insects through the host species [34,35,54-60]. For example, IAA and GA_3 added to the diet of *A. grisella* were shown to cause undesirable effects on some life history traits and biochemical parameters of the parasitoid *A. galleriae* [34,35,59]. Another parasitoid, *P. turionellae*, was also shown to be influenced by IAA and GA_3 in the host diet [54,55,58]. In the current study, the sex ratio of *A. grisella* adults was male-biased in all tested groups, independent of kinetin dose. This fact demonstrates that the considerable decline in the total number of F_1 progeny occurred because of the decline in both sexes. Similarly, kinetin and ABA added to the diet of *D. melanogaster* at different developmental stages did not change the sex ratio, which was female-biased [27]. Examination of the effects of kinetin on egg fecundity

of newly-emerged F_1 females revealed that hormone treatment caused insignificant changes. On the other hand, the total number of eggs and the fecundity percentage tended to increase parallel to the increment in kinetin doses. A study with *Aulocara elliotti* females (Thomas) (Orthoptera: Acrididae) fed with kinetin also demonstrated an increment in the mean number of eggs and in the number of viable eggs at 10 and 20 mg/L doses [19]. Similarly, it was found that kinetin added to the nutrient medium of *D. melanogaster*, especially at high doses (10^{-3} and 10^{-4} M), increased egg fecundity [25,26]. Moreover, the maximum egg number was observed at 100 ng/mL kinetin and 300 ng/mL GA_3 when applied to the silkworm *B. mori* topically [24]. In contrast, other studies showed that kinetin reduced the egg-laying capacity and the reproductive potential of fruit flies [13,29]. It can be concluded that kinetin influences various insect species differently in terms of egg fecundity, thus further studies should be conducted with different insect species to explore its effects in more detail.

Stress factors such as wounding, infection and chemical substances can cause some alterations in hemocyte counts in the circulation [36,43,44,61]. The THC of *A. grisella* decreased significantly in all control groups (null-injected, NaOH-injected and NaOH-PBS-injected) except for the PBS-injected group, when compared to untreated individuals. This result clearly showed stress association with the injection injury and the NaOH-induced effect. As the NaOH-PBS-injected group included the effects of all the other controls, the THC of kinetin-treated groups was statistically compared with only untreated and NaOH-PBS-injected controls. Comparing the counts for the two time periods, THC declined noticeably in all tested groups except for 5 mg/L at 3 h post-kinetin injection. However, the differences were only significant for 3 h in the NaOH-PBS-injected and 400 mg/L groups and for 24 h in the NaOH-PBS-injected, 25 and 3000 mg/L groups when compared to the untreated control. This reduction in the THC is thought to stem from the injection injury or NaOH-induced effect. Although the THC were markedly affected by the injection of the mixture of PBS and NaOH, the high rate of decline at 400 mg/L after 3 h (37%), and at 25 mg/L and 3000 mg/L doses after 24 h (34-44%) showed that kinetin also has a role in the decline in THC. Besides, THC considerably increased at 24 h compared to 3 h in all tested larvae, except for 25 mg/L. Kinetin is not likely to cause such differences between the two time points since the THC of the control groups also increased at 24 h compared to 3 h, similar to kinetin-injected larvae. It is known that insects exposed to different PGRs exhibit changes in hemocyte counts as a result of detoxification and immune responses [36,43,44]. The role played by hemocytes in the first line of the defense system against the toxicity of kinetin could be responsible for the observed decrease in THC in *A. grisella*. Consistent with our data, ABA was reported to cause a remarkable decrease in the number of circulating hemocytes in *G. mellonella* larvae at 4, 24 and 48 h post injection [44]. Unlike our results, an increase in the THC was shown in *G. mellonella* and *A. grisella* larvae when they were fed with GA_3- and IAA-treated diets, respectively [36, 43]. Thus, studies have shown that the extent to which PGRs affect hemocyte counts varies greatly depending on the type of hormone and insect species.

Extensive use of pesticides has led researchers to look for natural compounds, such as PGRs, to compensate for the harmful effects of these chemicals. However, the increasing usage of PGRs in agriculture has shown that over time these substances can be harmful. It is clear from the current study that one of the cytokinin hormones, kinetin, can interfere with normal developmental and immune processes of *A. grisella* by reducing the weight, the total number of progeny and the THC. Evidence from earlier studies mentioned here and our findings strongly indicate that kinetin can be unpredictably toxic to insects, affecting future generations. Therefore, further research is needed to reveal the long-term effects of kinetin and other PGRs on both host species and natural enemies used in IPM. It should be kept in mind that even natural compounds such as PGRs can have adverse effects on all living organisms and ultimately disrupt the ecological balance.

Funding: We did not receive financial support, we used the facilities available in our laboratory.

Author contributions: Olga Sak conceived the research. Olga Sak and Erinç Çelik conducted the experiments. Olga Sak and Erinç Çelik analyzed data and conducted statistical analyses. Olga Sak and Erinç Çelik wrote, read and approved the manuscript.

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

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