Research Article

Received: 5 August 2019 Revised: 6 March 2020 Accepted article published: 17 April 2020 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.5864

Determining baseline toxicity of ozone against an insecticide-susceptible strain of the common bed bug, *Cimex lectularius* L. under laboratory conditions

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Abstract

BACKGROUND: Ozone gas is commercially used for deodorization and microbial control. Its efficacy against stored product insect pests is well documented. In the midst of the common bed bug (*Cimex lectularius* L.) outbreak, claims were made that ozone gas was effective for their control. This study was conducted to determine baseline ozone concentrations and exposure times required for the control of an insecticide-susceptible *C. lectularius* strain under laboratory conditions. Dichlorvos (DDVP), an organophosphate class fumigant insecticide was used as a positive control.

RESULTS: Nymphs and adults were more susceptible to ozone than eggs. Complete (100%) nymph and adult mortality was achieved at an ozone concentration (C) of 1500 ppm and exposure time (T) of 180 min, or concentration × time product (CT) of 270 000 ppm-min, whereas eggs required an eightfold higher CT (2 040 000 ppm-min). DDVP vapor was 2070-, 2542- and 450-fold more potent than ozone, against nymphs, adults and eggs, respectively.

CONCLUSIONS: Baseline ozone toxicity data provide insights on the practicality of using this gas for the management of common bed bugs. High ozone CT products required for *C. lectularius* control, particularly eggs, suggest that its use for treating infested human dwellings is not feasible due to logistic, safety and monetary concerns.

Supporting information may be found in the online version of this article.

Keywords: *Cimex lectularius*; baseline toxicity; ozone; DDVP; comparative potency; IPM

1 INTRODUCTION

The common bed bug (*Cimex lectularius* L.) and tropical bed bug (*Cimex hemipterus* F.) are human ectoparasites that have resurged globally, except in Antarctica, over the past two decades.[1] This study was conducted with *C. lectularius*, which is more commonly found in temperate regions. The widespread presence of resistance to multiple insecticide classes in *C. lectularius* populations and their cryptic behavior are major hurdles to their successful control.[2–6] A multifaceted approach such as integrated pest management (IPM), which includes rational use of pesticides along with frequent monitoring and use of different alternative control measures, has proven effective for their control.[7,8] In addition to a plethora of insecticide efficacy reports, several studies have demonstrated and/or tested the effectiveness of alternative control methods such as heat treatments,[9] cold treatments,[10] steam,[11,12] desiccant dusts,[13] carbon dioxide and dry ice,[14,15] ultra-low oxygen and vacuum treatments,[16] insecticide-treated mattress encasements,[17] essential oils[18,19] and fumigants[20,21] for *C. lectularius* control. Unlike published reports on insecticide and alternative control techniques, only one preliminary study has assessed the ability of ozone (O_3) gas to kill *C. lectularius* adults;[22] the remainder of the non-empirical information on the efficacy of ozone can be found online (https://www.foreverozone.com/blogs/news/ozone-does-kill-bed-bugs-but-not-their-eggs and https://www.noai.org/does-ozone-kill-bedbugs). Moreover, anecdotal reports suggest that the public are using ozone generators as a do-it-yourself tool for the control of common bed bugs (Ashbrook AR, 2019, pers. comm.). Additional, anecdotal reports suggest that in some European countries, ozone was used for the

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control of *C. lectularius* and/or *C. hemipterus* infestations on yachts prior to 2003.

Ozone is a tri-atomic, highly unstable form of oxygen, found naturally in the upper atmosphere. The oxidative properties of ozone are greater than those of hypochlorous acid and chlorine.[23] At ground level, ozone is considered an air pollutant and is formed by the reaction between oxides of nitrogen and organic compounds that originate from volatile chemicals, automotive exhaust fumes and industrial emissions.[24] In humans, exposure to high concentrations of ground-level ozone may lead to adverse respiratory health problems including throat irritation, cough, reduced lung function, and aggravation of asthma.[24] However, the highly oxidative nature of ozone gas has led to its use in controlled settings as an antimicrobial agent for the treatment of agricultural produce and drinking water; it is also used as a disinfectant in hospitals.[25–28] Other commercial applications of ozone include its use in the odor remediation industry.[29,30] The United States Food and Drug Administration (FDA) and U.S. Environmental Protection Agency (EPA) have assigned 'GRAS' (Generally Recognized As Safe) status to gaseous and aqueous phases of ozone that are commercially used for microbial control and deodorization treatments.[31,32] The advantages associated with commercial use of ozone are that it can be artificially generated by several methods such as ultraviolet (UV) light and high-voltage corona discharge.[33,34] These methods can readily convert pure oxygen stored in tanks or oxygen found in atmospheric air into ozone. Commercial use of ozone does not aggravate the pollution problem because it is known to decompose into oxygen even under airtight and unventilated conditions.[33] Depending on the environmental conditions, the half-life of ozone can be as short as 39 min.[35]

In terms of insect pest control, ozone gas has been shown to be effective in controlling externally and internally feeding life stages of more than a dozen stored product pests, including *Plodia interpunctella* Hubner, *Sitophilus zeamais* Motschulsky and *Tribolium castaneum* Herbst.[36–44] It has also been shown to be effective against phosphine-resistant populations of four species of stored product pests.[45] A study by Kells *et al.*[37] showed that 92–100% mortality of *P. interpunctella*, *S. zeamais* and *T. castaneum* could be achieved by treating maize stored in a commercial grain bin (8.9 tons) with 50 ppm of ozone for 3 days. Using high concentrations of ozone and long exposure times, Hansen *et al.*[40] demonstrated that larvae of stored product pests feeding internally within the grain could be killed. The ability of ozone to kill stored product insects in large masses of maize[37] and internally feeding life stages[40] suggests that it can also be used to control bed bugs hiding in infested furniture or cracks and crevices. Another oxidative gas, chlorine dioxide (ClO_2), which has lower oxidative potential than ozone, has been shown to effectively control bed bugs under laboratory conditions at concentration (C) and exposure time (T) products (CT values) ranging from 30 000 to 181 000 ppm-min.[46] However, comprehensive studies on the effectiveness of ozone against different *C. lectularius* life stages have not been conducted. Thus, the objective of this work was to determine baseline ozone concentration (C) and exposure time (T) combinations or CT products required to control eggs, nymphs and adults of insecticide-susceptible *C. lectularius* placed in vented plastic tubes within a tabletop ozone chamber. The rationale for generating baseline ozone susceptibility data is that the CT product or lethal concentration (LC) values for different bed bug life stages will help determine the practicality of using this gas as a control agent for the management of common bed bugs.

Because this study was conducted with an insecticide-susceptible strain, the organophosphate insecticide, dichlorvos (DDVP), an active ingredient in a commercially available fumigant product registered for *C. lectularius* control, was included as a positive control. It should, however, be noted that DDVP may not serve as an appropriate positive control treatment for field strains because *C. lectularius* has developed resistance to multiple insecticide classes, including organophosphates.

2 MATERIALS AND METHODS

2.1 Insects

Insects were obtained from a laboratory-reared insecticide-susceptible Harlan strain colony. This *C. lectularius* strain has been maintained under laboratory conditions without exposure to any insecticide since 1973 and is thus susceptible to all insecticides tested to date. This *C. lectularius* strain was maintained in mixed age/sex colonies and fed weekly on defibrinated rabbit blood purchased from Hemostat Laboratories (Dixon, CA, USA). The membrane feeding method developed by Chin-Heady *et al.*[47] was used. Rearing was conducted in environmental chambers (Percival Scientific, Perry, IA, USA) at 25 °C temperature, 50 ± 15% relative humidity (RH) and a 12:12 h light/dark photoperiod. Insects with no prior ozone and DDVP exposure were used for all bioassays. Mixed sex adults (1:1 male : female ratio), third to fourth instar nymphs and 1–3-day-old eggs were used for bioassays. Adults and nymphs were fed 4–5 days before treatment. To obtain eggs, blood-fed males and females (1 : 4 ratio) were held in separate jars with folded index cards for 3 days. Eggs laid by females on index cards (1–3 days old) were used in bioassays.

2.2 Ozone production

Ozone was generated by passing 99% pure medical grade oxygen through a corona discharge ozone generator (Medizone International, Sausalito, CA, USA) (Fig. 1). Ozone gas then flowed (0.5 L min^{-1}) into an airtight polycarbonate treatment chamber (60 × 30 × 30 cm^3) connected to the ozone generator by Tygon flexible tubing (8.5 mm O.D./6 mm I.D.) (Saint-Gobain Performance Plastics, Beaverton, MI, USA). A 50-mm cooling fan (EC-4510 12 V 5000 rpm; EVERCOOL Thermal Corp. Ltd., New Taipei City, Taiwan) inside the chamber facilitated uniform gas distribution. To measure the ozone concentration in the treatment chamber, either a UV-100 (Eco Sensors, Newark, CA, USA) or L2-LC ozone analyzer (IN USA INC., Needham, MA, USA) was connected to a sampling port from the top of the chamber. Ozone was removed from the chamber (0.5 L min^{-1}) using suction and destroyed by an activated carbon filter column. For safety, all ozone treatments were performed under an approved fume hood or with ozone-destructive filter columns on all outlets.

2.3 Ozone and dichlorvos exposure bioassays

For ozone bioassays, adults, nymphs and eggs were placed separately in 50 mL plastic centrifuge tubes (BD Falcon, Franklin Lakes, NJ, USA). A folded index card was provided to adults and nymphs as harborage. Centrifuge tubes were modified by cutting the lower or tapered end and replacing it with a fine nylon mesh that allowed gaseous exchange within the tube while confining the insects. Tubes with ten adults, nymphs or eggs were placed in the airtight ozone chamber and exposed to a series of CT combinations (ozone concentrations and exposure times) that gave mortality rates between 0% and 100% (Table 1). In total, three replications were performed for each CT combination ($n = 30$). With a

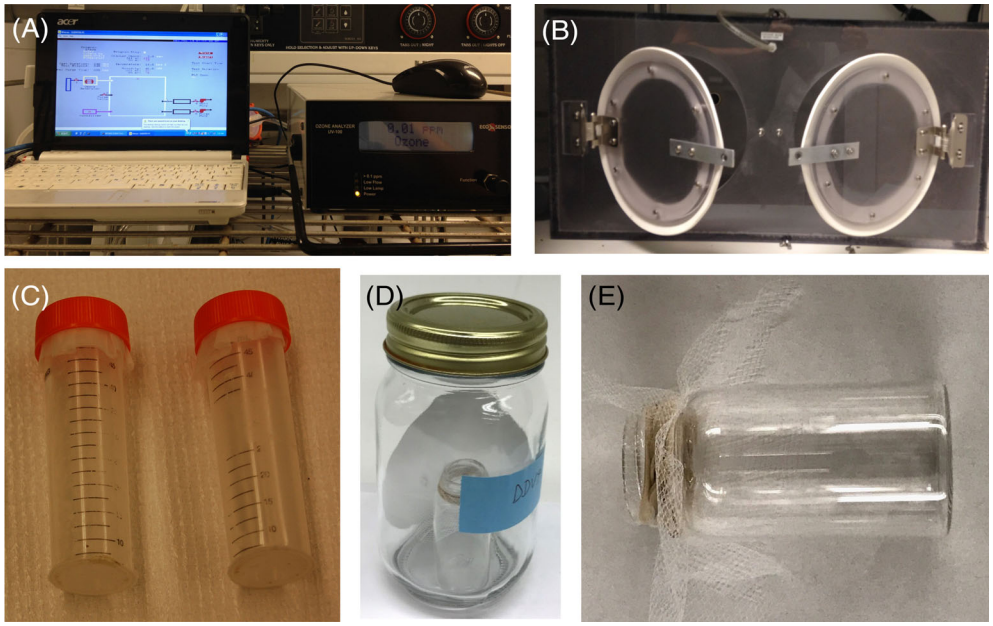

Figure 1 Set-up used for ozone (A–C) and dichlorvos (D–E) bioassays. (A) Ozone analyzer interfaced with computer-based software. (B) Polycarbonate ozone exposure chamber. (C) Modified 50 mL plastic centrifuge tubes with mesh bottom used to confine insects during ozone bioassays. (D) Sealed Mason jar with dichlorvos (DDVP)-treated filter paper and 20 mL scintillation vial with mesh cover. (E) Glass scintillation vials with mesh cover used to hold different insecticide-susceptible *Cimex lectularius* L. life stages during DDVP bioassays.

Table 1 Ozone and dichlorvos concentrations (C), exposure times (T) and resulting CT products (ppm-min) tested against insecticide-susceptible *Cimex lectularius* L. adults, nymphs and eggs

	Ozone			Dichlorvos (DDVP)		
Life stage	Concentration (C) (ppm)	Exposure time (T) (min)	CT product (ppm-min)	Concentration (ppm)	Exposure time (min)	CT product (ppm-min)
Adult	100	180	18 000	0.026	1440	37.44
	200	180	36 000	0.033	1440	47.52
	400	180	72 000	0.0396	1440	57.024
	600	180	108 000	0.0528	1440	76.032
	800	180	144 000	0.066	1440	95.04
	900	180	162 000	0.0792	1440	114.048
	1200	180	216 000	0.1057	1440	152.208
	1500	180	270 000	0.1585	1440	228.24
Nymph	100	180	18 000	0.013	1440	18.72
	200	180	36 000	0.0198	1440	28.512
	400	180	72 000	0.026	1440	37.44
	600	180	108 000	0.033	1440	47.52
	800	180	144 000	0.0396	1440	57.024
	900	180	162 000	0.0528	1440	76.032
	1200	180	216 000	0.066	1440	95.04
	1500	180	270 000	0.0792	1440	114.048
				0.1057	1440	152.208
Egg	400	180	72 000	0.1	1440	144
	900	180	162 000	0.3	1440	432
	1200	180	216 000	0.5	1440	720
	1500	180	270 000	1	1440	1440
	800	720	576 000	1.2	1440	1728
	850	1530	1 300 500	1.4	1440	2016
	850	2400	2 040 000	1.6	1440	2304
				1.8	1440	2592
				2	1440	2880

Table 2 Toxicity of ozone against insecticide-susceptible adults, nymphs and eggs *Cimex lectularius* L

Life stage	*n*	Slope ± SE	χ^2 (d.f.)	*P*-value	LC_{50} ppm-min (95% FL)[a]	Potency ratios (95% CI)[b]	LC_{90} ppm-min (95% FL)[a]
Nymph	270	2.34 ± 0.29	3.14 (6)	0.790	106 228 (94 465.1–117 208)	—	183 481 (162 796– 217 784)
Adult	270	1.82 ± 0.23	5.79 (6)	0.447	128 036 (113 546–143 198)	1.20 (0.85–1.75)	257 924 (219 235–330 167)
Egg	240	1.06 ± 0.14	4.26 (5)	0.512	253 947 (206 519–313 644)	3.11 (1.86–6.51)	848 377 (616 813–1 417 424)

[a]Lethal concentrations (LC) of ozone expressed as CT product (concentration × time) (ppm-min) required to achieve 50% (LC_{50}) and 90% (LC_{90}) mortality of different *C. lectularius* life stages. Values in parentheses represent 95% fiducial limits (FL).
[b]Potency ratios were obtained by performing relative median potency analysis.[48] The life stage with lowest LC_{50} estimate, i.e., nymphs, was used as the baseline. Values in parentheses denote 95% confidence intervals (CI). Ratios are considered statistically significant if the confidence intervals do not overlap with the number '1'.

total number of eight or nine CT combinations, including controls, and 30 insects or eggs per CT, 240–270 *C. lectularius* nymphs or adults or eggs were used for baseline toxicity determination (Table 2). Untreated controls included similarly aged adults, nymphs or eggs held separately in meshed centrifuge tubes under ambient room conditions (22 ± 2 °C and 40% RH) during ozone exposure. Mortality of ozone-treated and control insects was checked immediately after treatment and every 24 h thereafter up to 72 h. Bed bugs that did not show any body movement were scored as dead. However, only 24 h mortality data were used for further analysis and comparisons. Mortality data for eggs were collected by observing them under the microscope until 90–100% of the untreated control eggs had hatched, i.e., up to 7–10 days post exposure (Fig. 2). As described in the Introduction (Section 1), CT products (ppm-min) were obtained by multiplying the ozone concentration (ppm) by exposure time (min).[39]

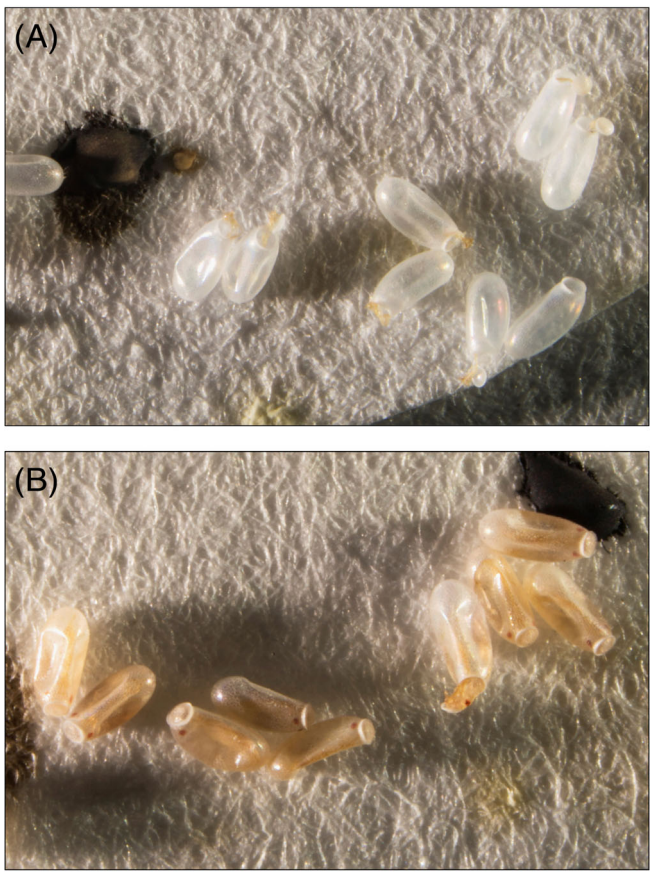

Figure 2 Example of eclosed (hatched) and uneclosed (dead) insecticide-susceptible *Cimex lectularius* L. eggs at 10 days after treatment. (A) Eclosed eggs from the untreated control (ambient air or acetone) group. Hatching is evident from open egg caps and transparent eggshells. (B) Eggs exposed to highest CT products (concentration × time) of ozone or dichlorvos (DDVP) that did not hatch appeared shriveled or dehydrated at 10 days after exposure.

To compare the efficacy of ozone, the conventional organophosphate class fumigant insecticide, DDVP, was used as a positive control. Technical grade DDVP (> 95% purity) was purchased from Chem Service (West Chester, PA, USA). Bioassays were conducted as per the protocol described by Gaire *et al.*[19] In brief, filter papers (Whatman™ #1; GE Healthcare, Little Chalfont, UK), 9 cm in diameter were treated with DDVP solution prepared in acetone (concentration 1.25–20 mg mL^{-1} and volume 5–100 µL) (Table 1). Analytical grade acetone was purchased from Fisher Scientific (Hampton, NH, USA). After complete evaporation of acetone (~ 1 min), treated papers were placed in 473 mL Mason jars (Anchor Glass Container Corporation, Tampa, FL, USA). Different life stages (either adults or nymphs or eggs) were held in individual mesh-covered glass scintillation vials (20 mL; W.W. Grainger, Inc., Lake Forest, IL, USA) with index card harborage in groups of ten, and the then placed in Mason jars along with treated filter papers. The Mason jar was then sealed completely and held in a growth chamber under environmental conditions similar to those used for bed bug rearing. Untreated control insects or eggs were exposed to acetone-treated filter papers. Three replicates (n = 30 nymphs or adults; n = 42–72 eggs) were performed for each concentration of DDVP. Collectively 270–300 nymphs or adults and 540 eggs were used for DDVP toxicity determination, as shown in Table 3. Morality scoring procedures (at 24 h) for different life stages were similar to those described for ozone bioassays.

2.4 Statistical analysis

Probit analysis was performed with the 24 h mortality data in Minitab Software Release 14.2 (Minitab Inc. State College, PA, USA) to determine the lethal concentration (LC) estimates at 50% and 90% mortality levels.[49] For each compound, life stage-dependent toxicity differences at the LC_{50} level were compared by performing relative median potency analysis.[48] The same statistical test was used to compare potency differences between ozone and DDVP at the LC_{50} level. For each compound, toxicity differences or ratios between life stages were calculated at the LC_{90} level by dividing LC_{90} value of the more tolerant life stage by the LC_{90} estimate of the least tolerant life stage.

Table 3 Toxicity of dichlorvos (DDVP) against insecticide-susceptible adults, nymphs and eggs of *Cimex lectularius* L

Life stage	*n*	Slope ± SE	χ^2 (d.f.)	*P*-value	LC_{50} ppm-min (95% FL)[a]	Potency ratios (95% CI)[b]	LC_{90} ppm-min (95% FL)[a]
Nymph	300	3.55 ± 0.42	22.25 (7)	0.002	42.81 (39.74–45.98)	—	61.40 (56.05–69.96)
Adult	270	3.21 ± 0.47	4.88 (6)	0.558	47.43 (43.19–51.2)	1.00 (0.54–1.74)	70.65 (64.03–82.59)
Egg	540	0.87 ± 0.07	34.14 (7)	<0.001	555.49 (442.65–666.56)	18.38 (3.50–593.49)	2418.29 (1997.94 – 3081.75)

[a]Lethal concentrations (LC) of DDVP expressed as CT product (concentration × time) (ppm-min) required to achieve 50% (LC_{50}) and 90% (LC_{90}) mortality of different *C. lectularius* life stages. Values in parentheses represent 95% fiducial limits (FL).
[b]Potency ratios were obtained by performing relative median potency analysis.[48] The life stage with lowest LC_{50} estimates, i.e., nymphs was used as the baseline. Values in parentheses denote 95% confidence intervals (CI). Ratios are considered statistically significant if the confidence intervals do not overlap with the number '1'.

3 RESULTS

3.1 Bed bug susceptibility to ozone

Ozone LC_{50} and LC_{90} values (expressed as CT products; ppm-min) for different life stages of bed bugs showed that nymphs and adults were the most susceptible, and the egg stage was the most tolerant (Table 2). Based on relative median potency analysis, LC_{50} values for nymphs and adults were statistically similar (potency ratio of 1.2 for nymphs; Table 2). The LC_{50} value for the egg stage was significantly higher (three times) in comparison with the nymphal stage (Table 2) as well as adults (2.6 times higher with confidence intervals of 1.65–4.90; Table S1). Although it was not possible to perform relative median potency analysis at the 90% mortality level, LC_{90} values of adults and eggs were 1.4 and 4.6 times higher, respectively, than the nymphal stage (Table 2). Based on the empirical bioassay data, complete (100%) mortality of nymphs and adults was observed at a concentration of 1500 ppm and exposure time of 180 min, or CT product of 270 000 ppm-min (Table 1 and Fig. 3). To achieve complete egg mortality, a CT product of just over 2×10^6 ppm-min (exposure for 2400 min at an ozone concentration of 850 ppm) was required (Table 1 and Fig. 3).

3.2 Bed bug susceptibility to DDVP

Similar to the trend observed with ozone, nymphs and adults were more susceptible to DDVP vapors in comparison with eggs (Table 3). Lethal concentrations of DDVP (expressed as CT product; ppm-min) against nymphs and adults at the LC_{50} level were statistically identical (Table 3). By contrast, the egg stage LC_{50} value was 18-fold higher in comparison with that of nymphs (Table 3). The difference between the potency of DDVP against bed bug eggs and adults was also 18-fold with confidence intervals ranging from 4 to 527 (Table S2). The DDVP LC_{90} value for the egg stage (2418.29 ppm-min) was 35–40-fold higher in comparison with LC_{90} estimates for nymphs and adults (Table 3). Empirical bioassay data showed that 100% mortality of nymphs and adults was first observed at a DDVP concentration of 0.066 ppm and exposure time of 1440 min (CT product 95.4 ppm-min) (Table 1 and Fig. 3). Eggs required a substantially higher DDVP concentration (2 ppm) or CT product (2880 ppm-min) (Table 1 and Fig. 3).

3.3 Comparison of ozone and DDVP susceptibility in bed bugs

Relative median potency analysis revealed that ozone LC_{50} estimates for all life stages were significantly higher than DDVP (Fig. 3 and Table S3). Lower CT products or LC estimates for DDVP indicated that it is a more potent mortality agent than ozone. Although DDVP was 2000–2500-fold more potent than ozone against nymphal and adult life stages, median potency differences for the egg stage were only 450-fold (Fig. 3 and Table S3).

4 DISCUSSION

Ozone concentrations and exposure times required to achieve complete (100%) mortality of different life stages of an insecticide-susceptible *C. lectularius* strain were determined under laboratory conditions. Here, differences in the efficacy of ozone against *C. lectularius* life stages are discussed in relation to data available for stored product insect pests.[36–44] We have also discussed comparative differences in efficacy between ozone and other alternative *C. lectularius* control methods such as carbon dioxide fumigation and low oxygen treatments.[14–16] Because ozone represents another alternative method that could be utilized in the future for the management of common bed bugs, potential challenges as well as opportunities associated with its use are also discussed.

Preliminary work performed by Feston *et al.*[22] showed that exposure of adult bed bugs to 1800 ppm ozone for 150 min in the presence of 1–2% hydrogen peroxide vapors resulted in 100% mortality (CT of 270 000 ppm-min). Although hydrogen peroxide was not used in combination with ozone in this study, 100% adult and nymph mortalities were achieved by exposure to 1500 ppm ozone for 180 min, which also resulted in a CT product of 270 000 ppm-min. Similarities in CT product required to achieve 100% mortality between the two ozone exposure experiments mentioned above suggest that concentration and exposure time can be manipulated to achieve the desired level of mortality. Data available from research on stored product pests have shown that mortality is dependent on not only ozone concentration, but also exposure duration.[34,39] The non-significant differences in the CT or LC estimates observed for late-instar nymphs and adults (Table 2) are likely due to the hemimetabolous lifecycle of bed bugs, wherein both life stages by and large exhibit similar morphological features except for body size, cuticular composition and maturity of reproductive organs.[50]

Because most of the research conducted with ozone has primarily focused on stored grain pests, the CT products or probit LC estimates determined for bed bugs in this study can be compared with lepidopteran and coleopteran insects such as, *S. zeamais*, *T. castaneum and P. interpunctella*. For the coleopteran species, the CT product (216 000 ppm-min; 1800 ppm for 120 min) required to obtain complete mortality of *T. castaneum* and *S. zeamais* adults[39] was slightly lower than the CT product required for active life stages of *C. lectularius* (Table 2). However,

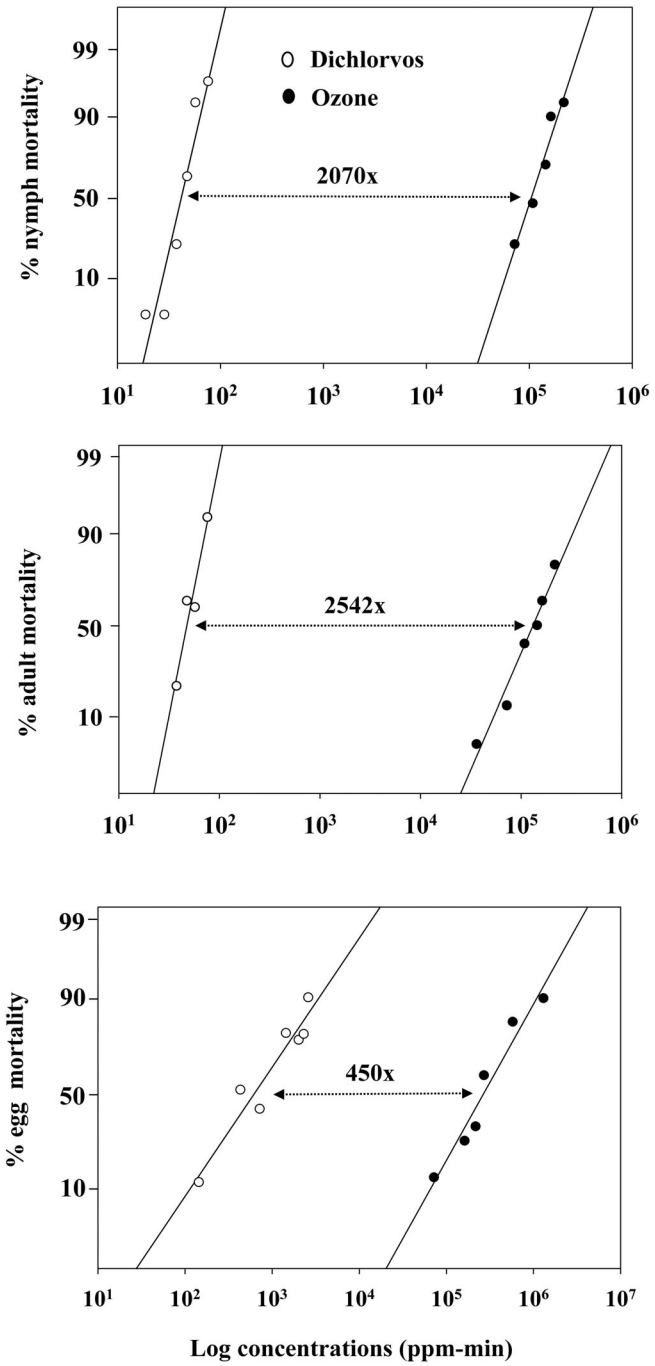

Figure 3 Percent mortality *versus* log concentration line graphs for dichlorvos (DDVP) and ozone. Data for nymphs, adults and eggs are shown in the upper, middle and lower panels, respectively. The numbers above the dashed line indicate potency ratios or differences between DDVP and ozone lethal concentration (LC) or (concentration × exposure time products (CT) estimates at the 50% mortality level. Potency ratios were calculated by performing relative median potency analysis.[48]

mortality of *P. interpunctella* adults approached 100% after just 60 min exposure at 500 ppm (CT product of 30 000 ppm-min)[39,51] which was ninefold lower than the CT value required to achieve 100% *C. lectularius* mortality. Although the specific causes of species-dependent variation in the insecticidal activity of ozone remain unknown, they may be caused by differences in body mass, respiration rate and/or oxidative stress response mechanisms, which likely vary between different groups and species of pest insects.[52,53] In comparison with the ozone CT product (270 000 ppm-min) required to achieve 100% mortality of insecticide-susceptible *C. lectularius* nymphs and adults, the minimum carbon dioxide concentration required to kill all mobile life stages of a field strain of *C. lectularius* was 30% or 300 000 ppm with an exposure time of 24 h or 1440 min,[14] which translates to a CT product of 432 000 000 ppm-min.

In congruence with previous reports on ozone, this research also showed that higher CT products are required for the egg stage in comparison with the immature and adult life stages. To achieve complete (100%) mortality of *P. interpunctella* eggs, a CT product of 324 000 ppm-min was required, which was approximately tenfold higher than the CT required for adults.[39] Bed bug CT products for eggs were five- to eight-fold higher in comparison with nymph and adult LC estimates at the 90–100% mortality level. Because ozone is known to exert its lethal effects against arthropods by promoting oxidative damage to the cuticle, respiratory spiracles and cell membranes,[54,55] the higher CT requirement for bed bug eggs is partially explained by the respiratory system morphology and physiology of this life stage. The egg stages of insects from the genus *Cimex* spp. (Cimicids) and *Rhodnius* spp. (kissing bugs) consist of a gas-impermeable outer chorion, and respiratory gas exchange takes place almost entirely through the egg cap.[56] Before reaching the embryo, atmospheric oxygen diffuses through the egg cap and its sealing structures. After that, air diffuses through another layer of spongy matrix containing pseudo-micropylic structures that lead to a plenum-like film of air surrounding the developing insect.[56–58] The number of diffusion events required for a gas such as ozone to reach the embryo suggest that it is likely reacting readily with egg structures before reaching the embryonic insect at significantly toxic levels. However, the large reactive surface area provided by the chorion and pseudo-micropyles may allow ozone to exert its oxidative effects on different egg structures. Similar to ozone, DDVP (a neurotoxic fumigant) LC estimates or CT products for the egg stage were 18–40-fold higher in comparison with the nymphal and adult stages. Again, large differences between egg and nymph or adult LC estimates for DDVP are likely because of the reduced gas permeability of the egg stage and the underdeveloped nervous system of the embryo. Previous controlled or modified atmosphere studies with bed bugs have shown that the egg stage is more tolerant to intermediate concentrations (50–80% for 8 h) of carbon dioxide than nymphs and adults.[14] By contrast, eggs are more susceptible than the active life stages to ultra-low oxygen and vacuum treatments.[16]

The baseline ozone toxicity levels for various life stages of an insecticide-susceptible *C. lectularius* strain also provide insights into the potential drawbacks of using this gas as a tool for bed bug management. The use of ozone for odor remediation, and water and agricultural produce disinfection requires low ozone concentrations and exposure periods (~ 1.5 ppm or less for 10–60 min).[54] However, for the control of stored product pests and *C. lectularius*, high ozone concentrations (35–1800 ppm) are required along with long exposure durations (a few hours to 7 d).[37,39,40,42,44,59–61] Realistically, it may not be feasible to achieve high ozone concentrations of 800–1000 ppm in bed bug-infested apartments, hotel rooms or homes. This is because the ozone output (in ppm) of generators meant for deodorization or other applications is likely well below insecticidal levels. Even if high-output generators capable of raising ozone concentrations to 800 or 1000 ppm in *C. lectularius*-infested apartments or structures are

employed, the potential of ozone to oxidize plastic, rubber and other surfaces of household items,[62,63] and its toxicity to indoor ornamental plants[24] will likely preclude its use for the control of common bed bugs. Also, because ozone is a gas, its use for treating a specific apartment in a multi-occupancy apartment building or hotel with multiple rooms is not advisable because at high concentrations it can easily escape from a vacant apartment that is being treated to adjacent apartments that are occupied. Similarly, ozone treatment of *C. lectularius*-infested single-family homes that are properly sealed to prevent gas escape (equivalent to tent fumigation for termites) may not be feasible due to the high cost associated with sealing an entire house within a tent.

Given the limitations associated with using ozone for bed bug management in single family homes, hotels and multi-occupancy apartment buildings, its potential use could be limited to portable ozonation chambers, or trailers, in which *C. lectularius*-infested items such as furniture, mattresses, books and other sensitive household items (paintings, sculptures, books, *etc.*) could be treated. Such ozonation chambers could also be useful in hospitals where pesticide use is largely restricted. To minimize the oxidative effects of ozone on household items, one option is to use lower ozone concentrations of ~ 50–80 ppm, for a prolonged period (24–48 h). In this regard, Feston *et al.*[22] showed that exposure of bed bug adults to 80 ppm ozone in presence of 1% or 2% hydrogen peroxide vapors for 24–48 h can provide 80–100% mortality under laboratory conditions. Field studies with stored product pests conducted in commercial grain bins (8.9 tons capacity) have used 25–50 ppm ozone concentrations for up to 5 days.[37] Although the use of lower ozone concentrations (50–80 ppm) in combination with long exposure times (> 48 h or 2 days) could be feasible for achieving *C. lectularius* control in an ozonation chamber, treatment duration and cost might limit the adoption of this technique by pest management professionals.

The potential future use of ozone chambers or trailers will depend upon development of this technology by the industry and field testing. If ozonation chambers are developed for treatment of *C. lectularius*-infested items, their use will be largely restricted by various regulatory agencies worldwide due to human safety concerns and pest management professionals may be required to carry fumigation licenses for using ozone. Under these regulations, entry of pest management professionals into ozonation chambers will be prohibited until ozone concentrations reach levels safe for human entry. Currently, in the USA, direct exposure of humans to 0.05–0.1 ppm of ozone for up to 8 h is permissible when it is used for microbial control and deodorization purposes (https://www.epa.gov/indoor-air-quality-iaq/ozone-generators-are-sold-air-cleaners#good-bad-ozone). While using ozone for *C. lectularius* control, pest management professionals will also be required to use respirators and other personal protective equipment and will have to comply with safe waiting period recommendations. Commercially available ozone sensors can be used by pest management professionals to monitor ozone concentrations during and after treatment. Interestingly, safety and waiting standards for using carbon dioxide fumigation for *C. lectularius* control[14] are not currently regulated by the EPA. However, regulations for pests such as beetles, lice and moths indicate that carbon dioxide concentrations in work areas cannot exceed 0.5% or 5000 pm (https://www.fsis.usda.gov/wps/wcm/connect/bf97edac-77be-4442-aea4-9d2615f376e0/Carbon-Dioxide.pdf?MOD=AJPERES).

5 CONCLUSIONS

By determining baseline ozone concentrations and exposure time data necessary to kill different life stages of insecticide-susceptible *C. lectularius* under laboratory conditions, this study provides insights into the utility of this technique in IPM programs. High ozone concentrations and long exposure times required to kill different *C. lectularius* life stages, particularly eggs, suggest that its use for treating entire apartments, hotel rooms and single-family homes may not be feasible due to several factors such as: (i) logistical limitations associated with producing and maintaining high ozone concentrations; and (ii) concerns related to adverse respiratory health effects caused by ozone exposure. However, pending future testing and regulatory approvals, treatment of bed bug-infested furniture, mattresses, books, etc. and specialty items (e.g., wall paintings, sculptures, medical devices) in portable ozonation chambers or trailers is likely possible. Lastly and most importantly, the efficacy of portable ozone chambers or trailers, the extent of safety-related restrictions imposed by regulatory agencies and the overall economics of this control technique will determine its future adoption as one of the IPM tool for the management of common bed bugs.

ACKNOWLEDGEMENTS

We thank Cecelia Foley and Aaron Ashbrook for assistance with bed bug rearing. We also thank John Lynn Obermyer for taking high-resolution photographs of hatched and unhatched bed bug eggs. We are grateful to Medizone International for providing ozone production equipment and sensors. SG was supported by merit-based scholarships from BASF and Norm Ehmann/Univar. This research was partially funded by Osmun Innovation Funds, Center for Urban and Industrial Pest Management and the Purdue University AgSEED grant program.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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