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Effect of Carbon dioxide (CO_2) on mortality and reproduction of *Anagasta kuehniella* (Zeller 1879), in mass rearing, aiming at the production of *Trichogramma* spp.

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ABSTRACT

Eggs of *Anagasta kuehniella* (Zeller 1879) are widely used for mass rearing of *Trichogramma* spp. and other parasitoids and predators, largely commercialized in many countries. The aim of this study is to evaluate the effect of carbon dioxide (CO_2) originated from larval metabolism on the biological parameters of *A. kuehniella*. For that purpose, we assess the production of carbon dioxide (CO_2) per rearing tray of *A. kuehniella* and the effect of CO_2 on the viability of egg-to-adult period and oviposition of *A. kuehniella*. Results allow to estimate that a rearing tray, containing 10,000 larvae between the 4th and 5th instars, produces an average of 30.67 mL of CO_2 per hour. The highest egg production of *A. kuehniella* was obtained when the larvae were kept in rooms with lower concentration of CO_2 (1,200 parts per million - ppm), producing 23% more eggs than in rooms with higher CO_2 concentrations. In rooms with high density of trays (70 trays/room), CO_2 concentration exceeded 4,400 ppm. The viability of the egg-to-adult period was not influenced by carbon dioxide.

Key words: mass rearing, factitious host, abiotic factor, carbon dioxide.

INTRODUCTION

Egg parasitoids *Trichogramma* are widely used in various parts of the world, with over 18 species being mass-reared for pest control in 16 countries, an area, estimated in the 90's, corresponding to around 18 million ha (Hassan 1997). In Russia alone, 3 to 10 million hectares were "treated" annually with *Trichogramma* spp. as a mean to control pests in different crops (van Lenteren 2008). Currently, in Brazil, an area of 500,000 ha of sugarcane has been treated with *Trichogramma galloi* Zucchi, 1988, for the control of *Diatraea saccharalis*. (Fabr 1794) (Parra 2010).

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The successful use of these parasitoids in pest control is attributed to the condition of rearing them on eggs of factitious hosts, since the number of insects required to rear them is very large and difficult to obtain in eggs of natural hosts. The flour-moth, *Anagasta kuehniella* (Zeller 1879), is one of the alternative hosts that provides desirable nutritional quality to their parasitoids (Lewis et al. 1976) and, due to its mass rearing condition, it is used in biological control programs in Europe and in Brazil (Parra 1997).

The mass rearing of this host requires control of temperature and relative humidity (RH), since temperature regulates the development of all stages of the insect, compromising reproduction, if not adequately controlled (Daumal and Boinel 1994).

High temperatures also favor the emergence of the parasitoid larvae *Habrobracon hebetor* (Say 1836), which is attracted by the frass produced by the larvae (Parra et al. 1996). Fungi and mites are commonly found in insect rearing, favored by high RH (Parra 1997).

One factor, not much taken into account, in mass rearing of *A. kuehniella* is carbon dioxide (CO_2), which accumulates in rearing rooms, released from the larval metabolism. Commercial laboratories use trays of several different sizes, containing a large number of larvae, so the density of trays (trays/m^2) inside of the rearing rooms is very high, which can lead to excessive accumulation of CO_2, and can ultimately cause losses in the rearing.

Given the characteristics of mass rearing of *A. kuehniella*, this paper aims to evaluate the CO_2 production per rearing tray of *A. kuehniella* and the effect of CO_2 on the viability of adult-egg period and oviposition of this moth.

MATERIALS AND METHODS

CARBON DIOXIDE (CO_2) PRODUCTION PER REARING TRAY OF *A. KUEHNIELLA*

The test to evaluate the production of CO_2 per rearing tray of *A. kuehniella* was developed at the Laboratory of Forest Ecology and Entomology, ESALQ/USP. To measure the CO_2 production per rearing tray of *A. kuehniella*, the trays remained in rooms of 3.60 m^2 or 9.79 m^3 with the temperature adjusted to 25 ± 3°C and a photophase of 14h. Ten, twenty, thirty, forty, sixty and seventy rearing trays (40 x 25 cm of base by eight inches tall) were kept in the rooms resulting in the following proportions: 1, 2, 3, 4, 6, 7 tray/m^3, respectively.

Each tray contained 1.4 kg of diet (97% whole wheat flour and 3% yeast) (Parra 1997), with the vast majority of larvae at the 5th instar. Each tray contained approximately 10,000 eggs, equivalent to 0.27 g of eggs (1g = 36,000 eggs).

Measurements were taken in these larvae in the 5th instar, since preliminary tests showed that peak CO_2 production occurs at this instar. After 24 hours, we measured the CO_2 concentration inside the rearing rooms, using a CO_2 environment meter Testo®, model 535/CO_2, and the CO_2 concentration was expressed in parts per million – ppm. An empty room without rearing trays was used as the control. We used the results to build a scatter plot, in which we related the CO_2 concentration with the number of trays per room in order to estimate the amount of CO_2 produced by a single tray of *A. kuehniella*.

To express the amount of CO_2 produced by trays in milliliters (mL), we made a conversion, where the amount of CO_2 produced in the room was subtracted by the amount of CO_2 in the control room. The remaining amount of CO_2 was converted into percentage using a rule of proportionality, considering that 1,000,000 ppm corresponds to 100% CO_2. Afterwards, the percentage of CO_2 in the room was transformed into mL, considering the volume in liters in the room and the percentage of CO_2 inside each room, to obtain the amount of CO_2 produced per hour. Once again, a rule of proportionality was used to quantify the amount CO_2 produced in 24 h and the quantity produced in 1h.

EFFECT OF CO_2, ON THE EGG-TO-ADULT VIABILITY AND OVIPOSITION OF *A. KUEHNIELLA*

The experiment to evaluate the effect of CO_2 on *A. kuehniella* was also carried out at the Laboratory of Forest Ecology and Entomology, ESALQ/USP. In this study, rearing trays of *A. kuehniella* (40 x 25 cm of base and eight inches tall), containing 1.4 kg of a diet (97% whole wheat flour and 3% yeast) and "inoculated" with approximately 10,000 eggs (Parra 1997) were kept in three rooms (3.60 m^2 or 9.79 m^3) permanently closed with temperatures adjusted to 25 ± 3°C and a photophase of 14h. In these rooms, 10, 35 and 70 rearing trays were placed, corresponding to 1.02, 3.57 and 7.15 trays/m^3, respectively.

We performed daily measurements of CO_2 in the rooms using a meter and the concentration was expressed in ppm. Measurements were made from the moment of placing the eggs into the trays until the emergence of the first adults. After emergence, five trays (randomly chosen) of each room were selected to determine the viability estimated for each treatment.

We collected adults emerged from each tray until the full emergence, at intervals of five days, using an adapted vacuum cleaner, and weighed the total of insect/tray on a scale in order to estimate the feasibility of each tray. We also estimated the number of eggs produced per tray, multiplying the estimated number of emerged females by the average number of eggs per female in each condition.

We evaluated the individual average weight for males and females of each collection day and we considered a gender ratio of 0.5 (Stein and Parra 1987), thus estimating the number of insects emerged in relation to the number of "inoculated" eggs.

We performed the assessment of the number of eggs laid and life span of adults by separating 20 couples on the first day of emergence of each treatment. For the daily egg counts, these couples of adult insects were kept in glass tubes (8 x 2 cm) in a room with temperature controlled at 25 ± 1°C, RH 60 ± 10%, photophase of 14h and atmospheric CO_2 concentration. Life span was assessed up until the death of all adults.

The viability of eggs laid was assessed by separating the eggs, from the second laying day onwards, into batches of 10 and placing them in Petri dishes (9.5 cm diameter) containing filter paper inside. The dishes were kept at a temperature of 25 ± 1°C, RH 60 ± 10%, photophase of 14h and atmospheric CO_2 concentration. The remaining eggs were stored for 10 days for subsequent weighing, separated into in batches of 50 and weighed on an analytical balance.

Statistical Analysis

Data on the number of eggs per female, individual weight of females on the first day of collection, viability of the trays, the total weight of insects per tray, estimated production of eggs, their weight and egg viability were analyzed in terms of normality, homoscedasticity and presence of outliers by the optimal transformation of Box-Cox (with the aid of statistical software SAS®). Subsequently, data were subjected to analysis of variance and means compared by Tukey test at the level of 5% probability.

The data on life span were analyzed using the survival curve, where the means and standard error were computed with the Kaplan-Meier of the survival function and corresponding duration, and averages compared by Log-rank ($p<0.05$).

RESULTS

Production of Carbon Dioxide (CO_2) Per Rearing Tray of *A. kuehniella*

The production of CO_2 per rearing tray of *A. kuehniella* showed a linear and proportional relation at an average temperature of 25 ± 3°C and a photophase of 14h, being that the greater the

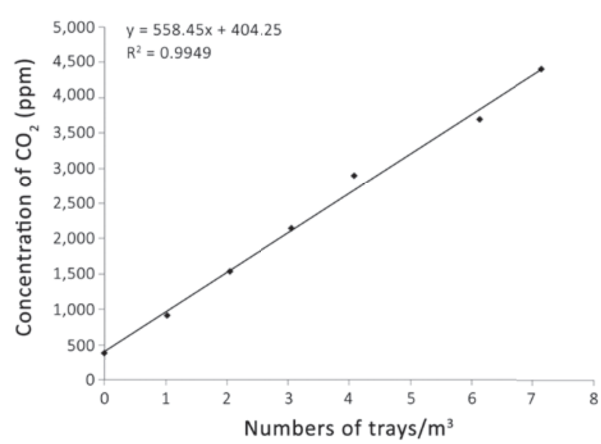

Figure 1 - Linear regression of CO_2 production at different densities of rearing trays of *A. kuehniella* (with approximately 10,000 larvae in the 5th instar) per m^3, 9.79 m^3 rooms, 25 ± 3°C, photophase: 14h.

number of trays, the higher the concentration of CO_2. Through the linear equation obtained with the collected data, we were able to estimate that a single rearing tray of *A. kuehniella*, with approximately 10,000 larvae in the 5th instar, increases the concentration of CO_2 in the rearing room to 9.79 m^3 at 75 ppm (Figure 1), i.e., a tray produces approximately 730 mL of CO_2 inside the room in a period of 24h. In the room with higher density of trays (7 trays/m^3), the amount of CO_2 produced was 39.40 L, thus increasing the concentration of CO_2 inside the room to 4,400 ppm (Table I).

TABLE I

Concentration of CO_2 (ppm) produced by trays of *A. kuehniella*, inside the rooms of 9.79 m^3, the amount of CO_2 into the rearing rooms (mL) and the amount of CO_2 produced per tray per hour (mL): Temperature: 25 ± 3°C, photophase: 14h.

Number of trays/ room	Proportion of trays/m^3	CO_2 (ppm)	CO_2 (mL)	mL CO_2/ tray/h
1	0.1*	75**	736**	30.67**
10	1	909	5,130	21.39
20	2	1,533	11,260	23.45
30	3	2,143	17,240	23.95
40	4	2,888	24,560	25.58
60	6	3,688	32,410	22.51
70	7	4,400	39,400	23.45

*proportion of one tray kept in a room of 9.79 m^3,
**Estimated values.

Effect of CO_2 on the Viability of the Egg-to-Adult Period and Oviposition of *A. kuehniella*

The concentration of CO_2 inside the rearing rooms was equal until the 8th day of larval development (450 to 550 ppm). From the 9th day onwards, the difference in the CO_2 concentration inside the rooms was more pronounced, rising gradually in each room as each instar increased, until the 25th day, considering that this time the larvae had reached the 5th instar, and were about to pupate. After the 25th day, the metabolism of insects decreased due to lower pupal metabolism (Chapman 1998) and sharply reduced the CO_2 concentration inside the rearing rooms (Figure 2). In the room containing 10 trays at maximum, the CO_2 concentration was 1,195 ppm, while in the room with 35 trays, the CO_2 concentration reached 1,763 ppm and in the room with 70 trays, the maximum CO_2 concentration was 4,425 ppm, a concentration 13 times higher than the atmospheric CO_2 concentration (approximately 300 ppm) (Figure 2).

Females of immature stages kept in rooms with lower CO_2 concentrations (1,195 ppm) produced, on average, 22% more eggs than females from rooms where the CO_2 concentration was higher than 1,500 ppm ($F=7.37$; $p<0.001$; $n=20$). Females in rooms where the CO_2 concentration reached 1,763 ppm and 4,424, laid the same number of eggs (Table II).

The weight of females, on the first day of collection, in rooms with different CO_2 concentrations were similar ($F=0.77$; $p=0.47$; $n=25$), likewise so were their life span ($T=2.2$; $p=0.338$; $n=20$) (Table II). Males that emerged in trays kept in rooms where the CO_2 concentration reached 4,425 ppm had a shorter life span than males emerged in trays kept in rooms where the CO_2 concentration reached 1,763 and 1,195 ppm ($T=13.3$; $p=0.001$; $n=20$) (Table II).

The total number of adults emerged, as well as their weight per tray of different CO_2 concentrations were similar in all treatments ($F=0.85$; $p=0.45$; $n=5$/ $F=3.37$; $p=0.071$; $n=5$), likewise so was the estimated viability of the egg-to-adult period for each treatment ($F=0.85$; $p=0.45$; $n=5$) (Table III). However, when estimating the number of eggs produced per tray, relating the number of eggs laid per female and number of emerged females, we could observe a higher production in trays maintained at concentrations below 1,200 ppm ($F=14.98$; $p=0.0005$; $n=5$). It can be inferred that these trays produce about 180,000 eggs, i.e., approximately 5 g more than those kept at concentrations above 1,200 ppm (Table III).

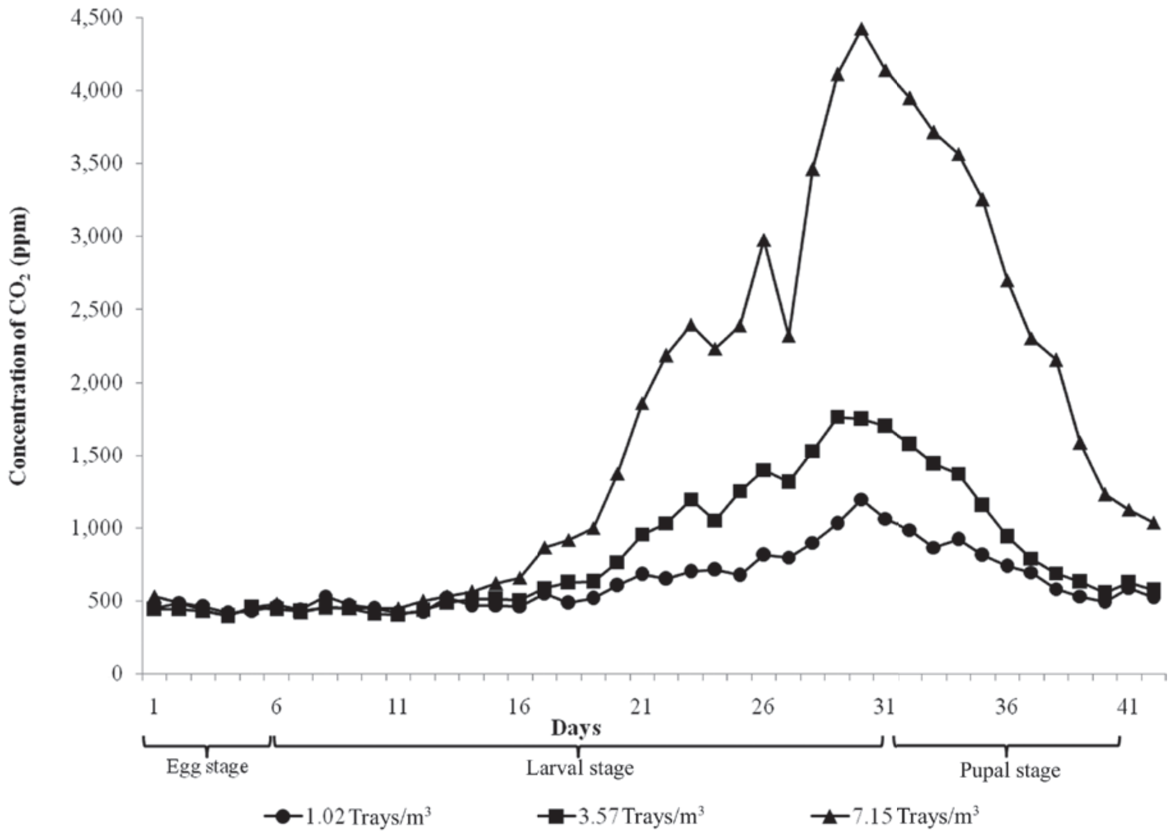

Figure 2 - Concentration of CO_2 (ppm) during the egg-to-adult period of *A. kuehniella*, inside the rearing rooms with different quantities of trays per m^3. Temp.: 25 ±3°C, photophase: 14h. The arrows indicate the maximum production of CO_2 in the three conditions studied, corresponding to the 5^{th} instar of the larval stage.

TABLE II

Number of trays per m^3, weight average of females, number of eggs per female, life span of ♂ and ♀ of *A. kuehniella*, emerged in rearing trays kept in rooms at different CO_2 concentration (metabolic) and maximum concentration of CO_2 (ppm) inside the rooms. Temperature: 25 ±1°C, RH: 60 ±10%, photophase: 14h, atmospheric CO_2 concentration.

Number of trays/room	Density of trays/m^3	Weight of females (mg)[1,5]±EP[4]	N° of eggs/ female[2]±EP[4]	Life span (days)		Maximum concentration of CO_2 (ppm)
				♂[3]±EP[4]	♀[1]±EP[4]	
10	1.02	21.08 ± 0.64	427.55 ± 23.67 a	12.25 ± 0.58 a	7.47 ± 0.24	1,195
35	3.57	22.04 ± 0.49	328.65 ± 14.07 b	13.50 ± 0.61 a	8.00 ± 0.44	1,763
70	7.14	21.89 ± 0.63	343.15 ± 20.09 b	7.70 ± 0.48 b	8.05 ± 0.41	4,425

[1]Data showed no statistical differences in the Tukey test, at 5% of probability. [2] Means followed by the same letter did not differ in the Tukey test at 5% of probability. [3] Means followed by the same letter did not differ in the Log-rank test. [4] Standard error of the mean. [5] Females on the first day of data collection.

TABLE III
Average of total weight of insects, estimated average of the number of insects, viability of the egg-to-adult period and number of eggs produced per tray, originated from *A. kuehniella* emerged in rearing trays kept in rooms with different CO_2 concentrations. Temperature 25 ±3°C, RH: 60 ±10%, photophase: 14h, atmospheric CO_2 concentration.

Number of trays/room	Total weight of insects/tray[1](g)±EP[3]	Estimated number of insects/tray[1]±EP[3]	Estimated viability/ tray[1]±EP[3]	Number of estimated eggs/tray[2]±EP[3]
10	64.78 ± 3.1	4,513 ± 218	45.1 ± 2.2	964.9 ± 46.71 a
35	63.8 ± 1.1	4,728 ± 95	47.3 ± 0.9	777.1 ± 15.72 b
70	67.38 ± 1.7	4,468 ± 107	44.7 ± 1.1	766.7 ± 18.52 b

[1] Data showed no statistical differences in the Tukey test, at 5% of probability. [2] Means followed by the same letter did not differ in the Tukey test at 5% of probability. [3] Standard error of the mean.

The viability of eggs laid by females maintained during the immature stages at different CO_2 concentrations were not affected, remaining equal for all concentrations and above 90% ($F=0.16$; $p=0.86$; $n=10$); nor was egg weight was affected by different CO_2 concentrations to which the immature eggs were exposed ($F=0.07$; $p=0.93$; $n=10$) (Table IV).

TABLE IV
Viability and weight of eggs from females of *A. kuehniella* emerged in rearing trays kept in rooms with different concentrations of CO_2. Temperature: 25 ± 3°C, RH 60 ± 10%, 14h photophase, atmospheric CO_2 concentration.

Number of trays/room	Viability of eggs (%)[1]± EP[2]	Egg weight (µg)[1] ± EP[2]
10	97 ± 1.5	24.0 ± 0.9
35	98 ± 1.3	24.4 ± 0.8
70	97 ± 1.5	24.4 ± 0.8

[1] Data showed no statistical differences in the Tukey test, at 5% of probability. [2] Standard error of the mean.

DISCUSSION

Production of Carbon Dioxide (CO_2) Per Rearing Tray of *A. kuehniella*

Analyzing the value of CO_2 production in a single tray found in this study, 0.73 L, for mass-rearing, in which about 10,000 trays are kept in the rearing room, 625 m^3, we can estimate a production of 7,300 liters of CO_2 per day, considering the 5th instar of larvae (Table I). However, we must consider that the 7,300 L of CO_2 provide a higher concentration inside the room of 625 m^3, because the density of trays in the room is 16 trays/m^3, resulting in a CO_2 concentration above 10,000 ppm, 30 times higher than the atmospheric air (which is approximately 300 ppm).

The CO_2 production showed an average rate of 23 mLCO_2/tray/h (Table I), much smaller than the CO_2 production of a human being at rest, which produces 12,000 mLCO_2/h, according to the National Institute for Occupational Safety and Health-NIOSH, USA (Williams 2009). Considering that each tray had 10,000 larvae, we can estimate a production of 0.0023 mLCO_2/larave/h or 2.3 µLCO_2 /larvae/h.

According to Williams (2009) exposure to high concentrations of CO_2 can cause to humans, visual disturbances, headaches, idleness, the sensation of breathlessness and dyspnea and induce narcosis, similar to nitrous oxide. Williams (2009) noted that human beings at rest can withstand, without restriction, concentrations of up to 15,000 ppm. Analogically, concentrations of 30,000 ppm are tolerated for up to 15h by humans at rest, while people running heavy duty can tolerate this concentration for just 30 minutes. When the CO_2 concentration is too high, about 70,000 ppm, the tolerance, even at rest, is 30 minutes, while people performing heavy work collapse and become unconscious for an undetermined period.

EFFECT OF CO_2 ON THE VIABILITY OF EGG-TO-ADULT PERIOD AND OVIPOSITION OF *A. KUEHNIELLA*

The effect of CO_2 on the fertility of *A. kuehniella* was reported in the literature by Janish (1924 apud Lum and Flaherty 1972) who reported that anesthesia with CO_2 reduces the frequency of copulation and oviposition of *A. kuehniella*. The smaller number of eggs laid by females in rearing rooms with higher CO_2 concentrations (up to 1,200 ppm) may be related to lower ovarian development. According to Press et al. (1973) exposure of females of *Tribolium castaneum* (Herbst 1797), newly emerged, to CO_2 concentrations of 96% (960,000 ppm), suppresses the development of the ovaries and the number of eggs laid reduces significantly, even when the insects are transferred to atmospheric concentrations of CO_2. Lum and Phillips (1972) reported that when *Plodia interpunctella* (Hübner 1813) is anesthetized with 96% of CO_2 (960,000 ppm), there is a 63% reduction in egg laying of the insect, when compared with those not treated with CO_2. The authors argued that the CO_2 can immobilize the sperms inside the female, which eliminates the stimulus for oviposition. CO_2 can also inhibit the development of eggs or block stimulus for oviposition when the oocytes are already mature. Kumar and Saxena (1978) reported mating delay of *Empoasca devastans* Distant, 1918 anesthetized with CO_2.

According to Schroeder et al. (2006), the increase of CO_2 in the soil to 550 ppm has a beneficial effect on egg production of *Diabrotica virgifera virgifera* (LeConte 1868) raising fertility by 50%. However, the authors concluded that CO_2 increases egg production because insects recognize the presence of their host by means of the CO_2 emitted by roots and, thus, the high concentration of CO_2 in the soil induces the insect to lay more eggs.

Under the conditions adopted in the present study, the different CO_2 concentrations during the immature stages did not affect the weight of females on the first day of collection, nor their life span (Table II). The shorter life span of males, in rooms where the CO_2 concentration reached 4,425 ppm, indicates that males have greater sensitivity to the conditions of CO_2 during the immature stages, and that is not possible to make such assessment in females, because they die soon after they oviposit. The results described are different from those reported by Edwards and Patton (1965) who observed that anesthesia with CO_2 concentrations above 600,000 ppm, even when the O_2 concentration is maintained similar to atmospheric conditions (210,000 ppm), has deleterious effects on size, weight and growth of nymphs of *Acheta domesticus* (Linnaeus, 1758). The authors also reported that the heartbeat of insects decreased with the increase of CO_2 concentration.

According to Perron et al. (1972) the anesthesia of CO_2 for 15 minutes affected the mortality, life span and fecundity of *Drosophila melanogaster* Meigen, 1830, with the most pronounced effect occurring in newly-emerged insects between 0 and 3 h. According to Hooper (1970) the use of CO_2 to anesthetize *Ceratitis capitata* Wiedemann, 1824, for a period of 30 minutes, causes unwanted adverse reactions such as increased mortality, decreased number of eggs per female and decreased viability of these eggs, when compared to the use cold and nitrogen which did not affect the insect. We should take into account that the concentrations of CO_2 to which the insects were subjected in the above mentioned studies are much higher than the concentrations of CO_2 of this work, since the authors cited aimed to anesthetize insects in their studies.

The viability and weight of the eggs found in this study showed no difference, but Lum and Phillips (1972) reported that when *Plodia interpunctella* (Hübner 1813) was anesthetized with 96% of CO_2 (960,000 ppm), the viability of eggs of this insect was reduced by 73%, compared with eggs laid by females not treated with CO_2. AliNiazee and Lindgren (1970) reported that eggs of *Tribolium confusum* Du Val, 1868 and *Tribolium castaneum* (Herbst 1763) from females kept in atmospheres with CO_2 above

25% (250,000 ppm) were not viable. The authors concluded that the decrease of oxygen and the increase of nitrogen had no effect on the incubation period and viability, but the increase of CO_2 concentration was effectively responsible for deleterious effects on the incubation period and viability. The authors argued that the accumulation of lactic acid resulting from anaerobic metabolism may possibly interfere with physiological processes of insects.

Thus, the accumulation of CO_2, yet little studied, should be considered for mass rearing of *A. kuehniella* for the production of *Trichogramma* spp. and other natural enemies, because the high concentration of CO_2 can reduce egg production and cause problems for the staff engaged in the daily handling of these insects in rooms with high concentrations of CO_2.

The CO_2 produced by the metabolism of the larvae interfered with oviposition of *A. kuehniella*, and females reared at low concentrations (1,195 ppm) laid more eggs than those kept at concentrations exceeding 1,200 ppm. For mass rearing of *A. kuehniella*, it is suggested that the CO_2 concentration inside the rooms be kept below 1,200 ppm in order to increase egg production.

However, in order to maintain the CO_2 at such levels, there is need for assessment on expenses involving electricity, air exchange and cooling of the room, to evaluate the cost/benefit ratio of such operations.

It is obvious that CO_2 is not the only factor affecting egg production of *A. kuehniella* in mass rearing for the production of *Trichogramma* spp. or predators, as Parra (1997) reported that temperature, humidity, the ectoparasitoid *Habrobracon hebetor* (Say 1836) and even ants, mites and fungi as other factors may be decisive in rearing such moth. Therefore, carbon dioxide is an additional parameter to be evaluated, especially in mass rearing that is becoming increasingly common, for the production and commercialization of parasitoids and predators in programs for Biological Control.

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RESUMO

Ovos de *Anagasta kuehniella* (Zeller, 1879) são muito utilizados para a criação massal de *Trichogramma* spp. e de outros parasitóides e predadores, sendo comercializados em muitos países. O objetivo deste trabalho foi avaliar o efeito do dióxido de carbono (CO_2), proveniente do metabolismo larval, em parâmetros biológicos de *A. kuehniella*, principalmente na postura. Para que este objetivo fosse atingido, foram avaliados a produção de dióxido de carbono (CO_2) por bandeja de criação de *A. kuehniella* e o efeito do CO_2 na viabilidade do período ovo-adulto e na postura de *A. kuehniella*. Por meio dos resultados obtidos pôde-se estimar que uma bandeja de criação, com lagartas entre o 4º e 5° ínstares, inoculada com 10.000 lagartas produz, em média, 30,67 ml de CO_2 por hora. A maior produção de ovos de *A. kuehniella* foi obtida quando as lagartas foram mantidas em salas com concentração de CO_2 inferior a 1.200 ppm, produzindo 23% mais ovos nesta condição em relação a de insetos provenientes de salas com concentrações maiores. Em salas com alta densidade de bandejas (70 bandejas/sala), a concentração de CO_2 ultrapassou 4400 ppm. A viabilidade do período ovo-adulto não foi influenciada pelo dióxido de carbono.

Palavras-chave: criação massal, hospedeiro alternativo, fator abiótico, dióxido de carbono.

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