Journal of Economic Entomology, 115(4), 2022, 1203–1212
https://doi.org/10.1093/jee/toac091
Advance Access Publication Date: 16 June 2022
Research

ENTOMOLOGICAL SOCIETY OF AMERICA
SHARING INSECT SCIENCE GLOBALLY

OXFORD

Horticultural Entomology

Exploring the Insecticidal Potential of Gaseous and Aqueous Ozone to Control Spotted-Wing Drosophila, *Drosophila suzukii* (Diptera: Drosophilidae)

Benjamin A. Savage,[1,3] Susan Masten,[2] Henry Chung,[1] and Matthew Grieshop[1]

[1]Department of Entomology, Michigan State University, MI 48824, USA, [2]Department of Civil and Environmental Engineering, Michigan State University, MI 48824, USA, and [3]Corresponding author, e-mail: benalexandersavage@msu.edu

Subject Editor: Thomas Phillips

Received 16 November 2021; Editorial decision 11 May 2022.

Abstract

Over the last decade, numerous companies have marketed aqueous ozone sprayers for insect and disease management, but little to no data has been published on their efficacy. Thus, we evaluated the potential of both gaseous and aqueous ozone as a potential preharvest insecticide against the adult life stage of the invasive fruit pest, spotted-wing drosophila, *Drosophila suzukii* (Matsumura). Gaseous ozone was applied at two dosages, 14,600 and 30,100 ppmv, for varying durations and the respective concentration-time (CT) exposure responses were modeled for sex-specific mortalities recorded at 0, 24, 48, and 72 h following treatment. We found that gaseous ozone primarily caused mortality immediately following exposure, with slight increases 72 h following ozone treatments. The female and male lethal concentration-time (LCT) 50 estimates were significantly different at 0, 24, 48, and 72 h after 30,100 ppmv treatments, where males observed an increased mortality response. However, the LCT 99 estimates confidence intervals (95%) of adult female and male *D. suzukii* were similar at 0, 24, 48, and 72 h after 14,600 or 30,100 ppmv ozone treatments. In contrast, ozone dissolved in distilled water at 18.52 ppm (mg/L) did not provide any mortality after total immersion of subjects for 30 s. While gaseous ozone may have some utility as a fumigant for *D. suzukii* in closed vessels where concentrations could be maintained, we did not identify any insecticidal potential for ozone dissolved in aqueous solution when simulating a preharvest treatment under optimal laboratory conditions.

Key words: fumigant, dissolved ozone, aqueous ozone, vinegar fly, exposure-response curves

Gaseous ozone has been reported to be a successful postharvest management insecticide, while aqueous ozone has not (Ebihara et al 2013, Grieshop et al. 2019). However, over the last decade, a variety of aqueous ozone sprayers have been developed and marketed to growers with the promise of both insect and disease control. These sprayers have been highlighted in trade literature and purport the benefits and effectiveness of using aqueous ozone as a preharvest pesticide treatment while very rarely providing field or laboratory efficacy data (Wood 2013, Adams 2014, Anon 2014, Puit 2014, McGregor 2018, Anon n.d.). A recent large-scale field evaluation of one commercially available ozone sprayer for management of the full complex of apple insect and diseases, demonstrated no discernible control (Grieshop et al. 2019). This study's objective was to confirm or dispel the notion of ozone as a preharvest insecticide. If effective, ozone could be a novel alternative to current organic and conventional insecticides.

Ozone is triatomic oxygen and has been used for microbial sanitation, food processing, odor reduction, waste-water treatment, pesticide degradation, and as a fumigant for stored product pest management (Kim et al. 1999, Kells et al. 2001, Rico et al. 2007). Ozone is a versatile oxidizing agent with biocidal potential that has a short residual in water at pH > 6 due to its rapid decomposition into nontoxic constituents within minutes at room temperature (~30 min) (Kim et al. 1999). Depending on its application, ozone is applied in the form of gas or dissolved in water or other liquids.

Ozone has been evaluated as a postharvest insect and disease control treatment for a variety of crops (Erdman 1980, Kells et al 2001, Mlikota-Gabler et al. 2010, Walse et al. 2017). For example,

grapes in a contained vessel can be treated with 5,000 ppmv ozone gas for one hour to control against gray mold, *Botrytis cinerea* Pers., with variable significant reduction dependent on grape varietal (Mlikota-Gabler et al. 2010). The western black widow spider, *Latrodectus hesperus* (Chamberlin and Ivie) (Araneae: Theridiidae), has a predicted control of 92% when exposed to 5,000 ppmv ozone gas for one hour (Walse et al. 2017). Thus, target pests can be controlled against when applying ozone, but the commodity may undergo damage as well. Little to no damage has been observed on the grape berry, however, the grape rachis can undergo browning when exposed to pesticide relevant concentrations of gaseous or aqueous ozone (Mlikota-Gabler and Smilanik 2001, Smilanick et al. 2002, Mlikota-Gabler et al. 2010). Understanding these variables and how they interact can inform the viability of a pesticide for use on a crop or commodity.

Ozone has been evaluated as a fumigant treatment for a variety of stored product insect pests to replace or use in conjunction with phosphine, due to insect resistance to phosphine fumigation (Chaudhry 2000, Pimentel et al. 2007). A study from Brazil in 2008 evaluated ozone application against the stored product pests *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae), and *Oryzaephilus surinamensis* (Linneaus) (Coleoptera: Silvanidae), and determined that no cross-resistance occurred between phosphine resistant populations to ozone (Sousa et al. 2008). The insect respiratory system is known to be the primary path for fumigation toxicity (Cotton 1932, Pimentel et al. 2007), however, Sousa et al (2008) showed that respiration rates and ozone susceptibility didn't correlate when compared to time–mortality curves of *T. castaneum, R. dominica*, and *O. surinamensis*. Currently, ozone's mode of action, has not been fully described and is hypothesized to be multifaceted and dependent on structurally-selective reactivity with biomolecules. Multiple environmental variables may affect a target organism's susceptibility to ozone toxicity including: temperature, humidity, and surface characteristics of surrounding materials (Isikber and Athanassiou 2015).

Ozone concentration and exposure time contribute to mortality, thus, a concentration*time (CT) product is sometimes used to quantify dose responses (Wickramanayake et al. 1984; McDonough et al. 2010, 2011, Feston et al. 2020). For example, the red flour beetle, *T. castaneum*, experienced 100% mortality after 1,800 ppmv gaseous ozone for 120 min in laboratory conditions (216,000 ppmv-min) and 47,000 ppmv gaseous ozone for 6 min in field conditions (282,000 ppmv-min) (McDonough et al. 2010). McDonough et al. (2011) reported that an ozone concentration of 1,800 ppmv significantly reduced the time to reach 100% mortality in *T. castaneum*, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae), and *Sitophilus oryzae* (Linneaus) (Coleoptera: Curculionidae), compared to that observed at 50 ppmv (McDonough et al 2011).

Studies have found ozone to have variable toxicity to insects, depending on species and environmental conditions. The adult red flour beetle, *T. castaneum*, adult maize weevil, *S. zeamais*, and larval Indian meal moth, *P. interpunctella* in a galvanized steel grain bin with approximately 9 tons of maize were treated with 25 ppmv ozone for 5 d as well as 50 ppmv ozone for 3 d and experienced >75% & >90% mortalities, respectively (Kells et al. 2001). Kells et al. (2001) provides evidence that the adult maize weevil had greater susceptibility to ozone than the adult red flour beetle or the larval Indian meal moth (Kells et al. 2001). In another study, *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) and *Tribolium confusum* (du Val) (Coleoptera: Tenebrionidae) experienced higher mortality from 13.9 mg/L of ozone when alone in a container than in 2 kg of wheat, providing evidence that ozone will kill insects less readily when in the presence of ozone susceptible organic molecules (Işikber and Öztekin 2009). However, a study testing ozone fumigation in a modified stainless steel screw conveyor (22.7 kg grain capacity) reported no significant change in 100% CT product mortality of *S. zeamais* and *T. castaneum* when compared to laboratory conditions, 216,000 ppmv-min and 286,920 ppmv-min respectively (McDonough et al. 2010). While we could not find literature on the potential lethality of gaseous ozone on *Drosophila* spp., Savage et al. (2021) demonstrated that *Drosophila suzukii* (Matsumura) (Diptera-Drosophilidae) exposed to 3,750 ppmv-min ozone were less resistant to desiccation than flies exposed to clean air or oxygen. These studies highlight the variable effects of environmental conditions on ozone toxicity to insects.

Dissolving ozone into aqueous solution is an alternative method of application. Insect mortality in response to aqueous ozone has not been as extensively studied as gaseous ozone exposure. Ebihara et al. developed an ozone-mist sprayer and reported >90% mortality of the red aphid, *Uroleucon nigrotuberculatum* (Olive) (Hemiptera: Aphididae) under greenhouse conditions (Ebihara et al. 2013). In this study, aphids were sprayed from a distance of 2–5 cm with gaseous ozone (8.4–3,200 ppmv) and water droplets combined at the nozzle, effectively making the experiment a gaseous ozone application. A second paper evaluated an axial fan radial airblast sprayer equipped with an ozone generator unit attachment that was marketed to control plant diseases and insects (Grieshop et al. 2019). This study reported no control of bacterial, fungal, and insect pests after a full season of applications at 0.75 ppm (mg/L) of ozone. However, none of these studies evaluated aqueous ozone induced insect mortality under laboratory conditions.

Drososphila suzukii, commonly known as spotted-wing Drosophila, is an invasive vinegar fly, first detected in California in 2008 (Bolda et al. 2010). *D. suzukii* quickly became a destructive invasive pest of soft fruits, due to its bladed ovipositor, short life cycle (~3 wk), and broad host range. The fly prefers laying eggs in ripening fruit (Mitsui et al. 2006), depositing its eggs using a serrated ovipositor. Spotted-wing drosophila completes an entire life cycle, from egg to adult, in 9–11 d at 25°C allowing dozens of generations per year (Kanzawa 1939). Spotted-wing drosophila readily reproduces in raspberries, blackberries, blueberries, strawberries, grapes, and cherries and the United States estimated fruit crop losses in California, Oregon, and Washington were $511 million annually (Walsh et al. 2011). Current management of this pest relies on repeated applications of broad-spectrum, contact insecticides from the pyrethroid, spinosyn, and organophosphate chemical classes (Bruck et al. 2011, Van Timmeren and Isaacs 2013). Certified organic producers rely almost entirely on organic formulations of spinosyns to control fly populations (Van Timmeren and Isaacs 2013). In 2019, low to medium spinosyn resistance was identified from a population of *D. suzukii* near Watsonville, CA (Gress and Zalom 2019). The development of novel control methods for *D. suzukii* populations is of growing concern to maintain usage of spinosyn insecticides (Gress and Zalom 2019).

Currently there are several ozonated water air blast sprayers on the market, primarily targeting perennial fruit growers, that claim insect and disease management efficacy. However, we could not find any peer reviewed laboratory or field data supporting these claims. Thus, the objectives of the study were to determine the potential of ozone as a *D. suzukii* management tool as either a 1) gas or 2) dissolved in water under controlled, laboratory conditions.

Materials and Methods

We conducted two experiments to determine the potential of ozone as an insecticide on *D. suzukii*. Experiment 1 evaluated gaseous ozone as an insecticide of *D. suzukii* by developing CT exposure response curves at two different ozone concentrations. Experiment 2 evaluated the potential of aqueous ozone by subjecting flies to a dip test.

Colony Details & Maintenance

D. suzukii (50–80) were reared on 5 ml of artificial diet described in Dalton et al. (2011) in 50-mL plastic vials (Lab Express, Cat. # 8002-cs) and maintained in a colony chamber at 23°C, 77% RH, and a photoperiod of 16:8(L:D) h. Flies were initially collected in 2015 from tart cherries at the Trevor-Nichols Research Center in Fennville, Michigan (Savage et al. 2021).

Drosophila Handling

Flies were transferred between colony rearing vials and aging vials after anesthetization with CO_2 delivered via a Fly Stuff Fly Pad (Genesee Scientific, San Diego, CA) and fly handling with forceps (BioQuip Products Inc., Rancho Dominguez, CA). Flies were held in 50 ml vials containing fresh diet during the aging period in the colony chamber (see Colony Details and Maintenance). Aged flies were anesthetized with CO_2 gas and then placed in groups (~10 female and ~10 male) into 5.33 cm diameter spherical exposure cages (Fu Store; Shenzhen, Guangdong, China; Model # 8,541,896,633) made of 304 grade stainless steel for aqueous ozone experiments. Gaseous ozone experiments used the same exposure cage, but modified to 2.16 cm diameter.

Flies in cages from a single treatment and time exposure were placed into 0.34 m × 0.34 m × 0.6 m mesh insect arenas (BioQuip Products Inc., Rancho Dominguez, CA) following treatment application. Flies were then collected via aspiration from an arena and placed into a new vial, which was then stored in the colony chamber (see Colony Details and Maintenance) for observation.

Ozone Generation and Handling

Ozone was generated using a corona-discharge Nano Ozone Generator (Absolute Ozone, Edmonton, Canada) fed with 99.5% oxygen. Gaseous ozone products were delivered to experimental arenas using polytetrafluoroethylene (PTFE) tubing and 316 stainless steel fittings. Stainless steel and PTFE were used due to their extremely low reactivity coefficient with ozone. Gaseous ozone concentrations were monitored using a Model 106-H Ozone Monitor (2B Technologies, Boulder, CO) (Fig. 1). The entire system was placed in a fume hood.

The two gaseous ozone concentrations evaluated (14,600 and 30,100 ppmv) were generated by feeding oxygen gas at 0.07 m^3/h (2.5 SCFH) and 4 psi through an ozone generator (Fig. 1). An independent feed leading from the oxygen tank to the application chamber allowed for the dilution of ozone concentration within the treatment chamber during gaseous ozone applications. The 14,600 and 30,100 ppmv ozone treatments were generated by diluting the ozone carrying gas with oxygen (99.5% purity) at 0.28 m^3/h (10 SCFH) and 0.112 m^3/h (4 SCFH), respectively. Aqueous ozone was generated by bubbling gaseous ozone, approximately 65,000 ppmv ozone, through a custom made PTFE diffusing ring (Ozone Solutions, Hull, IA) into 300 ml of distilled water (Dean Foods, El Paso, TX) inside a 473 ml glass jar (Ball Corporation, Broomfield, CO) for one hour (Fig. 1).

Experiment 1: Gaseous Ozone Mortality Response

Gaseous ozone exposure–response experiments were conducted on 4–8-d-old male and female *D. suzukii* adults. Treatments included a nontreated control, an oxygen treated control, 14,600 ppmv ozone treatment, and 30,100 ppmv treatment (Table 1). Fly mortality was evaluated using 14,600 and 30,100 ppmv ozone concentrations at different exposure times, resulting in the same concentration-time (CT) products, of: 5,000, 10,000, 20,000, 40,000, and 80,000 ppmv-min for each concentration (Table 1). Oxygen-treated flies were treated for 5.48 min, while nontreated control flies were left untouched. Concentration-time products at 14,600 and 30,100 ppmv and control treatments were replicated 10 times over the course of four trials, whereby each trial was performed on a different day (Table 1). In total, 2,381 male and female flies were utilized.

The gaseous ozone treatment apparatus (Fig. 1) attached a glass bubbler (Fig. 1-h) (150 mL of distilled water), a 2-neck glass

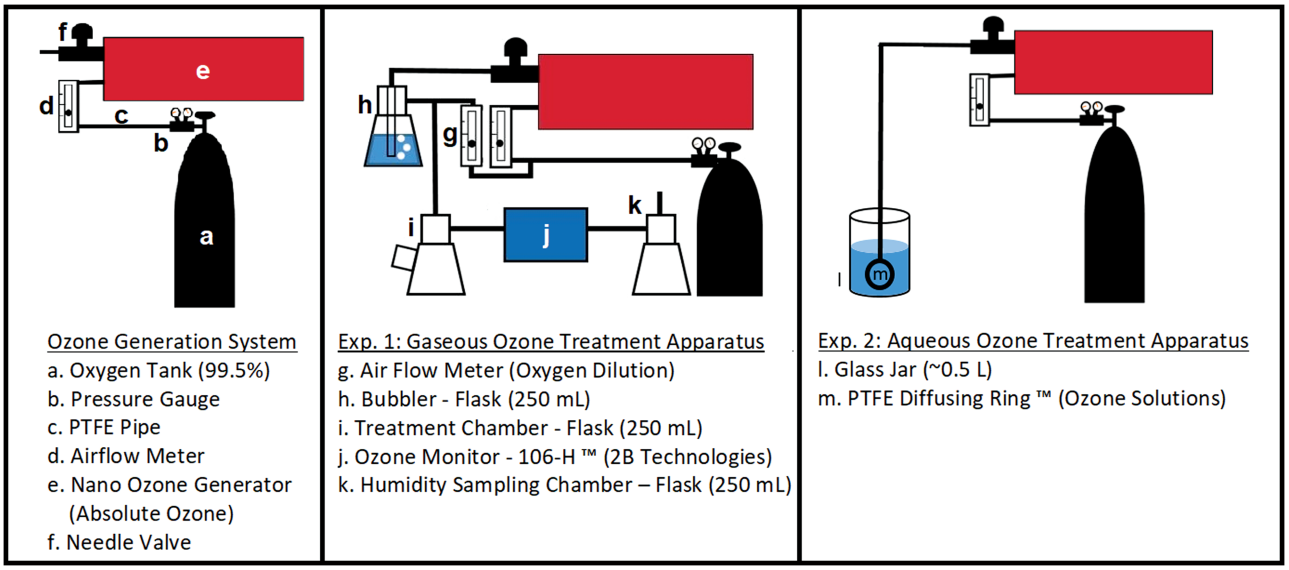

Fig. 1. A component breakdown of the ozone generation system and ozone treatment apparatuses. Flies were inserted into *k. Treatment Chamber – Flask (250 mL)* (Experiment 1) or submerged in a treatment within *l. Glass Jar* (Experiment 2).

Table 1. Experiment 1 and 2 ozone exposure treatments and parameters

Experiment 1: gaseous ozone mortality					
Treatments	Concentration (ppmv) 'Ozone: Oxygen'	Fly age (days)	Sample size (M,F)	CT product (ppmv-min)	Exposure time (s (min))
14,600 ppmv Ozone	14,600: 980,400	4–8	98,99	5,000	20.6 (0.34)
			99,100	10,000	41.1 (0.68)
			98,100	20,000	82.2 (1.37)
			99,100	40,000	164.4 (2.74)
			100,100	80,000	328.8 (5.48)
30,100 ppmv Ozone	30,100: 964,900	4–8	97,100	5,000	10 (0.17)
			99,100	10,000	19.9 (0.33)
			99,100	20,000	39.9 (0.66)
			101,99	40,000	79.7 (1.33)
			97,98	80,000	159.5 (2.66)
Oxygen	0: 995,000	4–8	99,99	0	328.8 (5.48)
Nontreated	–	4–8	100,100	–	–
Experiment 2: aqueous ozone mortality					
Treatment	**Constituents**	**Age (days)**	**Sample size (M,F)**	**CT product**	**Exposure time (s (min))**
Ozone	Distilled Water/18.52 ppm Ozone	5–8	80,80	9.25	30 (0.5)
Water	Distilled Water		80,80	0	30 (0.5)
Nontreated	–		80,80	–	–

Ozone/oxygen concentration, fly age, sample size, target concentration-time (CT) product, and exposure times are described for each treatment tested in experiments 1 and 2.
ppmv, parts per million volume; ppm, parts per million; M, F, male, female; CT, concentration-time.

treatment chamber (Fig. 1-i), an ozone monitor (Fig. 1-j), and a final glass humidity sampling chamber (Fig. 1-k) at the end for obtaining relative humidity values from exiting gas during oxygen treatments. Cages were attached to a hooked glass stopper and introduced to the treatment chamber (Fig. 1-i) via a separate neck on the side of the chamber once the proper concentration was maintained for at least 2 min. Cages remained in the treatment chamber until the target exposure time for a specific CT product was obtained. This process was repeated for all treatment applications in gaseous ozone. Gaseous ozone concentrations were measured during treatment applications for verification and consistency using the ozone monitor. Relative humidity and temperature of oxygen treatments of experiment 1 were measured inside a 250 ml glass flask (Fig. 1-k) during each trial using an electronic hygrometer sensor (Sensirion, Zurich, Switzerland) (Fig. 1). Relative humidity and temperature were not evaluated in ozone treatments due the possibility of sensor oxidation. Cages and forceps were hand-washed before and after each replicate with detergent (Alconox, White Plains, NY) in order to reduce possible contamination.

Experiment 1: Data Collection and Statistical Analyses

Mortality was measured every 24 h over a 72-h interval starting at 0 h after treatment application. Nontreated and oxygen controls were shared between 14,600 and 30,100 ppmv ozone treatments. Fly mortality of nontreated and oxygen controls was nonnormal at 0 h, 24 h, 48 h, and 72 h after treatment application. The 'kruskal.test' function in R version 3.5.1 was utilized to perform Kruskal–Wallis Rank Sum Tests to compare controls at 0 h, 24 h, 48 h, and 72 h (R Core Team 2015). Mortality of controls determined the *c* (lower limit) parameter of the following model log-logistic functions. Flies were considered dead if they were unable to return to a standing position after the vial was hand agitated.

Mortality of ozone treated flies was fitted to a two-parameter binomial log-logistic function using the 'drm' function in the R package 'DRC' (R Version 3.5.1) to create exposure response curves (Ritz et al. 2015, Ritz and Strebig 2016). Parameters *c* (lower limit) and *d* (upper limit) were constrained to 0 and 1, respectively, while the *b* (slope) and *e* (ED 50) parameters were allowed to vary. Exposure response models (8 models) were created for 14,600 ppmv and 30,100 ppmv at 0 h, 24 h, 48 h, and 72 h after treatment application. Two curves, female and male mortality, were fitted within all exposure response models.

Lethal concentration-time (LCT) estimates of 50 and 99 were compared between males and females within a model by using the 'EDcomp' function of DRC package in R version 3.5.1 (Ritz and Strebig 2016). Differences between LCT estimates were calculated using standard errors derived by the delta method (Ritz et al. 2015). Functions were weighted based upon the number of individuals treated during each application (8–11 male, 9–11 female). The LCT 50 and 99 estimates were rounded to three significant figures.

Experiment 2: Aqueous Ozone Mortality Response

The aqueous ozone experiment was conducted by exposing 10 male and 10 female *D. suzukii* adults (5–8 d old) to dissolved ozone in distilled water. Experimental treatments included a nontreated control, distilled water/0 ppm ozone control, distilled water/18.5 ppm ozone treatment, which was as high a concentration that could be developed using distilled water and a pure oxygen fed. The experiment was replicated 8 times during two trials and a total of 420 male and female flies were sampled (80 flies of a sex per treatment) (Table 1). Nontreated control flies were grouped into vials without receiving treatment. The water control and ozonated water flies were caged and submerged in 300 ml of distilled water or 300 ml of ozonated distilled water, respectively, for 30 s after which they were dried on a paper towel. Aqueous ozone concentrations were measured at the beginning and end of each trial using the I-2019 ozone

measuring kit (Chemetrics, Midland, VA). A 1:10 dilution (ozonated water:water) was performed prior to analysis and ozone concentration measurements were determined following manufacturer recommendations. Solution temperature and pH were measured using the 9107BNMD pH/ATC electrode (ThermoFisher Scientific, Waltham, MA) and Star A221 pH meter (ThermoFisher Scientific, Waltham, MA) before treatment application. Experimental apparatus was cleaned before and after each replicate with alconox detergent (Alconox, White Plains, NY) in order to reduce possible contamination.

Experiment 2: Data Collection and Statistical Analyses

Experimental subjects of a single treatment and replication were transferred to separate vials and mortality was measured every 24 h over a 72-hour interval starting at 0 h after treatment application. Flies were considered dead if they were unable to return to a standing position after the vial was hand agitated. Mortality data were nonparametric and were analyzed using a one-way Kruskal–Wallis test to determine the effect of treatment on female and male fly mortality at 0 h, 24 h, 48 h, and 72 h after treatment. The 'kruskal.test' function was used in R version 3.5.1 (R Core Team 2015).

Results

Female and male flies experienced high mortality during treatment application of 14,600 and 30,100 ppmv of gaseous ozone during experiment 1 (Fig. 2). Conversely, aqueous ozone caused very little to no mortality after treatment application during experiment 2 (Fig. 3). Both experiments 1 and 2 demonstrated increased mortality at 24 h, 48 h, and 72 h, with the highest mortalities observed at 72 h. Exposure response curve estimates, slopes, and 95% confidence intervals are reported in Tables 2 and 3.

Experiment 1: Gaseous Ozone Mortality Response

The temperature (mean ± SEM) and relative humidity (mean ± SEM) across oxygen treatments were 23.64 ± 0.08°C and 75.72 ± 1.25%. Kruskal–Wallis Rank Sum Tests between nontreated and oxygen treated flies were performed at 24 h, 48 h, and 72 h, but not at 0 h because no mortality was recorded (100% of subjects were alive in both treatments). Kruskal–Wallis Rank Sum Tests found no differences in mortality between nontreated and oxygen treated flies at 24 h, 48 h, and 72 h (Chisq = 0.765, df = 1, P = 0.382, Chisq = 0.169, df = 1, P = 0.681, Chisq = 0.606, df = 1, P = 0.435, respectively). The mortality proportions (mean ± SEM) of control flies (nontreated and oxygen treated) at 0 h, 24 h, 48 h, and 72 h were 0 ± 0, 0.015 ± 0.006, 0.018 ± 0.006 and 0.02 ± 0.006, respectively. Thus, the c parameter in exposure response models was set to zero.

In general, lethal concentration-time (LCT) 50 and 99 estimates (ppmv-min) decreased overtime after 14,600 ppmv ozone treatments to female and male flies (Fig. 2; Tables 2 and 3). The LCT 50 estimates (Mean ± SE) for females after 14,600 ppmv ozone treatments at 0 h, 24 h, 48 h, and 72 h were 19,100 ± 1,100, 16,600 ± 1,000, 15,400 ± 1,000, and 14,730 ± 920 ppmv-min, respectively (t = 16.93, P < 0.001, t = 16.05, P < 0.001, t = 16.23, P < 0.001, t = 15.97, P < 0.001, respectively) (Table 2). The LCT 50 estimates (mean ± SE)

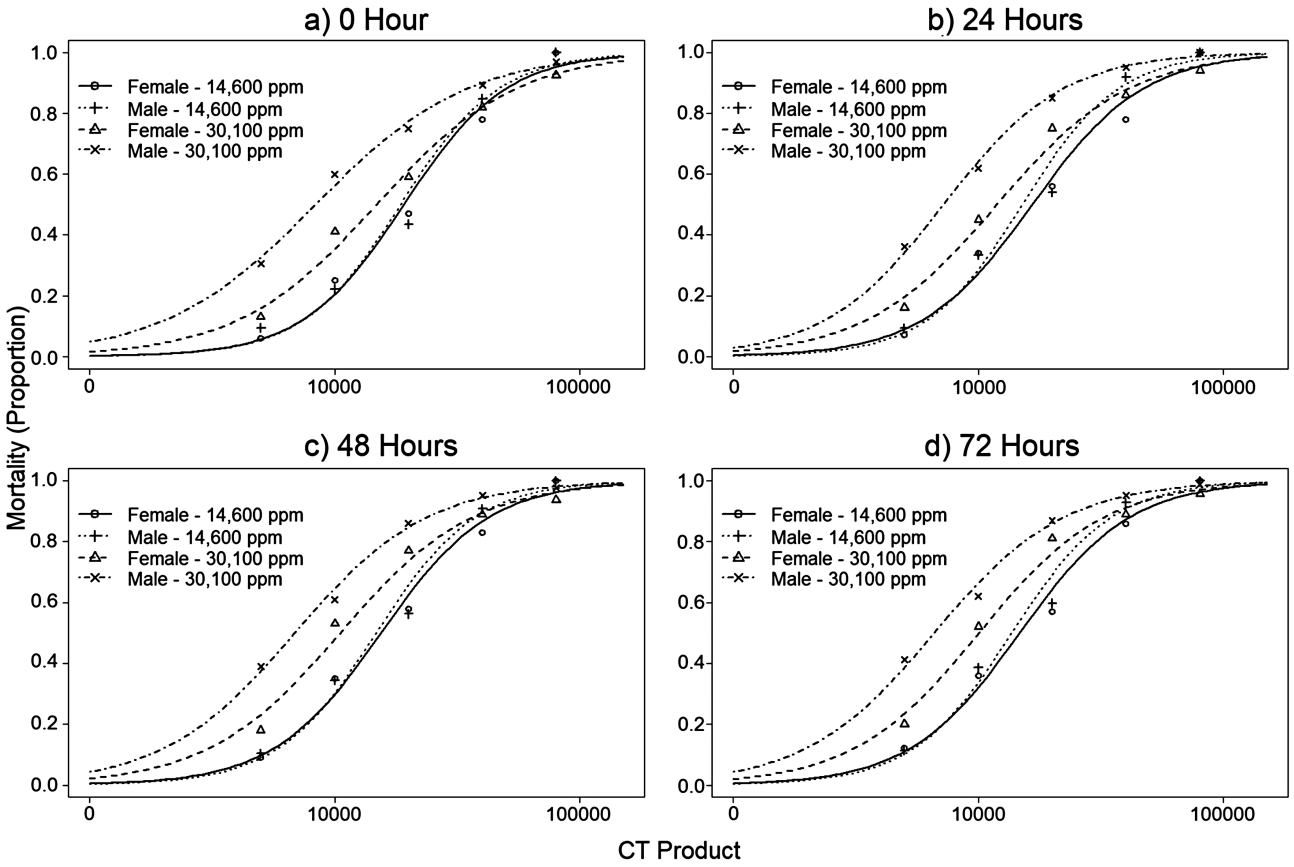

Fig. 2. Gaseous ozone concentration-time (CT) product response curves at 0, 24, 48, and 72 h after treatment for male and female *Drosophila suzukii*. Data were fit to a two-parameter binomial log-logistic function where the parameters c (lower limit) and d (upper limit) were constrained to 0 and 1.

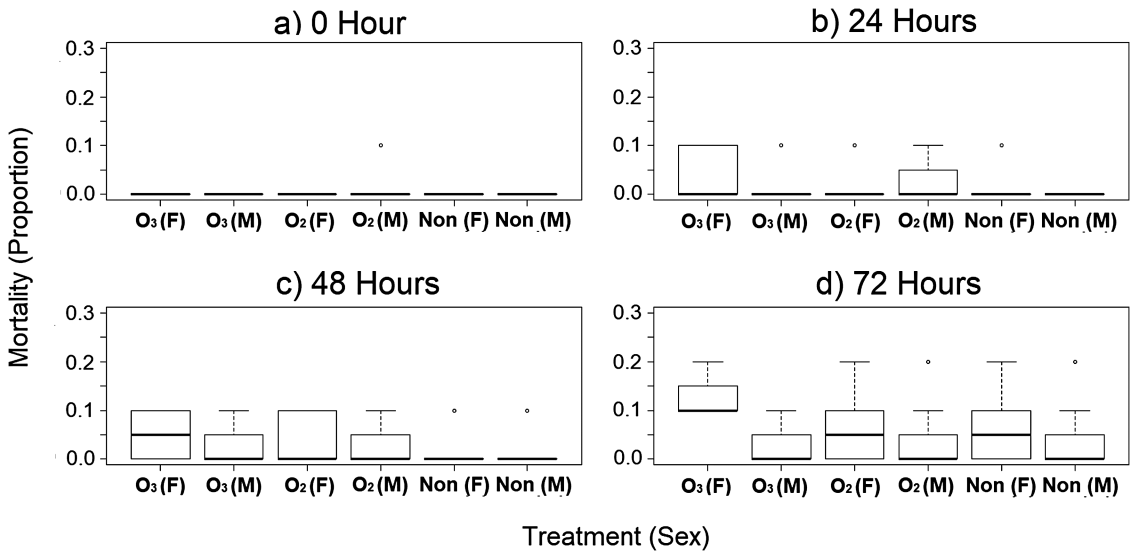

Fig. 3. Boxplots of adult male and female *Drosophila suzukii* mortality at 0, 24, 48, and 72 h after treatment with aqueous ozone. Treatment (Ozone (18.5 ± 0.8 ppm), Oxygen, Nontreated), and sex (M, F) are described on the x-axis and mortality as a proportion is described on the y-axis. The horizontal line is the median value of the data set. The lower whisker shows the extent of the 1st quartile of data, the lower half of the box is the second quartile of data, the top half of the box is the 3rd quartile, and the top whisker is the 4th quartile of the data?

for males after 14,600 ppmv ozone treatments at 0 h, 24 h, 48 h, and 72 h were 18,600 ± 1,100, 15,100 ± 900, 14,800 ± 900, and 13,700 ± 800 ppmv-min, respectively (t = 17.2, P < 0.001, t = 17.29, P < 0.001, t = 16.95, P < 0.001, t = 16.87, P < 0.001, respectively) (Table 2). The Delta method found no differences between female and male LCT 50 nor LCT 99 estimates when exposed to 14,600 ppmv ozone treatments (Table 4). The LCT 99 estimate for female and male flies at 72 h after 14,600 ppmv ozone treatments were 159,000 ± 32,600 and 117,000 ± 21,600 ppmv-min, respectively.

The CT products developed using 30,100 ppmv ozone showed lower LCT 50 and 99 estimates (ppmv-min) compared to CT products developed with 14,600 ppmv concentrations. The LCT 50 estimates (mean ± SE) for females after 30,100 ppmv ozone treatments at 0 h, 24 h, 48 h, and 72 h were 14,800 ± 1,100, 11,900 ± 900, 10,500 ± 800, and 10,000 ± 700 ppmv-min, respectively (t = 13.63, P < 0.001, t = 13.79, P < 0.001, t = 13.33, P < 0.001, t = 13.73, P < 0.001, respectively) (Table 2). The LCT 50 estimates (mean ± SE) for males after 30,100 ppmv ozone treatments at 0 h, 24 h, 48 h, and 72 h were 8,400 ± 800, 7,200 ± 600, 6,900 ± 600, and 6,600 ± 500 ppmv-min, respectively (t = 10.92, P < 0.001, t = 12.53, P < 0.001, t = 11.14, P < 0.001, t = 11.11, P < 0.001, respectively) (Table 2). The Delta method found significant differences between female and male LCT 50 estimates when exposed to 30,100 ppmv ozone treatments at 0 h, 24 h, 48 h, and 72 h with males experiencing a higher mortality compared to females (Table 4). For example, the estimated ratio, or relative potency, of the LCT 50 after 30,100 ppmv ozone treatments on male flies was 1.78 times greater than female flies at 0 h after treatment (Table 4). Overtime, the relative potencies of the LCT 50 at 30,100 ppmv from males to females at 24 h, 48 h, and 72 h were 1.65, 1.53, and 1.52, respectively (Table 4). Finally, the Delta method found no differences in female and male LCT 99 estimates (Table 4).

Experiment 2: Aqueous Ozone Mortality Response

The ozone concentration (mean ± SEM) of the ozonated distilled water treatment was 18.5 ± 0.8 ppm. The temperature (mean ± SEM) and pH (mean ± SEM) of the distilled water and ozonated distilled water treatments across trials were 23.45 ± 0.25°C and 6.38 ± 0.21, respectively. A Kruskal–Wallis rank sum test found no significant effect of the main effect, treatment, for female or male flies at 24 h (Chisq = 1.937, df = 2, P = 0.38, Chisq = 2.19, df = 2, P = 0.335, respectively), 48 h (Chisq = 2.516, df = 2, P = 0.284, Chisq = 0.484, df = 2, P = 0.785, respectively), and 72 h (Chisq = 4.6, df = 2, P = 0.1, Chisq = 0.026, df = 2, P = 0.987, respectively). At 0 h, no female fly mortality was recorded (0% mortality across treatments) and no significant effect was observed from treatment for males (Chisq = 2, df = 2, P = 0.368).

A single male fly died and no female flies died at 0 h after treatment application. The mean proportion of mortality for flies (females and males) observed in the nontreated, control, and ozone treatments at 72 h were 0.05, 0.05, and 0.08, respectively. Mean mortality at 72 h remained at or below 12.5% in all treatments of both sexes (Fig. 3).

Discussion

The goal of our study was to evaluate ozone, as a potential insecticide against the invasive fly species, *D. suzukii*. Our data suggest that gaseous ozone at 14,600 ppmv will instantly (0 h) cause 50% mortality of female and male flies after 1.16–1.46 min and 1.13–1.42 min, respectively (Fig. 3; Tables 2 and 3). Additionally, gaseous ozone at 30,100 ppmv will instantly (0 h) cause 50% mortality of females and males after 0.42–0.56 min and 0.23–0.33 min, respectively (Fig. 3; Tables 2 and 3). Ozone concentrations of 14,600 and 30,100 ppmv demonstrated overlapping LCT 99 lower and upper 95% confidence intervals, which indicates that both ozone concentrations attained 99% mortality of flies at similar CT products (Table 3). In contrast, while gaseous ozone shows potential as an insecticide, ozone dissolved in water is highly unlikely to have a lethal impact on *D. suzukii* (Fig. 3).

Across all ozone treatments, the majority of mortality was observed at 0 h after treatment with only a slight increase at 72 h after treatment. For example, male flies treated with 20,000 ppmv-min of 14,600 ppmv ozone observed mortality was 44% at 0 h and 60% at 72 h after treatment (Fig. 3a and d). Thus, gaseous ozone has a good

Table 2. Experiment 1: LCT 50 (ppmv-min) *Drosophila suzukii* mortality at 0 h, 24 h, 48 h, and 72 h after gaseous ozone treatment

			0 h			24 h			48 h			72 h		
Treatment	Sex	Sample Size	LCT 50 (SE)	b *(SE)*	95% CI	LCT 50 (SE)	b *(SE)*	95% CI	LCT 50 (SE)	b *(SE)*	95% CI	LCT 50 (SE)	b *(SE)*	95% CI
14,600 ppmv	Female	499	19,100 (1,100)	−2.09 (0.17)	16,900–21,400	16,600 (1,000)	−1.93 (0.16)	14,600–18,600	15,400 (1,000)	−1.97 (0.17)	13,600–17,300	14,700 (900)	−1.93 (0.16)	12,900–16,500
	Male	494	18,600 (1,100)	−2.17 (0.18)	16,500–20,700	15,100 (900)	−2.21 (0.19)	13,400–16,800	14,800 (900)	−2.15 (0.18)	13,100–16,500	13,700 (800)	−2.15 (0.18)	12,100–15,300
30,100 ppmv	Female	497	14,800 (1,100)	−1.53 (0.14)	12,700–17,000	11,900 (900)	−1.62 (0.15)	10,200–13,600	10,500 (800)	−1.61 (0.15)	9,000–12,100	10,000 (700)	−1.7 (0.16)	8,600–11,500
	Male	493	8,400 (800)	−1.41 (0.14)	6,900–9,900	7,200 (600)	−1.78 (0.18)	6,100–8,400	6,900 (600)	−1.61 (0.17)	5,700–8,100	6,600 (600)	−1.65 (0.18)	5,400–7,800

The LCT 50 estimates and 95% confidence intervals of female and male *D. suzukii* for the 14,600 and 30,100 ppmv ozone treatments were derived from CT product response curves (Fig. 3).
b, slope; CT, concentration-time; CI, confidence intervals; LCT, lethal concentration-time; ppmv, parts per million volume; SE, standard error.

Table 3. Experiment 1: LCT 99 (ppmv-min) *Drosophila suzukii* mortality at 0 h, 24 h, 48 h, and 72 h after gaseous ozone treatment

			0 h		24 h		48 h		72 h	
Treatment	Sex	Sample Size	LCT 99 (SE))	95% CI (Lower–Upper)	LCT 99 (SE)	95% CI (Lower–Upper)	LCT 99 (SE)	95% CI (Lower–Upper)	LCT 99 (SE)	95% CI (Lower–Upper)
14,600 ppmv	Female	499	172,000 (32,700)	108,000–237,000	181,000 (37,100)	108,000–253,000	159,000 (31,800)	96,500–221,000	159,000 (32,600)	95,400–223,000
	Male	494	154,000 (28,200)	98,900–210,000	120,000 (21,600)	78,000–163,000	126,000 (23,000)	80,200–171,000	117,000 (21,600)	74,400–159,000
30,100 ppmv	Female	497	300,000 (81,000)	141,000–458,000	203,000 (50,800)	103,000–302,000	183,000 (47,000)	91,800–274,000	151,000 (36,300)	79,900–222,000
	Male	493	217,000 (65,600)	88,000–345,000	94,800 (22,500)	51,000–139,000	119,000 (32,000)	57,000–181,000	108,000 (28,200)	52,500–163,000

The LCT 99 estimates and 95% confidence intervals of female and male *D. suzukii* for the 14,600 and 30,100 ppmv ozone treatments were derived from CT product response curves (Fig. 3).
CT, concentration-time; CI, confidence intervals; LCT, lethal concentration-time; ppmv, parts per million volume; SE, standard error.

Table 4. Female and male ozone LCT estimate comparisons

Treatment	Time (h)	Estimate (ratio of female to male)	Std. error	*T*	*P*
LCT 50 comparisons: 0, 24, 48, and 72 h					
14,600 ppmv	0	1.03	0.085	0.355	0.722
	24	1.097	0.093	1.044	0.297
	48	1.044	0.089	0.497	0.62
	72	1.074	0.093	0.804	0.422
30,100 ppmv	0	1.775	0.208	3.72	<0.001 (***)
	24	1.651	0.178	3.655	<0.001 (***)
	48	1.532	0.179	2.967	0.003 (**)
	72	1.521	0.176	2.957	0.003 (**)
LCT 99 comparisons: 0, 24, 48 and 72 h					
14,600 ppmv	0	1.119	0.295	0.402	0.688
	24	1.499	0.409	1.222	0.222
	48	1.264	0.344	0.766	0.444
	72	1.365	0.376	0.968	0.333
30,100 ppmv	0	1.385	0.562	0.685	0.493
	24	2.138	0.738	1.541	0.123
	48	1.538	0.566	0.951	0.342
	72	1.4	0.497	0.804	0.421

Delta method comparison of LCT 50 & 99 estimates between adult male and female *D. suzukii* at 14,600 and 30,100 ppmv ozone concentrations. Significance indicated with * < 0.05, ** < 0.01, *** < 0.001 alpha levels.

capacity for 'knock-down' activity at the tested concentrations with flies succumbing in a matter of minutes to 14,600 ppmv ozone and in mere seconds to 30,100 ppmv ozone. It has been reported that fast knock-down activity is a common characteristic of currently recommended insecticides for *D. suzukii* control (Isaacs et al. 2013). Thus, our results show that ozone has limited residual/latent insecticidal properties after initial exposure, but may be used as an effective fast knock-down insecticide to control *D. suzukii* populations when applied as a fumigant within a closed environment.

D. suzukii required a smaller CT product (males ~110,000 ppmv-min; females ~150,000 ppmv-min) to achieve 99% mortality, compared to *T. castaneum* and *S. zeamais*, which required a larger CT product (~216,000 ppmv-min) for 100% mortality (Kells et al. 2001). Sousa et al. (2008) noted that different species of stored product insects varied in their response to gaseous ozone and reported 95% mortality was achievable of *T. castaneum* and *R. dominica* with CT products ranging from 196,650 to 333,630 ppmv-min ozone as well as 95% mortality of *O. surinamensis* ranging from 99,270 to 168,480 ppmv-min ozone (Sousa et al. 2008). Thus, our results provide support for the hypothesis that different species and, possibly, different orders of arthropods require specific CT products to induce mortality.

Fly exposure to ozone dissolved in water did not provide a detectable increase in mortality compared to a water or nontreated control. The mean 18.5 ppm rate tested in this study is considerably higher than the 1–10 ppm produced by most commercial ozonation units used for sanitizing drinking water and wastewater (Oxidation Technologies, LLC 2017). Thus, we feel it is safe to conclude that aqueous ozone has very little potential to develop lethal activity in insects using current application methodologies in agricultural pest management. This finding is consistent with that of Grieshop et al. (2019), who evaluated a commercial airblast sprayer that delivered <1 ppm dissolved ozone concentration and concluded that it did not effectively manage insect nor disease pests of apples when used in a replicated, season-long experiment. Ozone susceptibility of egg and larval life stages of *D. suzukii* has not been recorded, and could be a future research area.

Differing LCT 50 estimates between gaseous ozone treatments were observed. The ozone treatments were generated by using the same oxygen flow rate, but had differing flow rates from a separate oxygen dilution feed into the treatment chamber. This may explain why we observed disparate LCT 50 estimates. Also, the male LCT 50 estimates were significantly lower than female estimates at 30,100 ppmv ozone, which coincides with similar mortality results observed by Savage et al. (2021) in regards to desiccation resistance after ozone exposure. Male and female flies experienced a 2 h (confidence intervals: 2–2 h) and 2.5 h median (confidence intervals: 2.5–5 h), respectively, time to mortality caused by desiccation (Savage et al. 2021). These results provide evidence that male and female flies experience variable toxicity and/or residual effects when ozone exposure is at or lower than the predicted LCT 50 estimate.

Our experiments provide definitive evidence that while gaseous ozone has insecticidal potential against adult *D. suzukii*, aqueous ozone does not. Our results show a negative correlation between ozone concentration and time when observing fly mortality, however, a comprehensive fumigation model of gaseous ozone against *D. suzukii* has yet to be fully described. Future applications of this technology should focus on systems where gaseous ozone concentrations can be maintained at suitable levels, e.g., on postharvest material stored in a closed vessel or perhaps in greenhouse production. Additionally, application of ozone concentrations outside of contained areas proves difficult because the ability to accumulate relative toxic concentrations of ozone is diminished. For example, *D. suzukii* adults are free-living outdoor flies that infest fruits in orchards and vineyards, so applying gaseous ozone at 14,600 or 30,100 ppmv would be impossible without confining a treatment area and investment in ozone generators with the required throughput. Application of ozone to a developing crop may damage the plant (Mlikota-Gabler and Smilanik 2001, Smilanick et al. 2002, Mlikota-Gabler et al. 2010), which could yield a diminished harvest or quality of commodity. Thus, this possibility would also need to be determined on a crop by crop basis.

In conclusion, this study found that ozone primarily causes mortality during direct ozone exposure, with only modest increase to

mortality over 72 h after ozone applications. The 14,600 ppmv ozone treatment demonstrated similar LCT 50 estimates across fly sex, but the 30,100 ppmv ozone treatment significantly increased mortality of males in comparison to females when comparing LCT 50 estimates. Overall, both the 14,600 and 30,100 ppmv ozone treatments produced similar LCT 99 estimates. Finally, 30 s of total immersion in aqueous ozone did not cause increased mortality of *D. suzukii*, supporting the hypothesis that ozone dissolved in water has little to no potential as an insecticide.

Acknowledgments

Experiments were made possible from the help of Charlotte Schuttler, Allison Fischer, Elizabeth Szcepanski and Kyle Akred. Funding came from the Organic Pest Management lab at Michigan State University directed by Matthew Grieshop, Ph.D. This research was funded by USDA SCRI project 2016-51181-25411. H.C. is supported by USDA NIFA via Michigan State University AgBioresearch (Umbrella project MICL02522).

References Cited

Adams, A. **2014**. Wider use for ozone in winemaking. https://winesvinesanalytics.com/news/article/135495/Wider-Use-for-Ozone-in-Winemaking. Accessed November 12, 2021.

Anon. **2014**. Bacigalupi vineyards experiments with AgriOzein to reduce pesticide, fungicide use. https://www.winebusiness.com/suppliernews/?go=getSupplierNewsArticle&dataId=142617. Accessed November 4, 2021.

Anon. **n.d**. Ozone pest control. http://www.absoluteozone.com/ozone-generator-pest-control.html. Accessed November 12, 2021.

Bolda, M. P., R. E. Goodhue, and F. G. Zalom. **2010**. Spotted wing drosophila: potential economic impact of a newly established pest. *Agric. Resour. Econ*. 13: 5–8.

Bruck, D. J., M. Bolda, L. Tanigoshi, J. Klick, J. Kleiber, J. DeFrancesco, B. Gerdeman, and H. Spitler. **2011**. Laboratory and field comparisons of insecticides to reduce infestation of Drosophila suzukii in berry crops. *Pest Manag. Sci*. 67: 1375–1385.

Chaudhry, M. Q. **2000**. Phosphine resistance. *Pestic. Outlook*. 11: 88–91.

Cotton, R. T. **1932**. The relation of respiratory metabolism of insects to their susceptibility to fumigants. *J. Econ. Entomol*. 25: 1088–1103.

Dalton, D. T., V. M. Walton, P. W. Shearer, D. B. Walsh, J. Caprile, and R. Isaacs. **2011**. Laboratory survival of Drosophila suzukii under simulated winter conditions of the Pacific Northwest and seasonal field trapping in five primary regions of small and stone fruit production in the United States. *Pest Manag. Sci*. 67: 1368–1374.

Ebihara, K., F. Mitsugi, T. Ikegami, N. Nakamura, Y. Hashimoto, Y. Yamashita, S. Baba, H. D. Stryczewska, J. Pawlat, S. Teii, *et al*. **2013**. Ozone-mist spray sterilization for pest control in agricultural management. *Eur. Phys. J. Appl. Phys*. 61: 24318.

Erdman, H. E. **1980**. Ozone toxicity during ontogeny of two species of flour beetle, Tribolium confusum and Tribolium castaneum. *Environ. Entomol*. 9: 16–17.

Feston, J., S. Gaire, M. Fardisi, L. J. Mason, and A. D. Gondhalekar. **2020**. Determining baseline toxicity of ozone against an insecticide-susceptible strain of the common bed bug, Cimex lectularius L. under laboratory conditions. *Pest Manag. Sci*. 76: 3108–3116. doi:10.1002/ps.5864

Gress, B. E., and F. G. Zalom. **2019**. Identification and risk assessment of spinosad resistance in a California population of Drosophila suzukii. *Pest Manag. Sci*. 75: 1270–1276.

Grieshop, M. J., K. Koonter, B. A. Savage, and J. Nye. **2019**. *Field scale operation evaluation of ozone for management of apple insect and disease pests*, vol. 11. The Michigan Apple Committee Newsletter, Apple Press.

Isaacs, R., B. Tritten, S. Van Timmeren, J. Wise, C. Garcia-Salazar, and M. Longstroth. **2013**. *Spotted wing drosophila management recommendations for Michigan raspberry and blackberry growers*, vol. 7. MSU Extension.

Işikber, A. A., and S. Öztekin. **2009**. Comparison of susceptibility of two stored-product insects, Ephestia kuehniella Zeller and Tribolium confusum du Val to gaseous ozone. *J. Stored Prod. Res*. 45: 159–164.

Isikber, A. A., and C. G. Athanassiou. **2015**. The use of ozone gas for the control of insects and micro-organisms in stored products. *J. Stored Prod. Res*. 64: 139–145.

Kanzawa, T. **1939**. *Studies on Drosophila suzukii. Yamanashi Kenritsu Nouji Shikenjo Gyomu Nenpo*. Yamanashi Prefecture Agricultural Institute, Kofu, Japan.

Kells, S. A., L. J. Mason, D. E. Maier, and C. P. Woloshuk. **2001**. Efficacy and fumigation characteristics of ozone in stored maize. *J. Stored Prod. Res*. 37: 371–382.

Kim, J. -G., A. E. Yousef, and S. Dave. **1999**. Application of ozone for enhancing the microbiological safety and quality of foods: a review. *J. Food Prot*. 62: 1071–1087.

McDonough, M. X., L. J. Mason, C. Woloshuk, and C. Campabadal. **2010**. Ozone technology in the post-harvest storage environment - a comparison of efficacy of high doses of ozone to insects treated under laboratory conditions and field conditions, pp. 386–388. In Proceedings, 10th international working conference on stored-product protection. Estoril, Portugal Julius-Kühn-Archiv, 425, Bundesforschungsinstitut für Kulturpflanzen, Berlin.

McDonough, M. X., L. J. Mason, and C. P. Woloshuk. **2011**. Susceptibility of stored product insects to high concentrations of ozone at different exposure intervals. *J. Stored Prod. Res*. 47: 306–310.

McGregor, N. **2018**. NZ: qpraying without chemicals, residues or health and safety issues. https://www.freshplaza.com/article/2193554/nz-spraying-without-chemicals-residues-or-health-and-safety-issues/. Accessed November 4, 2021.

Mlikota Gabler, F., and J. L. Smilanick. **2001**. Postharvest control of table grape gray mold on detached berries with carbonate and bicarbonate salts and disinfectants. *Am. J. Enol. Vitic*. 52: 12–20.

Mlikota Gabler, F., J. L. Smilanick, M. F. Mansour, and K. Karaca. **2010**. Influence of fumigation with high concentrations of ozone gas on postharvest gray mold, quality, and fungicide residues on table grapes. *Postharvest Biol. Technol*. 55: 85–90.

Mitsui, H., K. H. Takahashi, and M. T. Kimura. **2006**. Spatial distributions and clutch sizes of Drosophila species ovipositing on cherry fruits of different stages. *Popul. Ecol*. 48: 233–237.

Oxidation Technologies, LLC. **2017**. Ozone equipment manufacturer and ozone system integrators Ozone Water Systems Ozone Integration Experts. Oxid. Technol. LLC. (https://www.oxidationtech.com/products/ozone-water-system.html).

Pimentel, M. A. G., L. R. D. Faroni, M. R. Tótola, and R. N. C. Guedes. **2007**. Phosphine resistance, respiration rate and fitness consequences in stored-product insects. *Pest Manag. Sci*. 63: 876–881.

Puit, G. **2014**. Vineyard experiments with ozone to control pests and molds. https://www.record-eagle.com/news/local_news/vineyard-experiments-with-ozone-to-control-pests-and-molds/article_43bfbfe1-7589-5887-9b11-5a5d550029fe.html. Accessed November 4, 2021.

R Core Team. **2015**. *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Autria.

Rico, D., A. B. Martín-Diana, J. M. Barat, and C. Barry-Ryan. **2007**. Extending and measuring the quality of fresh-cut fruit and vegetables: a review. *Trends Food Sci. Technol*. 18: 373–386.

Ritz, C., and J. C. Strebig. **2016**. *drc: Analysis of dose-response curves*. R Package Version, 3.0–1. https://cran.r-project.org/package=drc. Accessed June 11, 2022.

Ritz, C., F. Baty, J. C. Streibig, and D. Gerhard. **2015**. Dose-response analysis using R. *PLoS One*. 10(12): e0146021.

Savage, B., Z. Wang, H. Chung, S. Masten, and M. Grieshop. **2021**. An ozonolysis based method and applications for the non-lethal modification of insect cuticular hydrocarbons. *J. Chem. Ecol*. 47: 628–641. doi:10.1007/s10886-021-01285-8

Smilanick, J. L., D. M. Margosan, and F. Mlikota Gabler. **2002**. Impact of ozonated water on the quality and shelf-life of fresh citrus fruit, stone fruit, and table grapes. *Ozone Sci. Eng*. 24:5: 343–356. doi:10.1080/01919510208901625

Sousa, A. H., L. R. D. Faroni, R. N. C. Guedes, M. R. Tótola, and W. I. Urruchi. **2008**. Ozone as a management alternative against phosphine-resistant insect pests of stored products. *J. Stored Prod. Res*. 44: 379–385.

Van Timmeren, S., and R. Isaacs. **2013**. Control of spotted wing drosophila, Drosophila suzukii, by specific insecticides and by conventional and organic crop protection programs. *Crop Prot*. 54: 126–133.

Walse, S. S., J. S. Tebbets, and L. G. Leesch. **2017**. Postharvest fumigation of California table grapes with ozone to control Western black widow spider, Latrodectus hesperus, (Araneae: Theridiidae). *Postharvest Biol. Technol*. 134: 1–4.

Walsh, D. B., M. P. Bolda, R. E. Goodhue, A. J. Dreves, J. Lee, D. J. Bruck, V. M. Walton, S. D. O'Neal, and F. G. Zalom. **2011**. Drosophila suzukii (Diptera: Drosophilidae): invasive pest of ripening soft fruit expanding its geographic range and damage potential. *J. Integr. Pest Manag*. 2: G1–G7.

Wickramanayake, G. B., A. J. Rubin, and O. J. Sproul. **1984**. Inactivation of Giardia lamblia cysts with ozone. *Appl. Environ. Microbiol*. 48: 671–672.

Wood, D. **2013**. Ozonated water: vineyard pest control without chemicals. https://midwestwinepress.com/2013/03/31/ozonated-water-vineyard-pest-control-without-chemicals/. Accessed November 4, 2021.