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The Acute Toxic Effects of Cyromazine and Hexaflumuron on Mosquito Larvae (*Aedes aegypti* L.)

Cyromazine ve Hexaflumuron'un Sivrisinek Larvaları (*Aedes aegypti* L.) Üzerindeki Akut Toksik Etkileri

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ABSTRACT

In this study, the acute toxic effects of cyromazine and hexaflumuron, two insect growth regulators used in vector control, were investigated on the larvae of the common vector mosquito, mortality rates of *Aedes aegypti* larvae exposed to six different concentrations of cyromazine and five different concentrations hexaflumuron were determined. Using the obtained data, the concentrations between LC_{10} and LC_{99} were calculated by applying the probit analysis method. The knockdown times were also measured based on concentration, and the LT_{50} was calculated for each concentration. The experiments revealed that the 14-days LC_{50} for cyromazine was 38.191 g a.i./ha (0.254 ppm) (95% Confidence Interval (CI): 33.296 g a.i./ha or 0.222 ppm and 43.497 g a.i./ha or 0.289 ppm). For hexaflumuron, the 14-days LC_{50} was 1.247 g a.i./ha (0.00831 ppm) (CI: 0.670 g a.i./ha or 0.00446 ppm and 1.736 g a.i./ha or 0.01157 ppm). For cyromazine, the LT_{50} values ranged from 5.002 days to 37,59 days, depending on the application doses between 30 g a.i/ha and 100 g a.i/ha, while for hexaflumuron, the LT_{50} values ranged from 5.677 days to 11.366 days, depending on the application doses between 1 g a.i/ha and 10 g a.i/ha. Hexaflumuron was found to be more toxic than cyromazine on mosquito larvae. It has been concluded that, for the effective use of both active ingredients in mosquito larval control, cyromazine should be applied at a dose of approximately 65.846 g a.i/ha (0.439 ppm) and hexaflumuron at a dose of 4.707 g a.i/ha (0.0313 ppm).

Key Words
Cyromazine, hexaflumuron, mosquito, chitin synthesis inhibitors.

ÖZ

Bu çalışmada vektör mücadelesinde kullanılan iki böcek büyüme düzenleyicisi olan siromazin ve hekzaflumuronun vektör sivrisinek larvaları üzerindeki akut toksik etkileri araştırılmış, siromazinin altı farklı konsantrasyonuna ve hekzaflumuronun beş farklı konsantrasyonuna maruz kalan *Aedes aegypti* larvalarının ölüm oranları belirlenmiştir. Elde edilen verilerle probit analiz yöntemi kullanılarak LC_{10} - LC_{99} arasındaki konsantrasyonları hesaplanmıştır. Ayrıca konsantrasyona bağlı olarak ölüm zamanları da ölçülerek her konsantrasyon için LT50 değeri hesaplanmıştır. Yapılan deneyler sonucunda %95'lik güven aralığında cyromazine için 14 günlük LC_{50}: 38,191 g a.i/ha (0,254 ppm) (Güven Aralığı (CI): 33,296 g a.i./ha veya 0,222 ppm ve 43,497 g a.i/ha veya 0,289 ppm) ve Hexaflumuron için 14 günlük LC_{50}: 1,247 g a.i/ha (0,00831 ppm) (CI: 0,670 g.a.i/ha veya 0,00446 ppm ve 1,736 g a.i/ha veya 0,01157 ppm) olarak bulunmuştur. Cyromazine için LT50 değerleri, 30 g a.i/ha ile 100 g a.i/ha arasındaki uygulama dozlarına bağlı olarak 5,002 gün ila 37,59 gün arasında, heksaflumuron için 1 g a.i/ha ile 10 g a.i/ha arasındaki uygulama dozlarına bağlı olarak 5,677 gün ila 11,366 gün arasında değişmiştir. Hexaflumuron sivrisinek larvalarında cyromazine göre daha toksik bulunmuştur. Her iki aktif maddenin sivrisinek larva mücadelesinde etkin olarak kullanılabilmesi için cyromazine aktif maddesinin 65,846 g a.i./ha (0,439 ppm) ve hexaflumuron aktif maddesinin ise 4,707g a.i/ha (0,0313 ppm) dozları civarında kullanılması gerektiği ortaya konulmuştur.

Anahtar Kelimeler
Cyromazine, hexaflumuron, sivrisinek, kitin sentez inhibitörleri.

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INTRODUCTION

There are approximately 2 700 species of mosquito in the world [1]. *Aedes aegypti* L. and *Culex pipiens* L. are among the world's most common vector mosquito species [2]. Mosquitoes are part of the terrestrial and aquatic ecosystem that can find habitat in different parts of the world, except for open oceans and permanently frozen areas [3]. However, mosquitoes, which breed rapidly in suitable habitats particularly under favorable temperature and humidity are vectors of serious human diseases, affecting millions of people in both urban and rural areas of developing countries [4]. *Aedes* and *Culex* many these genera species serve as vectors of important diseases, such as malaria, yellow fever, dengue fever, rift valley fever virus, viral encephalitis and bird malaria, elephantiasis and west Nile virus [1,4]. Malaria disease that still affects 200-450 million people annually in tropical regions and causes the death of approximately 2.7 million people [5].

Heavy rainfall and extreme weather events associated with climate change may lead to the emergence or spread of vector-borne diseases in areas where they were not previously endemic, by making non-endemic areas suitable for the development of a particular species [6]. Furthermore, in the absence of an effective vector control program or a lack of political will to implement existing programs, climate change can play a synergistic role in vector-borne diseases [7].

Mosquitoes are holometabolic insects that undergo complete metamorphosis, from egg to larva, from larva to pupa, and finally to adult, just as caterpillars turn into butterflies. Since the larval and pupal periods are spent in water, egg laying takes place in water or near water depending on the type of mosquito [8]. For this reason, larvae control is carried out in wetlands. It has become necessary to combat mosquito adults and mosquito larvae all over the world because they are vectors of many diseases and negatively affect social life. Vector struggle; it should be done using the lowest possible number of chemical pesticides after trying cultural, physical (mechanical) and biological control options [9]. Chemical control should be carried out using pesticides authorized by the relevant authorities in each country for use in the appropriate fields.

The commonly chemical method for controlling adult mosquitoes with pesticides is the application of ultra-low volume (ULV) field sprays, which provide insecticide formulation in minimum volume per unit area [10]. Conducting this application in open areas, where people and other non-target organisms live, poses a risk to the environment. If the pesticide settles on the ground and mixes with the soil shortly after application, it may lead to excessive exposure of pesticide to the non-target organisms, including humans. For this reason, before targeting adult mosquitoes, it is preferable to identify mosquito breeding areas and control population by managing larvae in appropriate periods. One advantage of targeting larvae is that they cannot escape from breeding sites until reaching the adult stage, and unlike adult mosquitoes, they cannot easily evade control measures [11].

Resistance to temephos, the primary larvicide used over the past 30 years to control mosquito larvae, has been reported in many Latin American countries [10]. Additionally, the repeated mass use of different organophosphate insecticides driven by their rapid degradation in the environment has posed a significant threat to non-target organisms such as fish, crabs and shrimp [12]. There has been increasing interest in developing different pesticides to combat mosquito larvae. Insect growth regulators (IGRs) are considered an alternative to conventional pesticides for pest control due to their high selectivity for target species and their safety for non-target organisms and are safer against mammals and the environment [10,13]. Compounds in IGR class are generally neither stomachic nor neurotoxic poisons. Instead, they act through distinct mechanism such as disrupting the molting process or cuticle formation in insects or by interfering with the hormonal balance of insects [14].

Insecticides that show high effectiveness at low doses on the target organism are important in terms of using less chemicals. Because exposure of non-target organisms in water to pollutants causes an increase in reactive oxygen species (ROS) and oxidative damage [12]. Cyromazine and hexaflumuron, both IGRs, have the same mechanism of action against the same target organism with the dose-related effectiveness. Cyromazine is believed to affect insect development by interfering with the ecdysone signaling pathway [15]. It interferes with the moulting process of larvae and pupation, resulting in deformed or/and dead larvae, pupal or adults [16]. Additionally, cyromazine treatment may also interfere with other signaling pathways such as the juvenile hormone (JH) pathway [15]. When housefly larvae are exposed to cyromazine, deformations can occur in the pupal stage, which result from interference with chitin digestion and synthesis [17]. Hexaflumuron as a benzoylphenylurea derivative controls the developmental stages of insects during molting by disrupting chitin synthesis [18] and impairs cuticle synthesis and development, causing abortive moult, (deformations in the cuticle) and hatching defects [19,20].

Cyromazine and hexaflumuron are authorized by the European Chemicals Agency (ECHA) as active ingredients in biocidal products for product type 18 (Insecticides, acaricides and products for the control of other arthropods). In the study conducted by Zhu et al. [21] to evaluate the toxicity of hexaflumuron on the larvae of the cutworm (*Spodoptera litura*), hemolymph samples were collected from larvae exposed to different concentrations of hexaflumuron to assess changes in hemocyte count, and developmental changes were examined based on measurements of length and weight. Similarly, Hassanen et al. [22] investigated the neurotoxicity of hexaflumuron and hymexazol in rats and reported that both pesticides could induce neurobehavioral changes, neuronal damage, and mitochondrial-mediated apoptotic pathways. Furthermore, Assar et al. [23] demonstrated that cyromazine reduced glucose, amino acids, alkaline phosphatase (ALP), and phenoloxidase levels in the homogenate of *Culex pipiens* larvae, while increasing protein content and acid phosphatase activity. Likewise, Martinez et al. [24] found that cyromazine exposure significantly reduced egg production in the Mexican fruit fly (*Diptera: Tephritidae*), highlighting its impact on oviposition.

Considering previous studies about these two technical active ingredients, either alone or together with synthetic pyrethroids, in a variety of species. However, no studies were found comparing these two pesticides. For this reason, the study aimed to compare the effectiveness of two different IGRs with the same mechanism of action (chitin synthesis inhibitor) on the same target organism by estimating their LC_{50} values.

MATERIALS and METHODS

Mosquito larva

Aedes aegypti mosquito larvae used in the present study was obtained from a susceptible population reared strain at the Hacettepe University, Pesticide Research and Reference Laboratory in Ankara, Türkiye. The population was stored at 25± 2°C and 50±5% relative humidity and 12 hours of light/12 hours of darkness. After the eggs collected from these mosquitoes were released into the water, the larvae hatched within 2-3 days were kept at 25±2°C, 50±5% humidity, 12 hours of light/12 hours of darkness, and fed with larvae food. 2nd and 3rd instar larvae formed within a week were used for toxicity testing.

Chemicals

Cyromazine (CGA 72662, N-cyclopropyl-1, 3, 5-triazine-2, 4, 6-triamine; $C_6H_{10}N6_{10,}$ CAS No: 66215-27-8) was supplied by LGC Dr. Ehrenstorfer with 99.32% (g/g) purity. Hexaflumuron(1-[3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy)phenyl]-3-(2,6-difluorobenzoyl)urea; $C_{16}H_8C_{l2}F_6N_2O_3$, CAS No: 86479-06-3), was supplied by HPC Standarts GmbH with 99.45 % (g/g) purity.

Experimental design

For the cyromazine and hexaflumuron, 1 mg of the chemical for each active was weighed and dissolved in 100 mL of distilled water to prepare a 10 ppm stock solution, which was used in the subsequent experiments. The experiments were conducted in triplicate at different concentrations by placing 300 mL of water and Stage 2 and 3 mosquito larvae into the 20 x10 cm experimental containers. Then, 6 concentrations for cyromazine (10 g a.i./ha or 0.066 ppm; 20 g a.i./ha or 0.133 ppm; 30 g a.i./ha or 0.2 ppm; 50 g a.i./ha or 0.33 ppm; 70 g a.i./ha or 0.46 ppm; 100 g a.i./ha or 0.66 ppm) were applied into the trial containers. Then, 5 concentrations (1 g a.i./ha or 0,0066 ppm; 2 g a.i./ha or 0,0133 ppm; 3 g a.i./ha or 0,02 ppm; 5 g a.i./ha or 0,033 ppm; 10 g a.i./ha or 0,066 ppm) for hexaflumuron were applied in the trial containers. After the application of concentrations, mosquito larvae were fed every other day, and the dead larvae were counted. At the end of 14 days, the larvae died or became flighted. Flying mosquitoes were placed in wire cages to prevent them from affecting the experimental environment.

Data Analysis

The LC_{50} values for cyromazine and hexaflumuron on *Aedes aegypti* larvae were calculated using the EPA Probit Analysis Program Version 1.5. The results obtained by measuring the mortality times of larvae based on application doses were analyzed using the t-test, and the Lethal Time 50% (LT_{50}) was calculated for each concentration within a 95% confidence interval. All statistical analyses were performed using the statistical analysis software SPSS.

Results

No mortality was observed in the cyromazine-treated test containers at concentrations of 10 and 20 g a.i/ha up to the second interval time. Up to the fourth interval time (10th day), there was no statistical difference in mortality rates between doses ($P > 0.05$). Mortality rates significantly differed between concentrations ($t = 2.856$, $P = 0.036$) starting with fifth interval time. The 70 g a.i/ha concentration ($t = 2.981$, $P = 0.041$) and the 100 g a.i/ha concentration ($t = 3.088$, $P = 0.037$) showed statistically significant differences from the other in terms of mortality rates. 100% mortality on the 13th day was recorded at 100 g a.i/ha and the measurements for all application doses were taken on the same day. The concentration except 10 g a.i/ha for cyromazine showed good linearity ($R^2 > 0.90$) by the mortality rate as shown at Figure 1. The lethal concentrations (LC) were calculated with probit analysis as shown at Table 1. LC_{50} was found 38.191 g a.i/ha for cyromazine. The lowest concentration (10 g a.i/ha) shown lower linearity ($R^2 = 0.74$) and was under the LC_1 value.

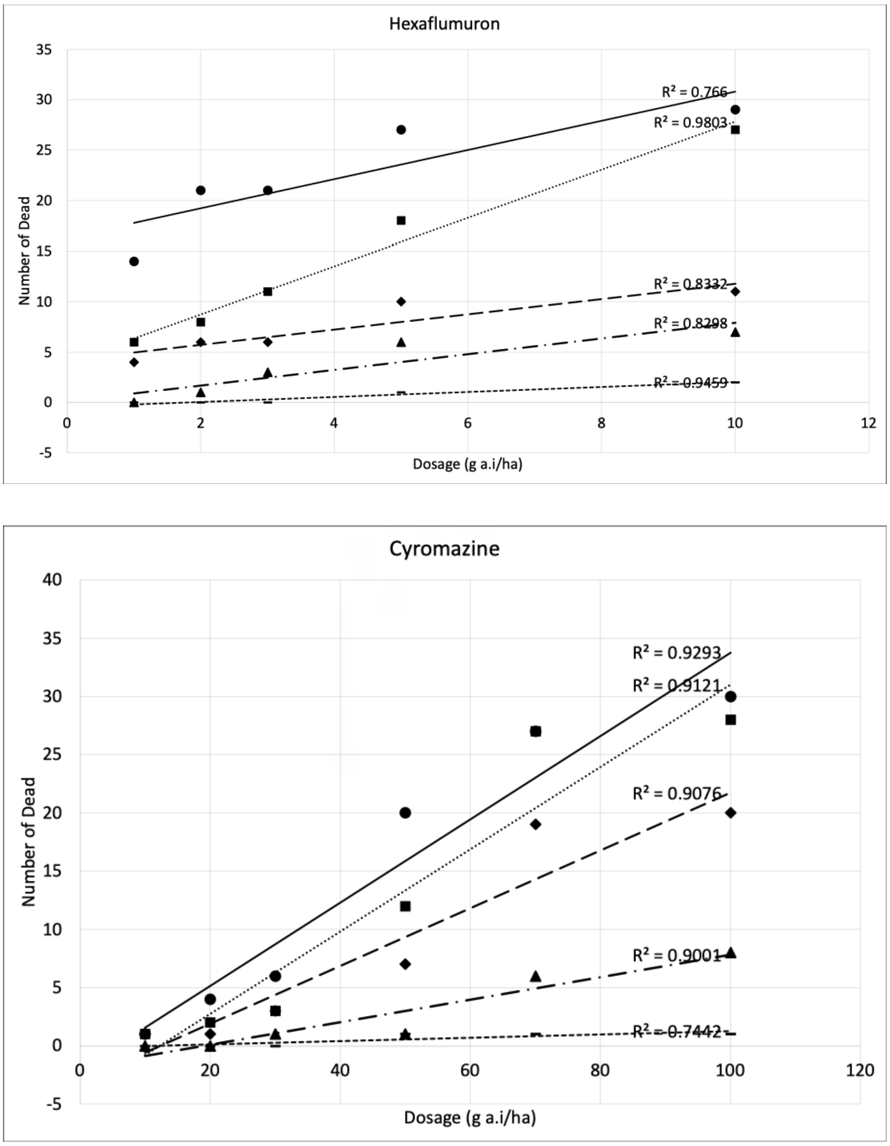

Figure 1. Linear Regression (R^2) Graphics of Dosage-Dependent Mortality in Target Larvae for Hexaflumuron and Cyromazine (Hyphen: 1st, Triangel: 2nd, Rectangel: 3rd, Circle: 4th and Square: 5th intervals).

No mortality was observed in the hexaflumuron-treated test containers at doses of 1, 2, and 3 g a.i./ha up to the first interval time (3rd day), and at the 1 g a.i./ha concentration up to the second interval time (4th day). Up to the third interval time (6th day), there was no statistical difference in mortality rates between doses ($P > 0.05$). The statistically significant differences were found by the mortality rates between the concentrations the fourth interval day (8th day) ($t = 3.651$, $P = 0.022$) and the fifth interval day (11th day) ($t = 8.491$, $P = 0.001$). 100% mortality on the 11th day was recorded at 10 g a.i/ha and the measurements for all application doses were taken on the same day. The 10 g a.i./ha concentration ($t = 2.801$, $P = 0.049$) showed a statistically significant difference in mortality rates compared to the other concentrations. Hexaflumuron showed good linearity ($R^2 > 0.90$) by the mortality rate at all doses as shonw at Figure 1. The lethal concentrations (LC) were calculated with probit analysis as shown at Table 1. LC_{50} was found 1,247 g a.i/ha for hexaflumuron.

LT_{50} values for all doses were calculated using probit analysis as shown at Table 2. The LT_{50} value for cyromazine was calculated between 37.59 and 5.002 days and LT_{50} value for hexaflumuron was found between 11.366 and 5.677 days. No statistically significant difference was found between the LT_{50} values of cyromazine concentrations of 20 and 30 g a.i/ha and those of 70 and 100 g a.i/ha (P>0.05). However, the 50 g a.i/ha concentration shows a statistically significant difference com-

Table 1. Log-dosage probit mortality regression analysis (LC50) for cyromazie and hetxaflumuron at *Aedes aegypti* larvae.

	Cyromazine				Hexaflumuron			
LC Value	Conc. (g a.i./ha)	X^2	Intercept	Slope	Conc. (g a.i./ha)	X^2	Intercept	Slope
1.00	11.245				0.063			
5.00	16.088				0.151			
10.00	19.473				0.241			
15.00	22.151				0.330			
50.00	38.191	9.488	-1.930	4.380	1.247	7.815	4.828	1.796
85.00	65.846				4.707			
90.00	74.903				6.446			
95.00	90.662				10.269			
99.00	129.707				24.598			

LC values of Cyromazine depending on exposure concentration.
g: gram, a.i: active ingredient, ha: hectare

Table 2. Log-time probit mortality regression analysis (LT_{50}) for cyromazie and hetxaflumuron at *Aedes aegypti* larvae.

Active Substance	Dosage (g a.i./ha)	KT_{50}	X^2	Intercept	Slope	95% Confidence Limits Lower	95% Confidence Limits Upper
Cyromanize	10	-	-	-	-	-	-
	20	-	-	-	-	-	-
	30	37.59		2.01	1.89	18.640	>1000
	50	10.728		1.71	3.19	8.945	14.09
	70	5.568		1.80	4.28	4.798	6.387
	100	5.002		1.31	5.27	4.364	5.658
Hexaflumuron	1	11.366	7.815	-0.196	4.92	9.747	15.391
	2	9.272		-0.175	5.35	8.253	10.953
	3	8.880		0.648	4.588	7.814	10.638
	5	6.723		0.958	4.883	6.003	7.605
	10	5.677		0.533	5.923	5.124	6.277

pared to all other concentrations (P <0.05) in terms of LT_{50}. For hexaflumuron, the 1, 2, and 3 g a.i/ha concentrations and the 5 and 10 g a.i/ha concentrations were not shown statistically significant differences in their LT_{50} values within their respective groups.

It was found that hexaflumuron was more toxic than cyromazine and hexaflumuron achieved 100% mortality two days earlier than Cyromazine. Additionally, for both pesticides, even at low doses, mosquito larvae that did not die showed darker coloration and a reduction in mobility. Furthermore, the time required for larvae to pupae and for pupae to transition into adults was longer compared to the control group.

DISCUSSION

The effectiveness of the pesticides on the target organisms is crucial for vector control. Determining the mortality rate and other effects on the target organism, depending on the exposure concentration, helps in deciding the appropriate amount of pesticide to use.

In our study, the LC_{50} value of cyromazine for *Aedes aegypti* mosquito larvae was found to be 0.254 ppm. In a study reported by Vazirianzadeh et al. [25] the LC_{50} value of cyromazine was found to be 0.207 mg/kg diet for the sensitive laboratory strain *Rentokil* and 0.216 mg/kg diet for a wild type of housefly collected from domestic chickens. These values are comparable to the LC_{50} value we found for mosquito larvae.

Al-Mekhlafi et al. [26] reported a decrease in reproductive rates with increasing application doses of cyromazine on the southern cowpea borer (*Callosobruchus maculatus F.*), while Khalid et al. [15] found that cyromazine significantly reduces the number of germ cells in the larval and adult ovaries of *Drosophila*. In our study, as the application dose increased, the time for the surviving larvae to pupate and transition to the adult stage also increased compared to the control group. Additionally, a decrease in the mobility of the adult mosquitoes was observed, with many dying within a few days. Based on the findings from all these studies, we can conclude that cyromazine not only causes the mortality of larvae but also reduces the quality of life of the surviving larvae.

Mirhaghparast et al. [19] conducted a study on the larvae of the Asian rice weevil (*C. suppressalis*), where the dose-dependent lethal concentrations of hexaflumuron after 48 hours were determined as LC_{10}, LC_{30} and LC_{50} to be 44.34, 179.94, and 474.74 µg/ml, respectively. These results are significantly higher than the LC_{50} value of 0.00831 ppm found in our study on *Aedes aegypti* mosquito larvae using hexaflumuron.

In the study conducted by Bashari et al. [27] on the elm leaf beetle *Xanthogaleruca luteola* (Col.: Chrysomelidae), LC_{30} and LC_{50} values of hexaflumurone were found to be 53.45 and 122.02 ppm, respectively. These results are higher than the LC_{50} value we found for *Aedes aegyyti* type mosquito larvae, but lower than the LC_{50} value found by Mirhaghparast et al. [19] for Asian rice weevil (*C. suppressalis*) larvae. The fact that the same pesticide has different LC_{50} values for different organisms shows the importance of determining the application dose.

In a study similar to ours by Kamalet al. [28]; the effects of pyriproxyfen and diflubenzuron, two different active substances with the same mechanism of action, on *Aedes aegypti* were studied and their IC_{50} values were found to be 0.0041 ppm and 0.00036 ppm, respectively. Considering the LC_{50} values, it was determined that diflubenzuron is more toxic to *Aedes aegypti* than pyriproxyfen. The LC_{50} values we obtained for cyromazine and hexaflumuron against the same species (*Aedes aegypti*) are higher compared to those of pyriproxyfen and diflubenzuron. Therefore, the toxicity ranking for *Aedes aegypti* mosquito larvae, from most toxic to least toxic, is as follows: diflubenzuron, pyriproxyfen, hexaflumuron, and cyromazine.

In a study conducted by Abada A. Assar et al. [20], the efficacy of cyromazine at different concentrations on various larval stages of *Culex pipiens* was examined, and the highest efficacy was observed in the 2nd and 3rd larval stages. This is consistent with our use of 2nd and 3rd instar larvae.

The World Health Organization and other studies recommend a minimum efficacy of 80% for the effective use of pesticides [29]. In this study, the LC_{85} values for the active ingredients cyromazine and hexaflumuron against *Aedes aegypti* were found to be 65.846 g a.i/ha and 4.707 g a.i/ha, respectively. The LT_{85} durations corresponding to these LC_{85} doses for both active ingredients are estimated to be approximately 6 days. Considering that the 2nd and 3rd larval stages of the species, which occur between the 4th and 12th days of the 15-day larval period, are affected by IGRs, it is evident that these doses are required for the active ingredients to exhibit their IGR effects. Therefore, it is conclulded that these doses be taken into consideration during application for both active substances to be used effectively.

Conclusion:

In conclusion; the acute toxic effects of two different chemicals with the same mechanism of action used in pest control in the field of public health on mosquito larvae of the species culex pipiens were evaluated and hexaflumuron was found to be more toxic than cyromazine. In addition, it was observed that mosquito larvae exposed to hexaflumuron reached 100% mortality rate earlier than mosquito larvae exposed to cyromazine. However, it was observed that the survival of mosquito larvae and the duration of their life cycles in mosquito larvae exposed to both chemicals changed compared to the control group.

Although both have the same mode of action, the fact that Hexaflumuron is effective at much lower concentrations than Cyromazine is a significant factor in mosquito larvae control. Based on the results of toxicological studies on non-target aquatic organisms, Hexaflumuron's lower toxicity to non-target aquatic organisms or the absence of a toxicity difference between the two pesticides would make Hexaflumuron a preferable option, as it would lead to reduced pesticide use.

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