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**Effect of ozone gas against life stages of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) in laboratory and a storehouse**

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(Article begins on next page)

1 **Effect of ozone gas against life stages of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)**  
2 **in laboratory and a storehouse**

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12

13 **Abstract**

14 Effects of gaseous ozone on *Ephesia kuehniella* eggs, larvae, pupae and adults were evaluated in  
15 laboratory and storehouse trials. In laboratory, all life stages were freely exposed in a test chamber  
16 to five ozone concentrations (5, 10, 20, 50 and 100 ppm) for 24 h to assess mortality, and also  
17 development for the treated eggs, larvae and pupae, and the number of eggs laid by the treated  
18 adults. In a storehouse, the effect of ozone at 50 ppm on adults was evaluated in comparison with  
19 routine treatment (pyrethrin application) and untreated control. In laboratory, all tested ozone  
20 concentrations significantly increased mortality of all *E. kuehniella* stages, in comparison with  
21 untreated control; however, complete mortality was observed only for adults starting from ozone  
22 concentration of 50 ppm. Time needed for the eggs to hatch and the larvae to pupate was delayed by  
23 ozone treatments, starting from ozone concentration of 10 or 5 ppm, respectively. Moreover, ozone  
24 affected also the number of eggs laid by surviving adults in the 24 h following ozone treatment: egg  
25 hatching rate was significantly lower at all concentrations. In the storehouse trials, the ozone  
26 treatment at 50 ppm killed almost all exposed adults as the pyrethrin treatment. Therefore, the  
27 results from this study suggest that ozone can be considered as an effective alternative to the current  
28 routine spray of pyrethrins during spring and summer.

29  
30 **Highlights**

- 31 · Ozone application increased mortality for all life stages compared with the control
- 32 · Complete mortality was observed only for adults, at concentrations  $\geq 50$  ppm
- 33 · Mortality of eggs laid by treated adults increased with the increase of ozone concentration
- 34 · Ozone concentration of 50 ppm killed 99% of exposed adults in a storehouse trials
- 35 · Ozone can be considered as an effective alternative to pyrethrin application

36  
37 **Key words:** Mediterranean flour moth; CT value; mortality; development time; hazelnut  
38 storehouse.

39

## 40 **1. Introduction**

41 Stored-product insects can cause serious postharvest losses, and they also contribute to  
42 contamination of foodstuffs through the presence of live insects, chemical excretions from their  
43 body, production of silk from mandibular glands, dead insects, and cast skins, general infestation of  
44 buildings and other storage structures (Hubert et al., 2018; Phillips and Throne, 2010). Chemical  
45 control is becoming increasingly difficult due to the development of insect resistance to insecticides  
46 (Boyer et al., 2012; Pimentel et al., 2007), the phase out of methyl bromide in 2005 as a fumigant,  
47 the environmental and safety concerns, and the consumers' demand for organic and pesticide  
48 residue-free food (Hansen et al., 2013; Işikber and Athanassiou, 2014; Jian et al., 2013; Phillips and  
49 Throne, 2010; Tiwari et al., 2010). Consequently, with the aim at implementing alternative  
50 measures to control stored-product insects, research has been recently focused on the application of  
51 ozone (O<sub>3</sub>) as an effective fumigant (Hansen et al., 2013, 2012; Işikber and Athanassiou, 2014; Jian  
52 et al., 2013; McDonough et al., 2011; Tiwari et al., 2010). Actually, applications of ozone are  
53 environmentally friendly because ozone can be easily generated with electricity and dried air on  
54 site, and it decomposes rapidly to molecular oxygen without leaving any residue on the commodity  
55 (Işikber and Athanassiou, 2014; Jian et al., 2013; Kells et al., 2001).

56 Ozone is a strong oxidant with a long history of safe use in many fields and mostly used in the  
57 industries of pharmaceuticals, synthetic lubricants, and many other organic compounds, and for  
58 various other environmental applications (Jian et al., 2013; Tiwari et al., 2010). It has been already  
59 widely applied as a powerful disinfectant in water treatment, for controlling microorganisms in  
60 water and air sources, eradicating water-borne parasites, and at present, many municipal water  
61 treatment systems control bacteria with ozone instead of chlorine oxide (Jian et al., 2013).  
62 Moreover, ozone has been successfully used as a sterilant or disinfectant and sanitizer for the  
63 treatment of agriculture products and equipment, such as fruit and vegetables, dairy and swine  
64 effluent, meat, gelatin, manufacturing equipment, packaging materials; it prevents microorganism  
65 activity on food surfaces, is effective on degradation of mycotoxins and pesticide residues on foods,  
66 and extends the shelf life of fruit and vegetables (Jian et al., 2013; Kamber et al., 2017;  
67 Pandiselvam et al., 2017; Tiwari et al., 2010; Trombete et al., 2016).

68 Application of ozone in stored-products is effective in controlling insects, microorganisms such as  
69 fungi and bacteria, and degrade pesticide residues (Al-Ahmadi et al., 2009; Işikber and Athanassiou,  
70 2014; Jian et al., 2013; Savi et al., 2016, 2015; Tiwari et al., 2010). Among stored-product insect  
71 pests, ozone is effective to control weevils, such as *Sitophilus* spp. (Coleoptera: Curculionidae), and  
72 other beetles, such as the lesser grain borer *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae),  
73 the drugstore beetle *Stegobium paniceum* (L.) (Coleoptera: Anobiidae), the merchant grain beetle  
74 *Oryzaephilus mercator* (Fauvel), the sawtoothed grain beetle *O. surinamensis* (L.) (Coleoptera:

75 Silvanidae), *Tribolium* spp. (Coleoptera: Tenebrionidae), the rusty grain beetle *Cryptolestes*  
76 *ferrugineus* (Stephens) (Coleoptera: Laemophloeidae), and moths, such as the Angoumois grain  
77 moth *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae), the Mediterranean flour moth  
78 *Ephestia kuehniella* Zeller and the Indian meal moth *Plodia interpunctella* (Hübner) (Lepidoptera:  
79 Pyralidae) (Bonjour et al., 2011; Hansen et al., 2013, 2012; Işikber and Athanassiou, 2014; Jian et  
80 al., 2013; Mahroof et al., 2018; McDonough et al., 2011; Sadeghi et al., 2017; Sousa et al., 2016;  
81 Subramanyam et al., 2017; Tiwari et al., 2010). At different concentrations and exposure times,  
82 ozone is effective to control both external and internal insect feeders (Hansen et al., 2012).  
83 For the control of stored-product moth pests, the effects of ozone application have been largely  
84 investigated on the Indian meal moth *P. interpunctella* (Bonjour et al., 2011; Hansen et al., 2013;  
85 Keivanloo et al., 2014, 2013; Kells et al., 2001; McDonough et al., 2011; Shaghaghian et al., 2014;  
86 Tiwari et al., 2010), whereas fewer studies are available for the genus *Ephestia*, including the  
87 Mediterranean flour moth *E. kuehniella*, which is one of the most important stored-product pests  
88 worldwide (Athanassiou et al., 2008; Yaman et al., 2015). Moreover, in these studies experimental  
89 conditions, such as exposure time and ozone concentration, modalities of exposure (i.e., specimens  
90 exposed freely or in presence of commodities), vary highly (Abo-El-Saad and Elshafie, 2011;  
91 Hansen et al., 2012; Husain et al., 2015; Işikber and Öztekin, 2009; Işikber et al., 2007; Sadeghi et  
92 al., 2017). Since the effectiveness of ozone depends on its application amount (i.e., concentrations  
93 and exposure times) but in a greater extent on its reaction with insects after the substrate organic  
94 matter oxidation (Jian et al., 2013), different effects of ozone on insects assessed in these studies  
95 might be caused by the presence or absence of the organic substrate (Jian et al., 2013).  
96 Therefore, this study was designed at improving the knowledge on the effects of ozone on all life  
97 stages of *E. kuehniella* (i.e., eggs, larvae, pupae and adults) that were freely exposed at different  
98 ozone concentrations in laboratory conditions to evaluate the lethal effects (i.e., direct mortality),  
99 and also sublethal effects such as developmental times of eggs, larvae and pupae, and hatching of  
100 eggs laid by the treated adults. Moreover, since in Piedmont, northwestern Italy, *E. kuehniella* is one  
101 of the main pests of stored hazelnuts in processing hazelnut factories, the study aimed at  
102 implementing alternative control measures; therefore, an application trial was conducted in a  
103 hazelnut storehouse to compare the efficacy of the routine treatment (weekly sprays of pyrethrins  
104 during spring and summer) with ozone treatment at the lowest lethal concentration on *E. kuehniella*  
105 adults evaluated under laboratory conditions.

106

## 107 **1. Materials and methods**

### 108 *2.1. Insect supply*

109 Eggs and larvae (2<sup>nd</sup>- and 4<sup>th</sup>-instars) of *E. kuehniella*, provided by Biotop (Livron-sur-Drôme,  
110 France), were used to start insect cultures. Colonies were reared on durum wheat flour plus dried  
111 brewer yeast (2%), and kept in climatic chambers under controlled conditions (25±1°C; 60±5% RH;  
112 L:D 16:8). Larvae were confined in glass containers (length×width×height 20×10×10 cm), opened  
113 on the top and covered with an insect-proof fine gauze (mesh size 0.3×0.4 mm), until adult  
114 emergence. Every 24 h, the new emerged adults were collected with a pneumatic aspirator (Vacuum  
115 Pump mod. 707, Asal s.r.l., Cernusco s/N, Milano, Italy); then, they were grouped in around 100  
116 specimens and placed in a Plexiglas cylinder to allow oviposition. Cylinders (height 20 cm,  
117 diameter 10 cm) were closed by an insect-proof net (mesh size 0.6×1 mm) at both extremities, and  
118 placed inverted over a Petri dish (diameter 13.5 cm), containing a black cardboard on the bottom to  
119 facilitate counting of the egg that fell on it. Every 24 h, eggs were collected with a fine brush, and  
120 stored in cold climatic chambers (5±1°C) no longer than one week. In fact, they were weekly used  
121 to start new colonies, in order to continuously obtain cohorts of the same age of larvae, pupae and  
122 adults.

### 123 2.2. Ozone generator and gas monitoring

124 In the trials, the ozone gas was produced using the mobile combined system (ozone and ozonated  
125 water production) COMBI.O3 (model C100-AG, O3Technology S.r.l., Pisogne, Brescia, Italy),  
126 ozone generator with corona discharge technology and nominal capacity 100 g/h. The oxygen  
127 supply for ozone generator was provided by two molecular sieves (model ATF-23, Chart Sequal  
128 Technologies Inc., Ball Ground, Georgia, USA) fed with surrounding air by a compressor (model  
129 2750CGHI60, Gardner Denver Thomas GmbH, Fürstenfeldbruck, Germany), and the power supply  
130 for ozone generator was provided by two power transformers 5.0-0-5.0 kV, 100mA. The ozone gas  
131 was released into a test chamber, which was different for the laboratory and storehouse trials, as  
132 below described. The ozone concentration was measured by means of a digital monitor-controller,  
133 and it was continuously monitored into the test chamber by the recirculation of ozone-enriched air  
134 from the chamber through a BMT 964 UV-photometric ozone analyzer (BMT Messtechnik GmbH,  
135 Berlin, Germany) that controlled the ozone generator output. The recirculation of ozone enriched air  
136 was assured by a ventilator (model MF401MF0.85M, P 0.85 KW). The movable machinery  
137 (length×width×height 128×98×110 cm) was also equipped with an electrochemical ozone detector  
138 (model EV49303PL, 0-5 ppmv, EDS, San Giovanni Lupatoto, Verona, Italy) to measure the ozone  
139 concentration in the environment.

### 140 2.3. Laboratory trials

141 The laboratory trials were carried out in a test chamber consisting of a Plexiglas (thickness 1 cm)  
142 chamber (length×width×height 150×60×120 cm, volume 1080 L), hermetically sealed and  
143 equipped, at one of the longer sides, with two openings for handling (diameter 11 cm), closable with

144 lids and gaskets. In one of the shorter sides, there were two openings (diameter 6 cm), in which the  
145 inlet and outlet flexible PVC pipes (diameter 6 cm, length 2.0m and 1.5m, respectively), connected  
146 with the ozone generator, were sealed with a multi-purpose silicone sealant. The inlet pipe was  
147 placed 15 cm above the outlet pipe, and inserted for 50 cm of the length into the test chamber in  
148 order to provide and maintain the recirculation of ozone-enriched air flow (pressure of 0.3 bar)  
149 inside the chamber. The following ozone concentrations were tested for 24 h of exposure time: 0  
150 (only airflow, no ozone), 5, 10, 20, 50, and 100 ppm. The ozone concentration (in ppm) was also  
151 multiplied by the exposure time (in minutes) to calculate the dosage [CT, concentration (ppm) ×  
152 time (minutes)] values (McDonough et al., 2011). The temperature inside the test chamber was  
153 measured through a column thermometer placed inside the test chamber during all the trials, and it  
154 always increased from the initial 25 °C-38 °C as the functioning of the machinery caused a heating  
155 of the output airflow, independently of ozone addition.

156 In laboratory trials, cohorts of eggs, larvae, pupae and adults of *E. kuehniella* were tested. Eggs  
157 were daily collected from adult rearing, and eggs not older than 24 h were used. Larvae (aged from  
158 2<sup>nd</sup>-to 3<sup>rd</sup>-instar) and pupae were collected from cohorts of the same age, and adults not older than  
159 24 h were used. Groups of 10 specimens of the same life stage were placed each one into an opened  
160 glass container (height 4 cm, 10.5 cm diameter), covered with an insect-proof net (mesh size  
161 0.6×1 mm) tightly sealed with a PVC tape. Eggs, larvae and pupae were transferred into their  
162 containers by means of a brush. In the containers with eggs a black filter paper was laid down on  
163 the bottom to facilitate the egg counting, while in the containers with larvae or pupae a small  
164 amount (5 ml) of durum wheat flour was added. Before using them, all eggs were observed by a  
165 stereomicroscope, and those ones showing alterations in colour (from light brown to black) and/or  
166 shape, or wrinkled looking were discarded, while all larvae and pupae were gently touched with a  
167 brush to check their vitality. For each life stage and treatment, overall 90 specimens were used as  
168 follows: 10 specimens were inserted into each container, three containers were arranged for each  
169 experiment (for a total of 30 eggs, 30 2<sup>nd</sup>-3<sup>rd</sup>-instar larvae, 30 pupae and 30 adults), and three  
170 experiments were performed for each ozone concentration (for a total of 90 eggs, 90 2<sup>nd</sup>-3<sup>rd</sup>-instar  
171 larvae, 90 pupae and 90 adults).

172 After the ozone exposure, eggs, larvae, pupae and adults were transferred to Petri dishes (diameter  
173 13.5 cm) containing the standard diet described above, and reared under controlled conditions  
174 (25±1°C; 60±5% RH; L:D 16:8). Newly emerged larvae were periodically transferred to other Petri  
175 dishes to avoid any cannibalism on eggs. Eggs, larvae and pupae were examined 1, 3, 7 and 10 days  
176 after the ozone treatment, when all vital eggs, larvae and pupae in the control treatment hatched,  
177 pupated or moulted to adults, respectively. Eggs showing alterations in colour, larvae that were  
178 immobile, and larvae and pupae changing colour and/or having stiff shrunken bodies were

179 considered as dead. Adults were checked for their mortality immediately and 24 h after the ozone  
180 treatment. Moreover, eggs laid by treated adults in the 24 h-period following the end of the  
181 treatment were counted; then, they were collected with a fine brush, transferred to Petri dishes and  
182 reared until their hatching.

#### 183 2.4. Storehouse trials

184 From late June to mid-July, the storehouse trials were conducted in a hazelnut processing factory  
185 located in Cortemilia (Cuneo, Piedmont, NW Italy). In the storehouse, three separate chambers  
186 (length×width×height 21×21×5m, volume 220 L) hermetically sealed were used as test chambers to  
187 compare the following treatments: ozone treatment; pyrethrin treatment (as routine treatment); no  
188 application (as untreated control). For ozone treatment, the lowest concentration which killed 100%  
189 of adults in the laboratory trials was chosen and applied in the chamber for 24 h, by using the ozone  
190 generator described above. For routine treatment, pyrethrins synergised with piperonyl butoxide  
191 (AquaPy®, Bayer CropScience srl, Environmental Science, Milano, Italy, containing 3 g of  
192 pyrethrins, 13.5 g of piperonyl butoxide, coformulants q.s. for 100 g) was nebulized using a ULV  
193 cold fogger (Vector Fog C20, Vectornate, Englewood Cliffs, NJ, USA) at 100 mL/L following the  
194 standard procedures.

195 In the storehouse trials, *E. kuehniella* adults were tested to compare the effectiveness of the ozone  
196 and pyrethrin treatments. Newly emerged adults (<1 day-old) were collected from laboratory mass  
197 rearing culture and transferred into Plexiglas opened cylinders (height 20 cm, diameter 10 cm),  
198 closed at both extremities with an insect-proof net (mesh size 0.6×1 mm). Overall 540 *E. kuehniella*  
199 adults were used as follows: 10 adults were released into each cylinder, six cylinders were placed in  
200 each test chamber for each experiment (for a total of 60 adults per treatment and experiment), and  
201 the experiment was repeated three times, on June 26, July 3 and July 10 (for a total of 180 adults per  
202 treatment). In each chamber, the six cylinders were placed two at each of three heights, i.e., 0.1 m,  
203 1.5 m and 2.0 m from the ground. Mortality of adults in each cylinder was checked 24 h after the  
204 ozone and pyrethrin treatments.

205 During all trials, temperature in the three chambers was recorded using temperature data loggers  
206 (Hobo® Pro Series, Onset Computer Corporation, Bourne, MA, USA), and it was similar ranging  
207 from 25 °C to 28 °C in the three replicates.

#### 208 2.5. Statistical analyses

209 In laboratory trials, proportions of dead specimens or unhatched eggs in each container were  
210 compared for each stage among ozone concentration via a binomial distribution model with a logit  
211 link function, using the general linear model (GLM) procedure of the software IBM SPSS®  
212 Statistics 24 (IBM Corp., NY, USA). Means were then separated at P<0.05 using the Bonferroni test  
213 under the GLM procedure. Mean times needed for eggs to hatch, for larvae to pupate, and for adults



214 to emerge from pupae after the treatment were compared among ozone concentrations with the Log-  
215 Rank test (IBM SPSS® Statistics 24, IBM Corp., NY, USA).

216 In storehouse trials, percentage mortality of *E. kuehniella* adults in the three treatments and at  
217 different heights in the chambers was compared via a binomial distribution model with a logit link  
218 function, using the GLM procedure of the software IBM SPSS® Statistics 24 (IBM Corp., NY,  
219 USA). Means were then separated at  $P < 0.05$  using the Bonferroni test under the GLM procedure.

220

### 221 **3. Results**

#### 222 *3.1. Laboratory trials*

223 Mean mortality of *E. kuehniella* eggs, larvae, pupae and adults after 24 h-exposure to ozone under  
224 laboratory conditions was significantly different among ozone concentrations for all tested stages  
225 (Tables 1 and 2). However, eggs, larvae, pupae and adults showed a variable susceptibility at the  
226 tested ozone concentrations, in terms of both mortality (Tables 1 and 2), and time needed for egg  
227 hatching and larval pupation (Fig. 1 and 2 and Table 3) or vitality of laid eggs for adults (Table 2).  
228 At 5 ppm ozone concentration (CT value = 7200 ppm min), mortality of all stages was significantly  
229 higher than that in the untreated control, even if not immediately but 24 h after the ozone treatment  
230 for adults (Tables 1 and 2). Then, for eggs, mortality did not differ significantly by increasing ozone  
231 concentration from 10 ppm (CT value = 14400 ppm min) to 100 ppm (CT value = 144000 ppm  
232 min), fluctuating from 52.2% to 62.2%, respectively (Table 1). However, there was a significant  
233 delay in hatching for eggs exposed to 10, 20 and 50 ppm, reaching an average of  $5.5 \pm 0.2$  days after  
234 the treatment, in comparison with eggs in the untreated control, which hatched on average  $3.2 \pm 0.2$   
235 days after the treatment (Fig. 1 and Table 3). Hatching time of eggs exposed to 100 ppm ( $3.6 \pm 0.5$   
236 days after the treatment) did not differ significantly from that of eggs in the untreated control and of  
237 eggs exposed to 50 ppm (Table 3). For larvae, mortality increased significantly lower than that at 20  
238 ppm, but significantly higher than that in the untreated control (Table 2). There was significant  
239 difference in hatching rate of eggs laid by females in the 24 h period after the ozone exposure  
240 between treatments, except for ozone concentration of 100 ppm in which at the end of ozone  
241 exposure all adults were dead and consequently no eggs were found. Also egg mortality was  
242 significantly lower in the untreated control than that at all ozone treatments; with some exceptions,  
243 there were significant differences in mortality among concentrations (Table 2).

#### 244 *3.2. Storehouse trials*

245 Since adult mortality did not significantly differ at the three tested heights (GLM: Wald  $\chi^2 = 1.40$ ; df  
246 = 2;  $P = 0.497$ ) and in the three dates of experiment replication (GLM: Wald  $\chi^2 = 1.40$ ; df = 2;  $P =$   
247  $0.497$ ), all the data obtained at different heights and in different dates were pooled. Almost all adults

248 were found dead after both ozone and pyrethrin treatments, while mortality of the adults was  
249 significantly lower with values below 10% in the untreated control (Table 4).

250

#### 251 **4. Discussion**

252 Although higher mortalities at the lowest ozone concentrations were observed for pupae, in the  
253 present study complete mortality was obtained only for adults, therefore showing a higher  
254 susceptibility to ozone exposure compared to the other stages. In fact, for adults, complete mortality  
255 was achieved at the CT value of 72000 (50 ppm for 24 h), whereas for the other stages it was not  
256 achieved even at the CT of 144000 (100 ppm for 24 h). These results are consistent with those  
257 obtained by Hansen et al. (2012); in their study, all *E. kuehniella* adults died at the lowest tested CT  
258 of above 150000 (20-21 ppm for 5 days), while eggs, larvae and pupae needed CT values higher  
259 than 300000 (35-36 ppm for 6 days) for complete mortality. On the other hand, complete mortality  
260 of adults, larvae and pupae, and high mortality of *E. kuehniella* eggs (above 85%) were obtained  
261 with ozone application at CT values just of 36000 (300 ppm for 2 h) in the study published by  
262 Işıkber et al. (2007), but the brief pressure drop to 100 mm Hg applied before ozone fumigation  
263 could have affected the results.

264 Results are indeed more variable when considering studies conducted on insects exposed to ozone  
265 not freely, but in the presence of commodities. Işıkber and Öztekin (2009) reported that higher  
266 values of CT of more than 800000 (13.88 mg/L, about 7000 ppm, for 2 h) were required for  
267 complete mortality of larvae, pupae and adults of *E. kuehniella* placed in 2 kg of wheat, but eggs,  
268 showing mean mortality of about 60%, proved to be the least susceptible stage to ozone gas.

269 Differently, a lower value of CT of 450 was enough to cause complete mortality of larvae placed in  
270 50 g of dried figs (Sadeghi et al., 2017). However, the presence of different commodities makes  
271 these results not comparable. Further CT should be evaluated on freely exposed each moth stage to  
272 establish their lethal exposure.

273 Similarly to the tested species, also adults and eggs of the almond moth *Ephestia cautella* (Walker)  
274 showed to be the most and least susceptible stage with mortality of 85% and 10% at the highest CT  
275 evaluated of 1440 (2 ppm for 12 h) when freely exposed to ozone, respectively (Abo-El-Saad and  
276 Elshafie, 2011). In the same family Pyralidae, also freely exposed stages of *P. interpunctella*  
277 showed similar susceptibility: complete mortality for adults and eggs required the lowest (30000,  
278 1800 ppm for 60 min) and the highest (324000, 1800 ppm for 180 min) CT values, respectively  
279 (McDonough et al., 2011).

280 In our study, ozone exposure did not cause only *E. kuehniella* mortality, but it also affected the time  
281 needed for the exposed eggs and larvae to hatch and pupate, respectively. In fact, eggs exposed at  
282 10, 20 and 50 ppm hatched later than eggs exposed at 0 and 100 ppm, and larvae exposed at 5, 10,

283 20, and 50 ppm pupated later than larvae exposed at 0 and 100 ppm. Larvae surviving after ozone  
284 treatment were therefore able to pupate, but later, perhaps due to a reduction of their feeding  
285 activity after ozone exposure. Our results showed that not only lethal, but also sublethal effects of  
286 ozone at sublethal concentrations can be effective in stored-product pest control, reducing both  
287 feeding damage by larvae and adult fecundity, as mortality of eggs laid by treated adults increased  
288 at increasing ozone concentration. Reduction in walking activity under sublethal ozone exposure  
289 was observed for populations of the maize weevil *Sitophilus zeamais* Motschulsky (Coleoptera:  
290 Curculionidae) (Sousa et al., 2017). Such effects might limit the insect ability to escape the ozone  
291 exposure, implicating in a low risk of resistance evolution in a short term (Sousa et al., 2017), and  
292 these aspects should be further evaluated also for *E. kuehniella* populations.

293 Currently, control of *E. kuehniella* infestations in storehouses is based on the use of conventional  
294 insecticides, depending on trap captures for decision-making; some alternative and biorational  
295 management tools such as temperature management, controlled and modified atmospheres, and use  
296 of natural enemies exist (Phillips and Throne, 2010). In order to respond to the consumers' demand  
297 for organic and pesticide residue-free food, ozone gas can be also an efficient substitute of chemical  
298 treatments. In the present study, the storehouse trials demonstrated that ozone gas was effective  
299 against adults of *E. kuehniella*, the most susceptible and the only freely exposable stage, and  
300 indirectly against eggs laid by treated adults. However, both hazelnuts and storehouse equipment  
301 could be damaged by excessive ozone exposure. Ozone is actually a powerful oxidant, second only  
302 to the hydroxyl-free radical. Therefore, it is capable of oxidizing many inorganic and organic  
303 compounds in air and water, and even if the effect of ozone on quality of grain and derived products  
304 is highly debated in the literature (Jian et al., 2013), lipid oxidation can affect kernel quality of  
305 shelled hazelnuts (Wang et al., 2018). Furthermore, ozone oxidizes most metals and reacts directly  
306 with some hydrocarbons such as aldehydes, polymers, or double bonds within its chain structure  
307 such as in natural rubber, wool, protein, and paint (Jian et al., 2013). Corrosion by ozone to  
308 materials used by the stored product industry is a potential disadvantage for ozone application and  
309 should be carefully evaluated. Ozone must be continuously supplied and might increase corrosion  
310 rates on metal components and degrade equipment such as rubber seals, and electrical equipment at  
311 unacceptable rates. Therefore, to reduce the corrosion, ozone should be used at low concentration,  
312 and contact between ozone and equipment should be minimized (Jian et al., 2013). Application of  
313 ozone at 50 ppm is the recommended concentration to be used in the fumigation of empty facilities  
314 and grain storage bins to suppress insect and microorganism populations and reduce mycotoxins  
315 (Abo-El-Saad and Elshafie, 2011; Jian et al., 2013). This concentration demonstrated to be effective  
316 on *E. kuehniella* adults in both laboratory and storehouse conditions.

317 In the present study, *E. kuehniella* adults resulted susceptible to the recommended concentration of  
318 50 ppm of ozone application in both laboratory and storehouse conditions. Furthermore, also eggs  
319 laid by adults survived in the following 24 h showed a mortality of more than 30%. Based on the  
320 results on lethal and sublethal effects of ozone gas on *E. kuehniella* in the present study, ozone can  
321 be considered as an effective alternative to routine application of synergised pyrethrin during spring  
322 and summer; however, further studies are required to test effect of ozone gas on quality parameters  
323 of the tested commodity.

324

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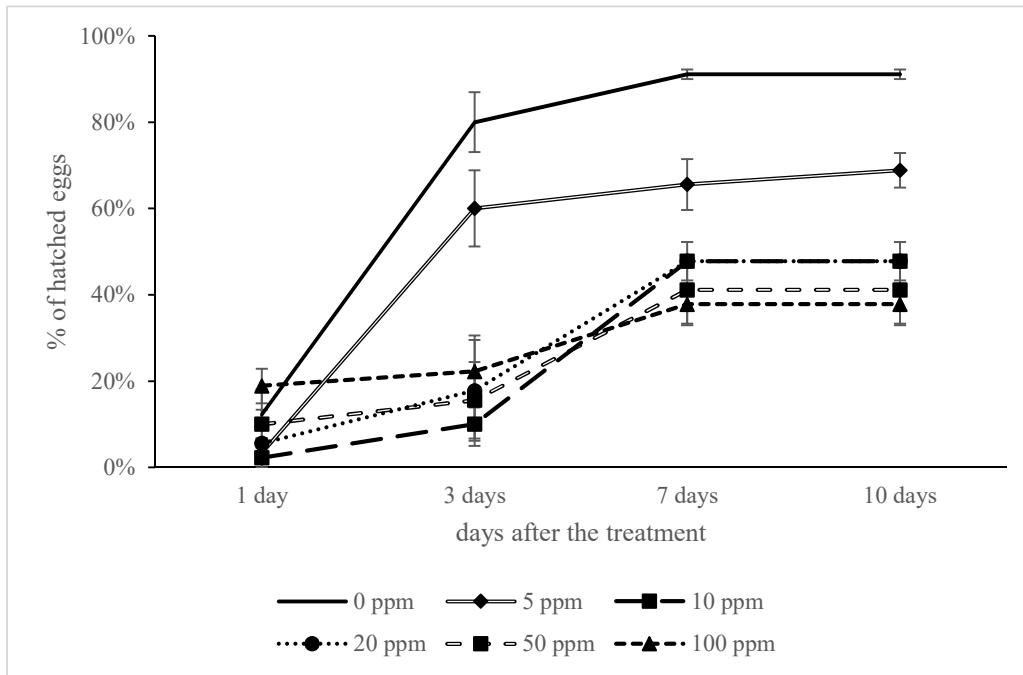
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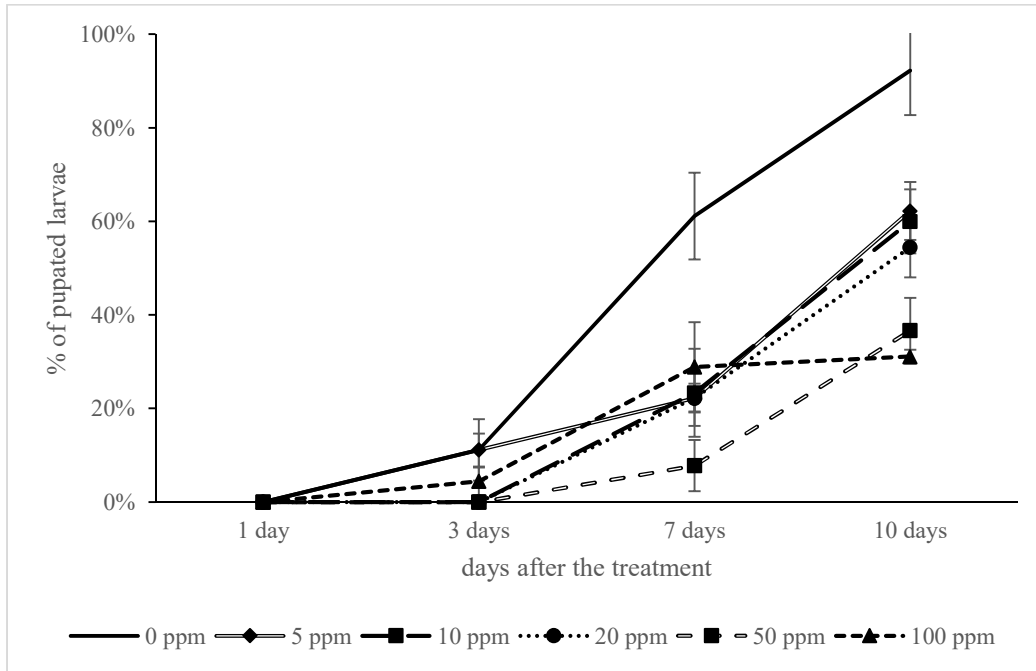


433

434 **Figure 1.** Cumulative mean percentages ( $\pm$ SE) of *Ephestia kuehniella* eggs hatched 1, 3, 7 and 10  
435 days after ozone treatment in the test chamber.

436





438

439 **Figure 2.** Cumulative mean percentages ( $\pm$ SE) of *Ephestia kuehniella* larvae pupated 1, 3, 7 and 10  
 440 days after ozone treatment in the test chamber.

441

442 **Table 1.** Mean percentages ( $\pm$ SE) of mortality of *Ephestia kuehniella* eggs, larvae and pupae freely  
 443 exposed to different gaseous ozone concentrations (ppm) for 24 hours in laboratory conditions.  
 444 Mortality was assessed 10 days after treatment, when no more eggs hatched, no more larvae pupate,  
 445 and no more adults emerged. In each column, values followed by the same letter are not significantly  
 446 different (Bonferroni test,  $P < 0.05$ , under GLM procedure with binomial distribution and logit link).

Dose (ppm)	CT product (ppm-min)	mortality (%)		
		eggs	larvae	pupae
0	0	8.33 $\pm$ 2.41 c	9.17 $\pm$ 2.60 c	8.33 $\pm$ 2.41 d
5	7200	31.11 $\pm$ 4.55 b	37.78 $\pm$ 3.64 b	61.11 $\pm$ 5.88 c
10	14400	52.22 $\pm$ 4.34 a	40.00 $\pm$ 10.67 b	64.44 $\pm$ 6.26 bc
20	28800	52.22 $\pm$ 2.78 a	45.56 $\pm$ 4.12 b	74.44 $\pm$ 2.42 b
50	72000	58.89 $\pm$ 4.84 a	63.33 $\pm$ 8.33 ab	76.67 $\pm$ 3.73 ab
100	144000	62.22 $\pm$ 5.47 a	68.89 $\pm$ 8.57 a	85.56 $\pm$ 6.69 a
Wald $\chi^2$		71.416	77.232	107.676
df		5	5	5
P		<0.001	<0.001	<0.001

447

448

449 **Table 2.** Mean percentages ( $\pm$ SE) of mortality of *Ephestia kuehniella* adults freely exposed to  
 450 different gaseous ozone concentrations (ppm) for 24 hours in laboratory conditions, immediately and  
 451 24 hours after the end of the trial, and mean percentages ( $\pm$ SE) of mortality of eggs laid in the 24  
 452 hour-period following the end of the treatment. In column, values followed by the same letter are not  
 453 significantly different (Bonferroni test,  $P < 0.05$ , under GLM procedure with binomial distribution and  
 454 logit link).

Dose (ppm)	CT product (ppm-min)	adult mortality (%)		laid egg	
		immediately	after 24 h	no.	mortality (%)
0	0	6.67 $\pm$ 1.42 d	11.67 $\pm$ 1.12 d	2830	5.28 $\pm$ 0.71 d
5	7200	17.78 $\pm$ 4.01 c	35.56 $\pm$ 6.48 c	1413	14.22 $\pm$ 3.15 c
10	14400	28.89 $\pm$ 7.16 c	41.11 $\pm$ 7.90 bc	1938	13.01 $\pm$ 2.21 c
20	28800	53.33 $\pm$ 5.53 b	61.11 $\pm$ 6.33 b	2235	20.84 $\pm$ 2.83 b
50	72000	94.44 $\pm$ 2.94 a	100.00 $\pm$ 0.00 a	470	33.01 $\pm$ 4.24 a
100	144000	100.00 $\pm$ 0.00 a			
Wald $\chi^2$		4540.645	14750.860		396.011
df		4	4		4
P		<0.001	<0.001		<0.001

455

456

457 **Table 3.** Mean time (days±SE) needed for *Ephestia kuehniella* eggs and larvae to hatch and pupate,  
 458 respectively, after the exposure to different gaseous ozone concentrations (ppm) for 24 hours in  
 459 laboratory conditions. In column, values followed by the same letter are not significantly different  
 460 (Log-Rank test, P<0.05).

Dose (ppm)	CT product (ppm-min)	Time (days) from the treatment to	
		Hatching for the exposed eggs	Pupation for the exposed larvae
0	0	3.22±0.17 c	7.53±0.24 b
5	7200	3.11±0.14 c	8.21±0.36 a
10	14400	6.07±0.29 a	8.83±0.20 a
20	28800	5.28±0.36 a	8.78±0.21 a
50	72000	5.00±0.44 ab	9.36±0.22 a
100	144000	3.65±0.50 bc	6.64±0.32 b
$\chi^2$		67.678	48.655
df		5	5
P		<0.001	<0.001

461

462

463 **Table 4.** Mean percentages ( $\pm$ SE) of mortality of *Ephestia kuehniella* adults freely exposed to  
464 different treatments for 24 hours in a hazelnut storehouse. Values followed by the same letter are not  
465 significantly different (Bonferroni test,  $P < 0.05$ , under GLM procedure with binomial distribution  
466 and logit link).

Treatment	mortality (%)
Untreated	9.44 $\pm$ 2.21 b
Pyrethrum (100mL/L)	98.89 $\pm$ 0.76 a
Ozone (50 ppm)	99.44 $\pm$ 0.56 a
Wald $\chi^2$	118.772
df	2
P	<0.001

467