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Determination of Effect of Three Different Atmospheric Carbon Dioxide Applications on Egg-Hatching Ratio and Hatching Times of *Halyomorpha halys* (Stål 1855) (Hemiptera: Pentatomidae)

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ABSTRACT: This research has been conducted to determine the effect of three different carbon dioxide (CO_2) applications on the egg hatch ratio and hatching times of brown marmorated stink bug, *Halyomorpha halys* (Stål, 1855), (Hemiptera: Pentatomidae). In the study, doses of 600 and 670 ppm of CO_2 were applied to newly laid eggs, and the hatching ratios of eggs left in this environment for 24 hours were compared statistically. According to the results of the study, at the highest dose of 670 ppm, yellowing and then darkening were observed in the eggs, and at doses of 600 ppm, more yellowing color change was observed. It was determined that 234 eggs out of 261 eggs were opened in the control applications, 218 eggs out of 261 eggs opened in the 600 ppm dose application, and 188 eggs out of 260 eggs opened in the 670 ppm application. In terms of egg--hatching times, a statistically significant difference was found between the control (400 ppm), 600 ppm and 670 ppm group ($p<0.05$). In the 670 ppm group of applications, it was determined that the egg-hatching times of the bugs were significantly lower than the egg--hatching times of the bugs in the control and 600 ppm groups ($p <0.05$). As a result of the chi-square test performed for the application of 600 ppm according to egg hatching, the difference between the 600 ppm application and the control was found to

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be statistically significant (x^2 = 4.224, $p<0.05$). This result showed that the use of 600 ppm reduced egg-hatching compared to the control at a statistical significance level of 5%. As a result of the chi-square test performed for the application of 670 ppm according to egg hatching, the difference between 670 ppm and control was found to be statistically significant (x^2 = 26.174, $p<0.05$). This result showed that the use of 670 ppm reduced egg-hatching compared to the control at a statistical significance level of 5 %. The difference between 600 ppm and 670 ppm was found to be statistically significant ($p<0.05$). It was also determined that the use of 670 ppm reduced egg- hatching compared to the use of 600 ppm at a statistical significance level of 5 %, and the chi-square value between these two applications was calculated as x^2 = 9.975. As a result, it was determined that CO_2 applications affected the egg-hatching time and hatching ratios of the bugs. These results are important in terms of pest reproduction predictions during global warming periods and improving the use of elevated CO_2 applications in bug control.

KEYWORDS: *Halyomorpha halys*, Carbon dioxide, Hatch Ratio, Hatching Time

INTRODUCTION

Halyomorpha halys is native to East Asia. With rapid dispersal ability (Wiman et al., 2015), this invasive insect species has been spreading to hundreds of countries in Asia and Europe, especially the United States since 1998 (Rider, 2006; Mityushev, 2016). After being first detected in Istanbul and Artvin in Turkey in 2017, it spread throughout the Black Sea by 2020 (Çerçi & Koçak, 2017; Göktürk & Tozlu, 2019; Göktürk et al., 2018). It goes through five nymphal stages, and the most characteristic feature of nymphs is the presence of spines on their pronotum in later stages (Hoebeke & Carter, 2003). Although *H. halys* can produce 1-5 offspring per year depending on temperature and photoperiod (Niva & Takeda, 2003; Lee 2015), in Eastern Black Sea Region, in Artvin province, it mostly produces 1 generation per year and sometimes 2 in coastal areas.

In this study, it is examined what kind of changes global warming and atmospheric carbon dioxide cause on pests. It is determined what kind of effects this change has on the bug and its host in terms of both nutrition and damage levels. Although there have been some studies on pest-host and natural enemy species of atmospheric gases in previous studies, there is no comprehensive research on the effect of gas exchange on pests. The effects that will be determined need to be supported by simulation studies on the behavior of harmful insects at the tropical level. It should be determined to what extent changes in atmospheric gas levels, especially temperature changes, affect the reproductive parameters that may cause pests to form different biotopes. In this study, it was aimed to determine the effects of two different CO_2 applications on the egg-hatching time and egg-hatching rates of *H. halys*.

MATERIAL AND METHODS

Mass Rearing of *Halyomorpha halys*

Individuals were isolated in a climate chamber where 16/8 photoperiod conditions were maintained at 28±1 °C and 60±10 % relative humidity. Since the pest is polyphagous, it is fed with carrots, soybeans, kiwi, apples, and peppers. The main culture was created with adult *H.halys* individuals collected from Black Sea hazelnut areas where the pest has a high population. Approximately 60 male and 60 female individuals, not in diapause, were collected from the Black Sea provinces (Artin, Samsun, Trabzon) on 12-13/01/2024. The studies were arried out in climate chambers with approximately 45±5 % humidity conditions at 28±1 °C and 16/8 (light/ dark) conditions (Figure 1). The eggs photographs were taken with an Olympus SZX7 stereobinocular microscope and 8 X magnification was used.

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Figure 1. The Climate cabins where *Halyamorpha halys* individuals are reproduction

The pest was kept under room conditions for 2 days, healthy individuals were separated and kept in culture cages, and 5 male and 5 female individuals were kept in 12 different culture containers, a total of 10 individuals. 10 adult individuals of *H. halys* were placed in a circular container with a diameter of 25 cm × 9 cm in height and four 5 cm diameter ventilation holes opened along the edges of the container to provide air circulation. Other individuals were placed in a square-shaped container of 30 cm length × 23 cm width × 10 cm height. Paper was placed inside the containers to provide hiding places and additional surface area for adults to lay eggs comfortably, and filter paper was also placed in the holes opened on the side of the containers. The open end of the filter paper was placed in pure water and the closed end in the container, and the water needs of the individuals were met.

Culture containers were checked regularly three times a day to ensure that adult individuals brought from nature adapted to the environmental conditions. Whether the humidity conditions in the container remained stable and whether the adult individuals drank water regularly through the filter paper and water pores were checked. During this period, dead individuals in the containers were collected daily and care was taken to ensure that the male and female ratio in the container remained 1/1. Egg packets left daily were collected by hand. To prevent damage to the eggs by the adults, the eggs were collected regularly daily (Figure 2). The experiment was started with the eggs given by the new generation adults of the pest. Eggs were taken from adult individuals brought from nature, approximately 1 month later, on 08.2.2024. The eggs were placed in smaller containers and, following hatching, they were fed with carrots, soybeans, kiwi, apples, and peppers to ensure the development of the nymphs. After the new generation of adults were

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obtained, they were brought together to mate and the atmospheric CO_2 dose was applied intermittently at two different rates to the eggs they laid. The first new generation adult emergence occurred on 25.03 2024, and the 10 new generation adult individuals were brought together as 5 male and 5 female individuals. The hatching rates of the eggs were determined by applying two different doses of CO_2 to the new-generation eggs taken from the new-generation adults on 06.04.2024. Each dose was applied to the eggs of 26 egg packs that hatched on the same day. For each dose, the experiments were carried out with 10 replicates and a total of 260 eggs in each dose. Trial studies were carried out approximately with a total of 780 eggs.

Figure 2. Freshly hatched *Halyamorpha halys* eggs prepared for applications

Controlled carbon dioxide applications

The test room – a controllable and traceable atmosphere – consisted of two parts. The first piece was made of plexiglass material and forms the header (65x50x60 cm^3). The second part was, the base (15x50x65 cm^3), made of stainless steel, containing sensors and electronic circuits, and having inlets and outlets to provide gas control and sample collection. By combining the two parts, a gas-tight room and controllable environment were created. A fan was placed at the bottom of the room to ensure the complete mixing of CO_2-laden air in the room. To minimize gas leakage, parts of the test chamber were combined using a Neoprene gasket. During the experiments, the air humidity was kept constant by reducing the surface temperature of the heat exchanger; thus, condensing excess water. Inlets on the floor of the test chamber were used to collect air samples. Therefore, there was a sorption tube with 100 ng each of cyclooctane and cyclododecane added according to current standards. Additionally, gas-tight syringes, fittings, and screw-on septum caps were used.

The carbon dioxide concentration in the cabin was measured using the MHZ-19 carbon dioxide sensor. The sensor, which makes instantaneous measurements, was also tested with the gas chromatog-

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raphy technique. Gas chromatography (GC) measurements were carried out with a Q-bond column on a Thermo GC Trace 1300 model TCD device (Figure 3). Gas was used as the carrier gas at a flow rate of 0.1 mL/min. GC experiments were conducted with a gas sample volume of 1 mL, column temperature of 40 °C, injection temperature of 50 °C, and TCD temperature of 200 °C.

Figure 3. Gas chromatography (GC) measurements

In the study, the effects of 2 different CO_2 doses on egg hatching of *H. halys* were determined. Air samples in the cabin were taken within 24 hours and the changes in gas rates were determined. Two different doses of carbon dioxide were applied intermittently in the cabin (Figure 4). These doses were applied as 600 and 670 ppm. Once the gas was introduced, the eggs were kept in the cabin for 24 hours, and the eggs were, then, taken into the conditioning cabin. The cabin temperatures of the cabin where the eggs were kept and the air conditioning cabinet were kept at the same temperature within 24 hours. In the study, the first air sample was taken at the 8th minute, and the other samples were taken at the 4th, 18th, 21st and 24th hours. For each treatment, 10 individuals were left as controls. The control medium was not treated with any gas. After the application, the egg-hatching status was monitored in the air conditioning cabinet.

Figure 4. The newly improved cabin for dosing applications

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Statistical analysis

Shapiro Wilk Goodness of Fit and Kolmogorov Smirnov tests were used to analyze whether the obtained data conformed to normal distribution. In the categorical data in the study, frequency, and percentage (%) values are given, and in continuous data, descriptive statistics such as mean and standard deviation are given. Since normality assumptions were met, ANOVA (variance analysis) was used to compare the groups. Chi-square analysis was performed for the number of opened and unopened eggs in dose treatments and control group. In the analyses, the statistical significance level was accepted as $p<0.05$. Statistical analyses were performed using SPSS (Statistical Package for Social Sciences; SPSS Inc., Chicago, IL) 22 package program.

RESULTS AND DISCUSSIONS

The current conditions of the eggs after carbon dioxide applications are shown in Figs.5. At the highest dose of 670 ppm. Yellowing, and then, darkening were observed in the eggs. In addition, at doses of 600 ppm, color change in the form of yellowing was observed. It was observed that the eggs were healthy and the anchor marks were prominent at the dose of 430 ppm with normal carbon dioxide concentration in the control treatments (Figure 5). It is seen that the morphological appearance of the eggs changed with the decrease in the carbon dioxide dose in the application cabin (Figures 5). Especially at the dose of 600 ppm, it was observed that the eggs shrivelled and the colour of the eggs changed, and at 670 ppm, the colour was darker than normal (Figure 5).

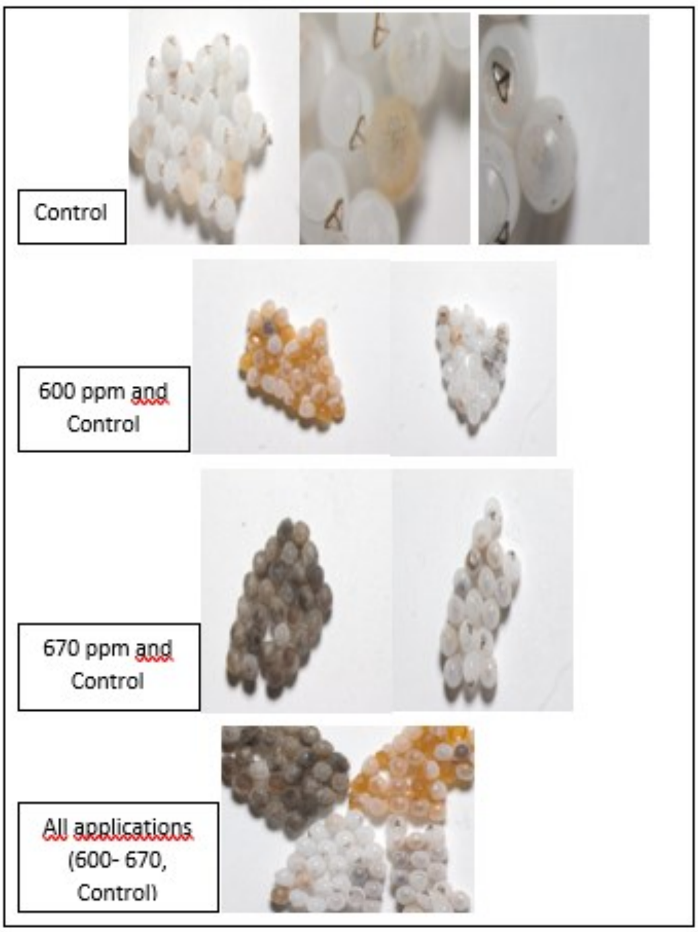

Figure 5. The all of Applications

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In the study, egg-hatching rates were monitored daily, no gas was applied in the control application, and egg-openings were monitored only in the conditioning room. It was determined that 234 eggs out of 261 eggs hatched in the control applications, 218 eggs out of 261 eggs hatched in the 600 ppm dose application, and 188 eggs out of 261 eggs hatched in the 670 ppm application (Figure 9). It has been observed that the ratio of unhatching of egg packages is consistent in the packages left at a time, in other words, the packages are generally opened or not opened in bulk. It is important for the reliability and sustainability of the study that the climatic conditions of the gas supplied environment and the climatic conditions of the air-conditioning cabinet where production is carried out are optimal. In the next stage of the studies, the scenario of the temperature increase predicted in global warming will be planned with dose increases. The differences in egg-hatching times of eggs opened after two different doses of carbon dioxide compared to the control group (400-430 ppm) are shown in Table 1.

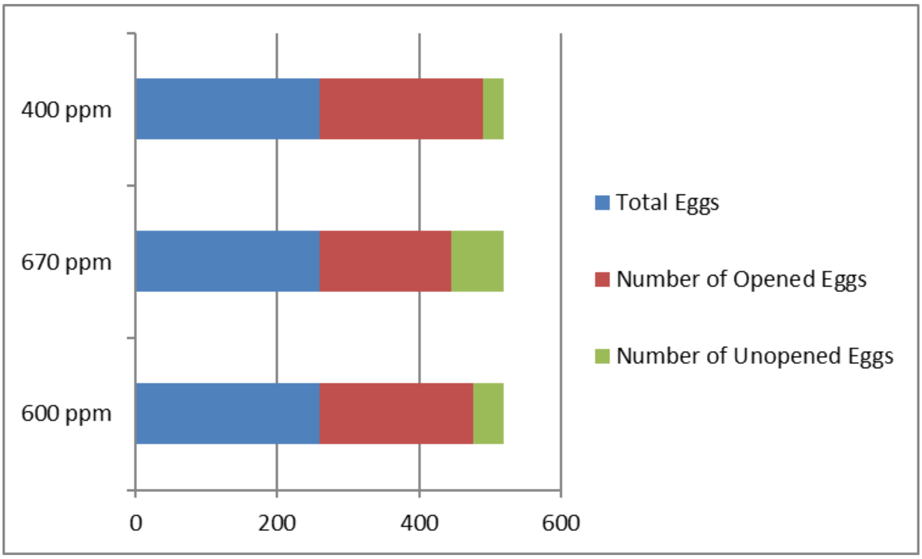

Figure 6. Differences between carbon dioxide applications

Table 1. Statistical evaluation of egg hatching times of *Halyamorphahalys* species between different CO_2 dose applications and the control group (400-430 ppm)

Groups	Number	Mean	Std. Dev.	F	P-value
400-430 ppm	230	4.4304	0.49622	5.465	**0.004**
600 ppm	216	4.4167	0.49415		
670 ppm	186	4.2849	0.45261		

A statistically significant difference was found between the egg hatching times of control, 600 ppm and 670 ppm dose treatments ($p<0.05$).

The hatching time of 670 ppm dose treated pests was significantly lower than the hatching time of the control group and 600 ppm dose treated pests ($p<0.05$).

In the study that was conducted to determine the effects of carbon dioxide applications on *H. halys* egg-hatching, the total of opened and unopened eggs in 600 ppm applications are given in Table 2.

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Table 2. Number of opened and unopened eggs compared to the control group after 600 ppm carbon dioxide application to *Halyamorpha halys* eggs

Application	Opened	Unopened	Total
Control (400-430 ppm)	234	27	261
600 ppm	218	43	261
Total	452	70	522

The difference between 600 ppm and control (400 ppm) was found to be statistically significant (x^2 = 4.224, $p<0.05$), showing that the use of 600 ppm reduces egg opening compared to the control at a statistical significance level of 5 %. In the study conducted to determine the effects of carbon dioxide applications on *Halyamorpha halys* egg-hatching, the total of opened and unopened eggs in 670 ppm applications is expressed in Table 3.

Table 3. Number of opened and unopened eggs compared to the control group (400-430 ppm) after 670 ppm carbon dioxide application to *H. halys* eggs

Application	Opened	Unopened	Total
Control (400-430 ppm)	234	27	261
670 ppm	188	73	261
Total	422	100	522

The difference between the 670 ppm application and the control group was found to be statistically significant (x^2 = 26.174, $p<0.05$), suggesting that 670 ppm application reduces egg hatching compared to the control at a statistical significance level of 5 %. In the study conducted to determine the effects of carbon dioxide applications on *H. halys* egg opening, the total of opened and unopened eggs in 600 ppm and 670 ppm applications is given in Table 4.

Table 4. Number of opened and unopened eggs after 670 ppm carbon dioxide application to *H. halys* eggs compared to 600 ppm application

Application	Opened	Unopened	Total
600 ppm	218	43	261
670 ppm	188	73	261
Total	406	116	522

The difference between 600 ppm and 670 ppm was found to be statistically significant (x^2 = 9.975, $p<0.05$). This result shows that the use of 670 ppm reduces egg opening compared to the use of 600 ppm at a statistical significance level of 5 %.

CONCLUSION

In this study, the effect of three different CO_2 applications on the egg opening ratio and time of *H. halys*, commonly known as the bug (brown marmorated stink bug), was investigated. The findings revealed significant effects of high CO_2 levels on both opening ratios and incubation times of pests. At the highest CO_2 dose of 670 ppm, observable changes, such as yellowing and darkening of eggs, were noted. There was a subsequent reduction in opening ratios

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compared to lower CO_2 concentrations and the control group. Similarly, the 600 ppm application caused a decrease in opening ratios compared to the control group (400 ppm), although less than the 670 ppm application. Additionally, while no significant difference was observed in terms of incubation times between the control and 670 ppm groups, a significant decrease in egg-hatching times was noted in the 670 ppm group. This points to possible behavioral changes in response to high CO_2 levels.

According to statistical analysis, both 600 ppm and 670 ppm treatments showed decreased opening ratios compared to the control group. Besides, the difference between the two CO_2 concentrations was also statistically significant, demonstrating the dose-dependent effect of CO_2 on bug reproduction. Overall, these results highlight the importance of considering atmospheric CO_2 levels in predicting pest reproductive patterns, especially in the context of global warming scenarios. It is also anticipated that potential implications for insect control strategies can be developed through high CO_2 applications and optimization studies. In this pest axis, atmospheric carbon dioxide changes will constitute a source of basic data in creating a strategy for combating atmospheric carbon dioxide changes.

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