Effect of packaging chickpeas with CO_2 modified atmospheres on mortality of *Callosobruchus chinensis* (Coleoptera: Chrysomelidae)

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ABSTRACT

High CO_2 modified atmosphere packaging (MAP) is a sustainable alternative for pest control in stored products. The effectiveness of this method varies depending on the CO_2 concentration used, insect exposure time, temperature, humidity, the tested insect species, and the insect's stage of development. One factor that substantially influences the concentration of CO_2 available inside the packages, and therefore the effectiveness of the treatment, is the sorption of gas in the commodity. This study evaluated the impacts of packaging chickpeas in modified atmospheres of 50% and 90% CO_2 with filling ratios (proportion of grain relative to the volume of the package) of 1% and 96% grain on the mortality of *C. chinensis* eggs and pupae and on the fecundity of the females emerging from the surviving individuals. In packages with a 96% filling ratio, CO_2 concentration in the headspace of packages reduced to 60% and 30% from initial concentrations of 90% and 50% (respectively) in the first 24 hours of exposure. Despite this reduction in CO_2 concentrations, no differences in the mortality of *C. chinensis* eggs and pupae were observed between these packages and those with a 1% filling ratio. The estimated exposure time to achieve 95% mortality (LT_{95}) of the eggs ranged from 38 to 68 hours; for pupae, it ranged from 142 to 248 hours. The fecundity of females that emerged from the surviving pupae decreased after 48 hours of exposure to CO_2, but the fecundity of females that emerged from surviving eggs was not affected by exposure time, MAPs, or filling ratio. Therefore, effective pest control can be accomplished with the use of sufficient treatment times to eradicate the most tolerant developmental stages, regardless of whether the packages are full or not.

Keywords

Packaged chickpeas; Modified atmosphere; Insect pests; Control; Oviposition

1. Introduction

Callosobruchus chinensis (L) (Coleoptera: Chrysomelidae) is one of the most voracious insects that attacks legume grains (Dick & Credland, 1984; Desroches et al., 1995; Ahmed et al., 2003). This species has a short life cycle and a high breeding capacity, which makes it a dangerous pest during the long storage periods that are typical for legumes. Fumigants such as phosphine (PH_3) or residual insecticides are used to prevent and control pests in legumes (Wong-Corral et al., 2013). However, the toxicity of these products, their negative impact on the environment, and the resistance that insects have developed to them are reasons to limit their continued use to control pests (Flora et al., 2006; Nayak et al., 2020).

Currently, modified or controlled atmospheres are a safe and sustainable alternative for insect pest control in many types of stored grains (Riudavets et al., 2009; Navarro, 2012; Cui et al., 2016). Studies of three species of weevils (*Callosobruchus maculatus* (Fabricius), *Zabrotes subfasciatus* (Boheman), and *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae) demonstrate the efficacy of atmospheres with low oxygen (O_2) concentrations and high concentrations of carbon dioxide (CO_2) on the mortality of these insects (Wong-Corral et al., 2013; Iturralde-García et al., 2016). The most tolerant stages to hypercarbia are the egg and pupal stages; adults are the least tolerant (Mbata et al., 2000; Wong-Corral et al., 2013). In addition to mortality, Dawson (1995) demonstrated that modified atmospheres can also affect the reproductive potential of the surviving females of *C. maculatus.*

When using modified atmospheres with a high concentration of CO_2, it is important to note that the grains absorb the gas, thereby decreasing the CO_2 concentration in the headspace of the grain's packaging. This sorption generates a negative pressure that can collapse the package and eventually break it (Navarro, 1997; Cofie-Agblor et al., 1998; Jian et al., 2014). The ability of packaged chickpeas to absorb CO_2 at different package-filling ratios (proportion of grain relative to the volume of the package) was measured by Iturralde-García et al. (2019). However, that study did not determine whether the amount of gas in the headspace of a package after sorption is sufficient to achieve insect mortality. Iturralde-García et al. (2020) showed that a decrease in CO_2 concentration due to sorption in modified atmospheres with a 50% or higher concentration of CO_2 results in reduced mortality of the eggs and adults of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), an occasional chickpea pest. However, this decrease in CO_2 concentration due to sorption does not result in a decrease in mortality in the larvae and pupae of this pest, which develop inside the grain.

C. chinensis, the key pest of stored chickpeas, has a similar pattern of development to *R. dominica*: The eggs and adults are located outside the grain, and the larvae and pupae are concealed inside it. However, unlike *R. dominica*, *C. chinensis* does not lay loose eggs; rather, the eggs are attached to the grain's surface, and the newly emerged larvae bore directly into the grain and develop inside it until adulthood. As mentioned before, Chrysomelidae is most tolerant of CO_2 during the egg and pupal stages. A question that arises, therefore, is whether CO_2 sorption in chickpea packages at high filling ratios (which are typical for commercial products) decrease the efficacy of lethal CO_2 concentrations to the most CO_2-tolerant stages of *C. chinensis*, as is the case with the egg stage of *R. dominica*.

Therefore, for the present study, it was hypothesized that the mortality of *C. chinensis* eggs and pupae in packaged chickpeas with modified atmospheres would increase as the exposure period and the initial concentration of CO_2 increased. It was further hypothesized that mortality would decrease for eggs as the filling ratio of containers increased, that mortality would stay the same for pupae regardless of filling ratio, and that fecundity would be decreased at high CO_2 concentrations. To verify these hypotheses, we aimed to assess the impact of packaging chickpeas in modified atmospheres of 50% and 90% CO_2 and with filling ratios of 1% and 96% grain-to-volume on the mortality of *C. chinensis* eggs and pupae and on the fecundity of surviving individuals.

2. Materials and Methods

Eggs and pupae of *C. chinensis* were obtained from cultures maintained in a climatic chamber at 28 ± 2°C, 70 ± 5% R.H., and a photoperiod of 16:8 (L:D) hours. Chickpeas (200 g) were placed in plastic jars and infested with 200 *C. chinensis* adults for 24 hours. The adults were removed, and chickpeas with a desired number of eggs attached to their surface were selected and placed in ventilated incubation cages. The eggs were then incubated for a period of one day; pupae were incubated for 20 days. Five grains infested with three eggs or pupae were placed in woven bags for each replicate.

2.1. *Mortality of C. chinensis*

Semi-rigid plastic packages with a capacity of 710 mL and a thickness of 500 mm (polyethylene terephthalate, PET) were used. The packages were filled with chickpeas at two different filling ratios: a 1% filling ratio (6 g of chickpeas) and a 96% filling ratio (500 g of chickpeas). The packages also contained the abovementioned woven bags containing grains infested with the eggs or pupae of *C. chinensis*. Next, the packages were filled with the desired modified atmosphere, which was previously prepared in a gas mixer (Witt Km 100-3M gas mixer/MEM, Witt Gasetechnick, Witten, Germany). Two types of modified atmosphere packaging (MAPs) were tested with different initial concentrations of gases: The first MAP contained 50% CO_2, 10% O_2, and 40% N_2; the second contained 90% CO_2, 3% O_2, and 7% N_2. A gas analyzer (OXYBABY® Witt Gasetechnick, Witten, Germany) was used to verify the amount of CO_2 and O_2 inside the packages, and the gas levels were measured at the beginning and end of each exposure period. The tested exposure periods were 6, 18, 24, 30, 48, 72, and 96 hours for eggs and 6, 24, 48, 72, and 120 hours for pupae. After exposure to the MAPs, the containers were opened, and the infested chickpeas were stored in the climatic chamber until adults emerged in order to assess mortality. Three replicates of each MAP, filling ratio, and exposure time were conducted. A control treatment with the infested grains but without MAP was also included. To test the permeability of the packages, three containers without grain were included for each of the two MAPs tested.

2.2. *Fecundity of C. chinensis*

Emerging adults of *C. chinensis* that survived the MAPs treatments were sexed and placed in new plastic cages with 50 g chickpeas for seven days to allow them to mate and lay eggs. After the seven-day interval, the cages were opened, the adults were removed, and the newly deposited eggs were counted. Only replicates that initially contained more than four females were included in the analysis.

2.3. *Data analysis*

After 48 hours of exposure, the available gas volume in the semi-rigid package, the volume of CO_2 sorbed by chickpeas, and the negative pressure (vacuum) produced at the filling ratio of 96% were calculated using the equations described in Iturralde-García et al. (2019). Sorption was calculated using the equation $S = (\rho CO_2 V_S) / M_{chickpea}$, where S is sorption of CO_2 (g) per mass of chickpea (kg); ρCO_2 is the CO_2 density of 0.00182952176 g/mL, according to the equation for the density of gases; V_S is volume of CO_2 sorbed by the chickpeas (mL); and $M_{chickpeas}$ is chickpeas mass (kg). Negative pressure was calculated using the equation $P_f = ((m\ R\ T) / (V_{gas}\ M_{CO2})) - P_i$ (2), where P_f is final pressure (kPa); R is the universal gas constant (8.314472 L kPa/K mol); T is temperature (K); V_{gas} is the gas volume available in the container (L); M_{CO2} is the molar mass of the CO_2 (g/mol); P_i is the initial pressure (Kpa); and m is total mass of CO_2 sorbed in the container (g) (obtained from m = S $M_{chickpeas}$). The percentages of egg and pupae mortality were calculated using the initial number of individuals placed in each cage and analyzed with a Probit analysis (Poloplus, LeOra Software). The estimated exposure times to achieve 50% and 95% mortality (LT_{50} and LT_{95}) were compared using fiducial limits (95% confidence interval) for the ratio of LTs, which was the criterion used to identify significant differences in egg and pupae mortality for different filling ratios and MAPs (Robertson et al., 2017). The mean fecundity rates for adults emerging from eggs and pupae were compared using a three-way ANOVA; exposure time, CO_2 concentration, and filling ratio were used as independent factors. The statistical analysis was performed using JMP® 14.2.0 (SAS Institute Inc. 2018). A nominal significance level of 5% ($P < 0.05$) was used.

3. Results

3.1. *Changes in gas concentration*

Fig. 1 shows the CO_2 concentration inside the packages with chickpeas throughout the tested exposure times. The CO_2 concentration in the empty packages (without chickpeas) reduced by a maximum of 1% at the end of the experiment for both MAPs, indicating that the containers were very airtight. In the packages with a filling ratio of 1% chickpeas, CO_2 concentration reduced by no more than 1%, as in the empty packages. However, in packages with a filling ratio of 96% chickpeas, CO_2 concentration reduced by up to 20-30% (Fig. 1). In the full packages, the final CO_2 concentration reduced from 90% to 60% and from 50% to 30% by the end of the exposure time. The largest decrease in CO_2 concentration occurred during the first few hours of exposure.

Table 1 shows the CO_2 sorption by chickpeas and the negative pressure inside the packages after 48 hours of exposure (to 50% or to 90% CO_2 at 1% or 95% filling ratios). In a previous study (Iturralde-García et al., 2019), chickpea sorption levels stabilized after 48 hours of exposure. In our packages with 1% filling ratios, CO_2 sorption varied from 1.9 to 2.9 g per kg of chickpea; almost no negative pressure was observed. However, at the 96% filling ratio, sorption was much lower (0.2 to 0.35 g per kg of chickpeas), and a notable amount of negative pressure was generated inside the packages (73.9 to 58.6 kPa).

3.2. *Mortality of C. chinensis*

When eggs were exposed to 50% and 90% CO_2, total mortality was achieved after 48 hours at both filling ratios (1% and 96%) (Fig. 2). When LT_{50} was calculated for *C. chinensis* eggs, no significant differences were observed between different CO_2 concentrations at the 96% filling ratio. However, when confidence intervals were compared, significant differences were observed at the 1% filling ratio; LT_{50} was higher at 90% CO_2 than at 50% CO_2 (Table 2). No significant differences in LT_{95} values were observed at the 1% filling ratio, regardless of CO_2 concentration. However, at the 96% filling ratio, a higher LT_{95} was obtained at 50% than at 90% CO_2.

Pupae treated with 50% and 90% CO_2 were all killed after 120 hours of exposure at both filling ratios (Fig. 3). No significant differences in LT_{50} or LT_{95} values were found when the confidence intervals between different MAPs and filling ratios were compared (Table 3).

3.3. *Fecundity of C. chinensis*

To assess whether fecundity was altered by the treatments, the number of surviving females from exposure times under 30 hours for eggs and under 48 hours for pupae were tested. After longer exposure periods, too few individuals survived to estimate female fecundity. Females that developed from the eggs (Fig. 4) exposed to the two MAPs produced

41 and 94 eggs on average per female; no significant differences were observed between different exposure times, initial CO_2 concentrations, or filling ratios ($F = 1.3577$; df = 19, 76; $P = 0.1747$).

The fecundity of females emerging from treated pupae differed significantly for different treatments ($F = 4,4974$; df = 15, 68; $P < 0.0001$). Females exposed to high CO_2 MAPs for 48 hours produced an average of 21 eggs, significantly fewer than those exposed for shorter periods, which produced an average of 67 eggs ($F = 17,7280$; df = 3; $P < 0.0001$) (Fig. 5). However, no significant differences were observed between different initial concentrations of CO_2 ($F = 0.9608$; df = 1; $P = 0.3305$), filling ratios ($F = 0.1456$; df = 1; $P = 0.7040$). There were also no significant differences in the interactions between exposure time and CO_2 concentration ($F = 0.9701$; df = 3; $P = 0.4120$), between exposure time and filling ratio ($F = 0.7704$; df = 3; $P = 0.5146$), between CO_2 concentration and filling ratio ($F = 2.4048$; df = 1; $P = 0.1256$), or among all three factors ($F = 2.4752$; df = 3; $P = 0.0688$).

4. Discussion

The susceptibility of *C. chinensis* to the various MAPs differed for the two developmental stages tested. The eggs were more susceptible to the treatment, reaching 100% mortality after 48 hours of exposure. On the other hand, pupae were more tolerant to the treatment, reaching the same mortality after 120 hours of exposure. In both cases, mortality increased as the time of exposure to CO_2 increased. The eggs of a related species, *C. maculatus*, are also more susceptible than the pupae (Wong-Corral et al., 2013; Iturralde-García et al., 2016). For *C. maculatus* eggs, LT_{99} ranges from 2.5 to 4.1 days, depending on CO_2 concentration (70% and 50%, respectively). For pupae, a minimum of nine days are necessary to obtain 100% mortality with CO_2 concentrations greater than 50% (Wong-Corral et al., 2013). Two other bruchid species, *A. obtectus* and *Z. subfasciatus*, require nine days of exposure to achieve total mortality (Wong-Corral et al., 2013). In contrast, in the present study, *C. chinensis* reached 100% mortality after five days of exposure, suggesting that *C. chinensis* is more susceptible to high CO_2 concentration modified atmospheres than the species used in previous studies. Similarly, pupae of *Callosobruchus subinnotatus* Pic (Coleoptera: Bruchidae) reached 100% mortality after seven days of exposure to hypercarbic atmospheres (99% CO_2). With hypoxic atmospheres (2% O_2), more time (10 days) was required to achieve similar mortality (Mbata et al., 2000). Furthermore, in stored horse grams (*Macrotyloma uniflorum* (Lam.) Verdc.) and pigeon peas (*Cajanus cajan* (L.) Huth) infested with *C. chinensis*, no insect damage was recorded after six months and one year of exposure to concentrations of 40% and 50% CO_2, respectively (Divya et al., 2016; Padmasri et al., 2017).

However, variations in filling ratios had little effect on the egg mortality and no effect on pupae mortality for *C. chinensis*. In comparison, Iturralde-García et al. (2020) observed a decrease in *R. dominica* egg mortality when chickpeas were packaged with a filling ratio of 96%. In that study, for the internal developmental stages of larvae and pupae, susceptibility to CO_2 did not change based on filling ratio. Eggs are more susceptible to modified atmospheres because young eggs tend to lose oxygen and water; atmosphere impacts older eggs because the larvae's respiratory activity increases shortly before it emerges (Mbata et al., 2004). Pupae have been shown to have lower metabolic rates and therefore lower oxygen demand. Therefore, pupae can continue developing in an environment with a high CO_2 concentration, which enables them to survive longer periods of exposure (Mbata et al., 2000).

The decrease in the CO_2 concentration observed in the headspace of the packages with a 96% filling ratio was related to the sorption of gas into the chickpea grains. This decrease was not observed in packages with a 1% filling ratio, where there were too few chickpeas to absorb enough CO_2 to reduce the CO_2 concentration inside the packages. Sorption occurs in two phases. The first, physical sorption, involves Van der Waals forces. The second, chemisorption, involves electron transfer between the gas and the product (Brunauer, 1943). In both MAPs, the most important reduction in CO_2 concentration occurred during the first few hours of exposure; this can be attributed to physical sorption. The amount of negative pressure produced by the CO_2 sorption differed for the different treatments; it was highest at 90% CO_2 and a 96% filling ratio. According to Iturralde-García et al. (2019), the CO_2 sorption of chickpeas increases with a higher initial concentration of CO_2 and a lower filling ratio (due to increased availability of gas in the package headspace). However, the highest negative pressure occurs with a higher initial concentration of CO_2 and a higher filling ratio (due to the low amount of gas available in the package headspace).

The fecundity of *C. chinensis* females emerging from treated eggs was not affected by MAP concentration levels, filling ratios, or exposure time. Similarly, the fecundity of females emerging from treated pupae was not affected by the concentration of CO_2 or the filling ratio. However, the fecundity of females emerging from treated pupae was reduced after exposure times of 48 hours. According to Pascua et al. (2021), exposing eggs of *C. maculatus* to high CO_2 modified atmosphere (MA) conditions disrupts the structure of the eggs, leading to reduced development duration and longevity and increased mortality for the surviving adults that emerge. Other studies on the fecundity of *Tribolium castaneum* (Herbst) and *Tribolium confusum* Duval (Coleoptera: Tenebrionidae) in modified atmospheres (hypoxia and hypoxia/hypercarbia) report increased fecundity in these species after exposure to 5-20% oxygen and 10% CO_2 (Spratt, 1984). Cheng et al. (2013) quantified genes encoding protease enzymes in *C. maculatus* under conditions of hypoxia and hypoxia/hypercarbia; in the latter set of conditions, significantly higher proteolysis was detected after the insects returned to normoxia. This suggests that, in states of hypoxia/hypercarbia, CO_2 may somehow encourage metabolic recovery and promote the development of insects.

In conclusion, a total of five days (120 hours) of exposure to either 50% or 90% CO_2 killed both the eggs and pupae of *C. chinensis*. However, no differences in the treatment times required to achieve 100% mortality would be expected between the tested MAPs, between eggs and pupae, or between different filling ratios. The fecundity of females that emerged from the eggs that survived MAP exposure was not affected, but females that emerged from pupae exposed to MAPs for at least 48 hours demonstrated reduced fecundity. This suggests that if packages lose CO_2-tightness during packaging or if exposure time is too short, then the surviving insects could continue to develop and reproduce and, consequently, could damage the stored chickpeas.

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Table 1. CO_2 sorption and negative pressure inside packages with two different filling ratios of chickpeas after 48 hours of exposure to two different MAPs.

CO_2 (%)	Filling ratio (%)	Loss of CO_2 (%)	CO_2 sorption (g/Kg chickpea)	Negative pressure (kPa)
50	1	1.43	1.91209	99.3
	96	19.53	0.22177	73.9
90	1	2.15	2.86813	98.3
	96	30.38	0.34495	58.6

Table 2. Probit analysis of the toxicity of two MAPs (50 and 90% CO_2) in two package filling ratios (1% and 96%) on *C. chinensis* eggs.

CO_2 (%)	Filling Ratio (%)	Slope (SE)	LT$_{50}$ (h)^{a}	95% fiducial limits	LT$_{95}$ (h)^{a}	95% fiducial limits	χ^{2b} (df)
50	1	8.33 (1.58)	26.34b	23.22-29.21	41.50ab	35.77-57.72	21.90 (19)
	96	5.37 (0.82)	33.62ab	27.85-38.73	68.03a	56.53-94.63	22.11 (19)
90	1	22.25 (5.20)	34.31a	31.47-36.03	40.67b	38.45-46.16	22.44 (22)
	96	20.18 (5.32)	31.86ab	27.19-34.00	38.44b	35.63-52.71	34.57 (22)

[a] Values with different letters in the same column are significantly different ($P < 0.05$, confidence interval for the ratio of LTs).

[b] Chi-square testing the linearity of dose-dependent mortality indicated that normal distribution provided an adequate fit for the model in all cases ($P < 0.05$).

Table 3. Probit analysis of the toxicity of two MAPs (50 and 90% CO_2) in two package filling ratios (1 and 96%) on *C. chinensis* pupae.

CO_2 (%)	**Filling Ratio (%)**	**Slope (SE)**	**LT_{50} (h)[a]**	**95% fiducial limits**	**LT_{95} (h)[a]**	**95% fiducial limits**	**χ^{2b} (df)**
50	1	3.81(0.69)	35.08a	26.41-42.08	142.79a	75.54-142.44	8.59 (13)
	96	3.51 (0.65)	48.52a	37.32-58.02	222.90a	109.26-239.70	11.73 (13)
90	1	3.28(0.85)	48.15a	22.00-63.99	246.04a	103.15-733.74	17.40 (13)
	96	3.00 (0.64)	41.82a	28.41-52.85	248.26a	105.71-302.76	12.30 (13)

[a] Values with different letters in the same column are significantly different ($P < 0.05$, confidence interval for the ratio of LTs).

[b] Chi-square testing the linearity of dose-dependent mortality indicated that normal distribution provided an adequate fit for the model in all cases ($P < 0.05$).

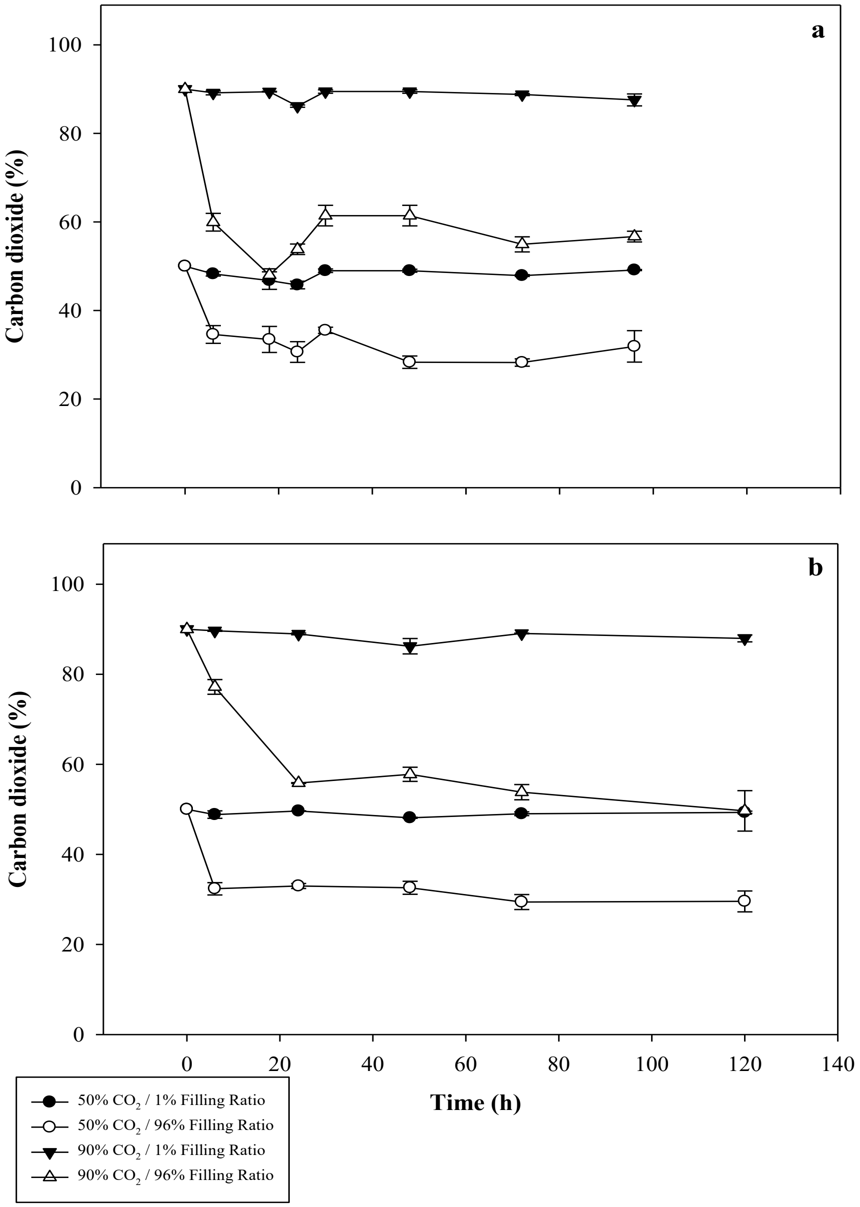

Fig 1. Mean (± standard error) CO_2 concentration within the sealed packages after different exposure times for eggs (a) and pupae (b) with two different modified atmospheres (50% and 90% CO_2) and two different filling ratios (1% and 96%).

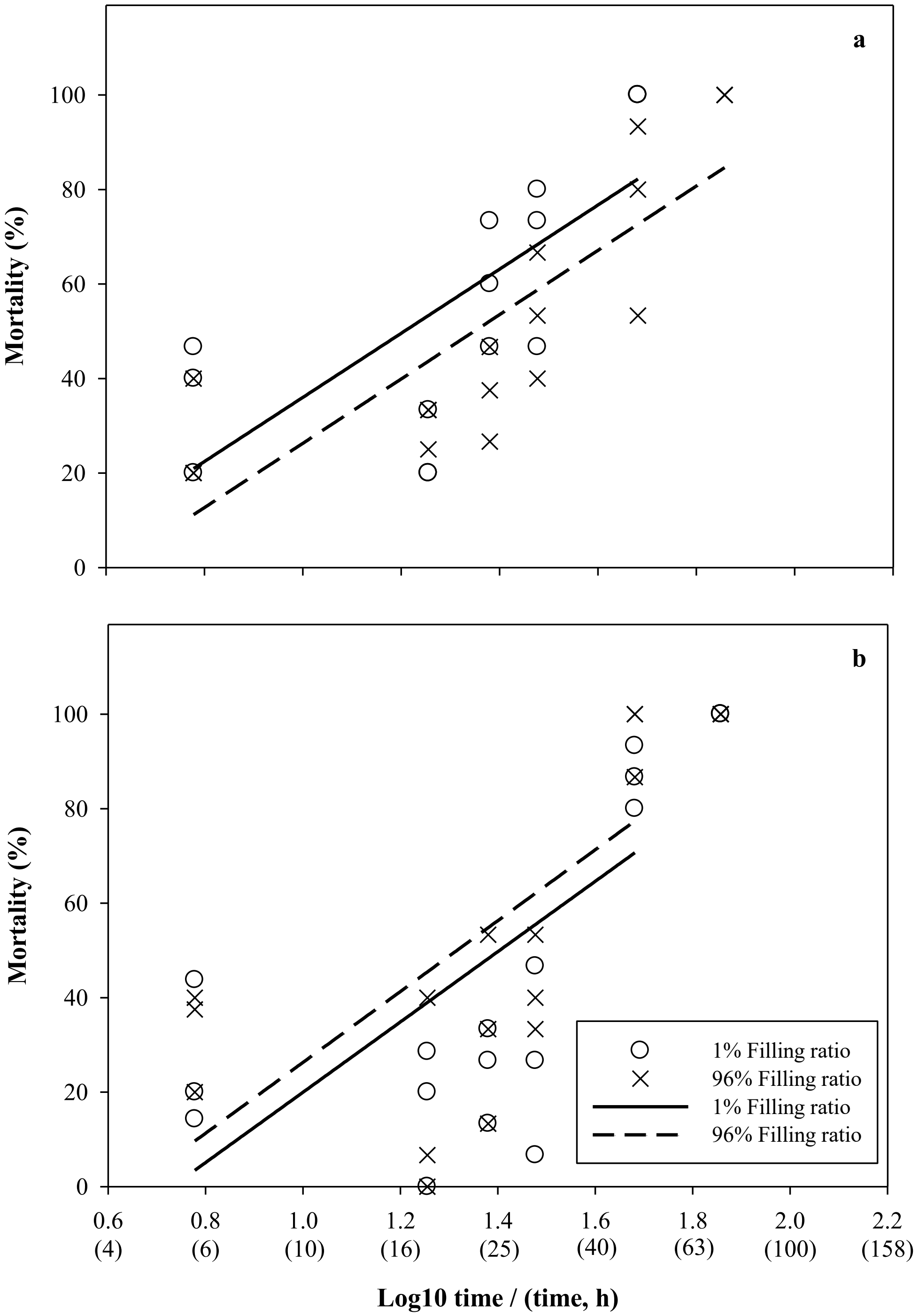

Fig 2. Mortality (on Log10 and on a linear scale) for eggs exposed to two different MAPs (a: 50% CO_2; b: 90% CO_2) in packages of chickpeas with two filling ratios (1% and 96%) after 6 to 96 hours of exposure.

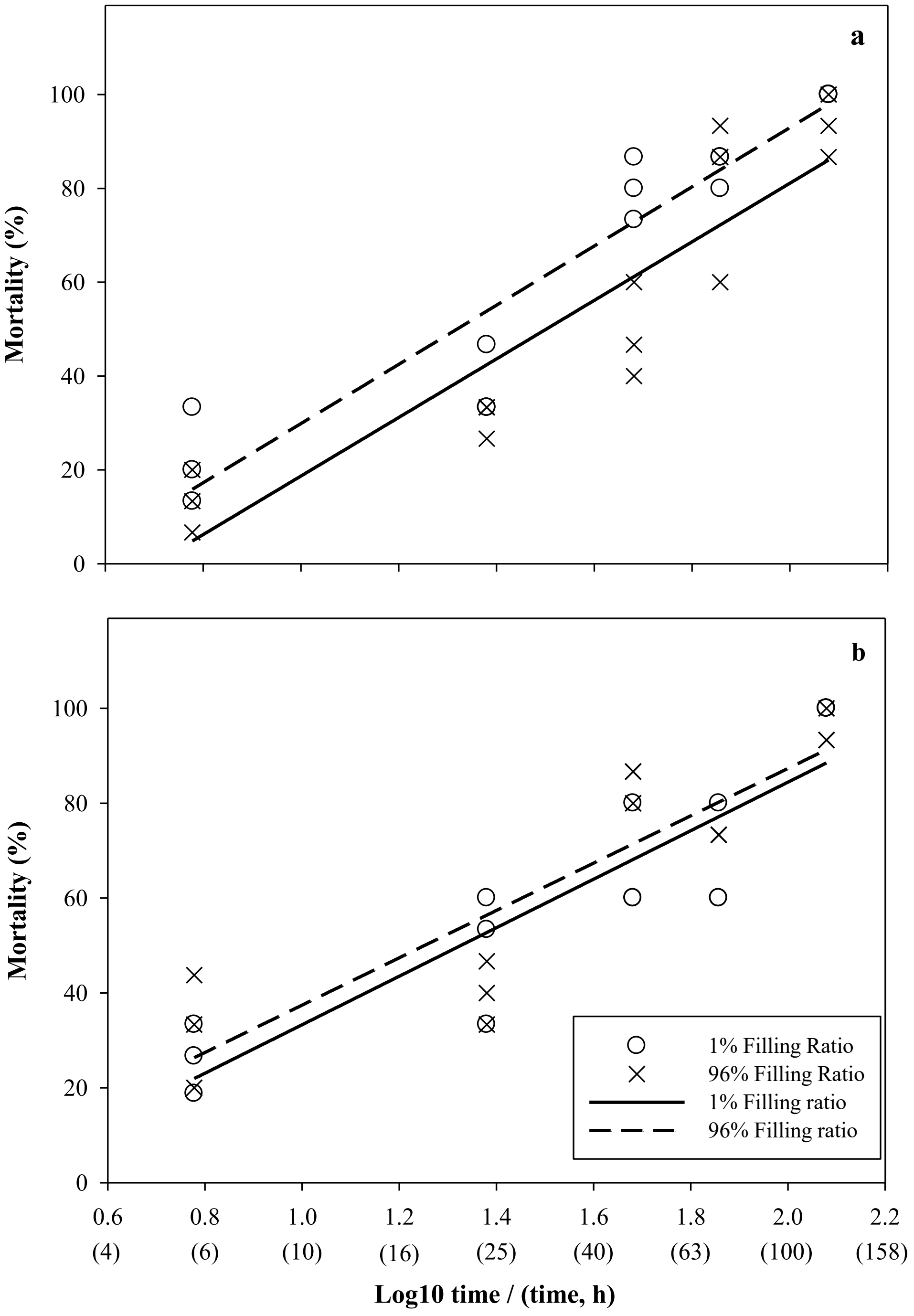

Fig 3. Mortality (on Log10 and on a linear scale) for pupae exposed to two different MAPs (a: 50% CO_2; b: 90% CO_2) in packages of chickpeas with two filling ratios (1% and 96%) after 6 to 120 hours of exposure.

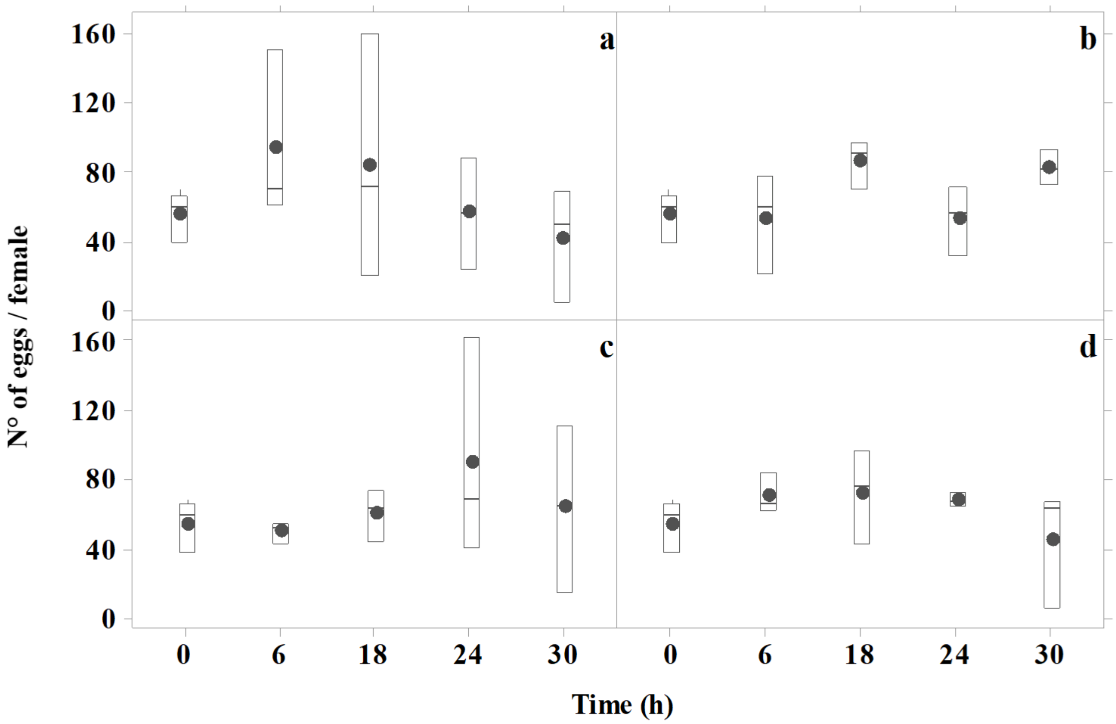

Fig. 4. Number of eggs laid by females that emerged from eggs treated with 50% (a, b) or 90% CO_2 (c, d), and with 1% (a, c) or 96% (b, d) filling ratios. The boxplot shows the mean (solid circle), the median (solid line), the inter-quartile range (box length), and the minimum and maximum values (whiskers).

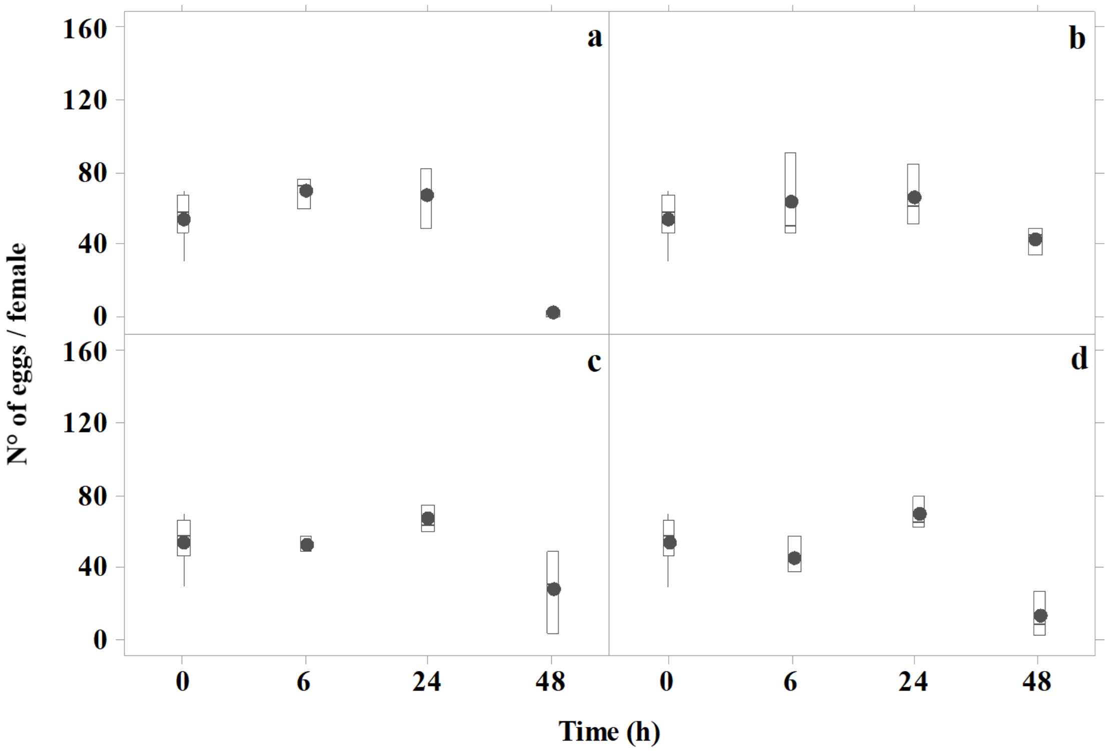

Fig. 5. Number of eggs laid by females that emerged from pupae treated with 50% (a, b) or 90% CO_2 (c, d), and with 1% (a, c) or 96% (b, d) filling ratios. The boxplot shows the mean (solid circle), the median (solid line), the inter-quartile range (box length), and the minimum and maximum values (whiskers).