Potential of Ozone as a Fumigant to Control Pests in Honey Bee (Hymenoptera: Apidae) Hives

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ABSTRACT Ozone is a powerful oxidant capable of killing insects and microorganisms, and eliminating odors, taste, and color. Thus, it could be useful as a fumigant to decontaminate honey comb between uses. The experiments here are intended to determine the exposure levels required to kill an insect pest and spore forming bee pathogens. Ozone was effective against greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), even on naturally infested comb. Neonates and adults were the easiest life stages to kill, requiring only a few hours of exposure, whereas eggs required a 48-h exposure (at 460–920 mg O_3/m^3). Two honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), pathogens, *Ascosphaera apis* (a fungus that causes chalkbrood) and *Paenibacillus larvae* (a bacterium that causes American foulbrood), also were killed with ozone. These pathogens required much higher concentrations (3,200 and 8,560 mg O_3/m^3, respectively) and longer exposure periods (3 d) than needed to control the insects. *P. larvae* was effectively sterilized only when these conditions were combined with high temperature (50°C) and humidity (≥75% RH). Thus, ozone shows potential as a fumigant for bee nesting materials, but further research is needed to evaluate its acceptability and efficacy in the field. The need for a reliable method to decontaminate honey bee nesting materials as part of an overall bee health management system is discussed.

KEY WORDS chalkbrood, foulbrood, honey bees, ozone, pathogens

Many pests and diseases of honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), persist or "hide away" in old comb after honey has been extracted or a colony has died, and reusing such comb may spread the problem to a new colony. Other pests, such as greater wax moths, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), directly infest and destroy stored comb. Fumigants have been used in beekeeping, such as ethylene dibromid, paradichlorobenzene, hydrogen cyanide, and methyl bromide, mainly for greater wax moth control. However, only paradichlorobezene is actually registered for use on honey comb. Methyl bromide is a common agricultural fumigant, but an international treaty has called for the discontinuation of its use because it depletes the ozone layer in the upper atmosphere, potentially affecting current world climate conditions (U.S. Environmental Protection Agency 1999, WMO 2007). It is moderately effective at killing chalkbrood spores, reducing viability by 99.0% (James 2005a). A renewed interest in methyl oxide and gamma radiation has recently occurred, and these treatments can be very effective at killing insect pests and diseases (Tomio et al. 2001). However, the treatments are also expensive, heavily regulated due to their potential health and environmental effects, and are generally only available to beekeepers who operate near a treatment facility.

Ozone is a form of oxygen (O_3) that is a powerful oxidant. It has the ability to disinfect; eliminate odors, taste, and color; and is commonly used as a treatment for both drinking and swimming water (U.S. Environmental Protection Agency 1999). Recently, it has been developed as an agricultural fumigant, for example, it is used on stored potatoes (*Solanum tuberosum* L.) to prevent rot, and it kills insect and fungal pests in stored grains (Kells et al. 2001, Allen et al. 2003, Mendez et al. 2003). It works for these purposes because, as a strong oxidizer, it is highly toxic to living organisms at high concentrations. Thus, it also has acute toxicity to humans and must be applied in a sealed fumigation chamber, away from workers; however, ozone breaks down quickly into oxygen (O_2), so it will not persist in the air, nor on wax comb, wood, and plastic. Although ozone is not currently registered as a pesticide, the registration requirements are relatively simple if ozone is generated directly from air and applied in a fumigation chamber without a product being involved (C. Grable, personal communication).

The objective of the research reported here was to determine whether ozone can kill greater wax moths and spore forming pathogens, and if so, to develop the

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response curves relative to different ozone concentrations. Honey bees can be infected by a variety of pathogens, but the spore-forming pathogens are the pathogens most likely to be transmitted on comb due to their long persistence. Spores are also the most difficult life stage to kill because they are in a dormant state with very low metabolic activity. Two spore-forming pathogens were tested here, *Ascosphaera apis* (the cause of chalkbrood) and *Paenibacillus larvae* (the cause of foulbrood). Bacterial spores can be extremely difficult to kill, being resilient to both heat and chemical treatments. *P. larvae* is resistant to many antibiotics (Kochansky et al. 2001), and the spores can withstand temperatures of >80°C (Forsgren et al. 2008, Genersch 2008). This pathogen can survive for years on hive material (Lindström 2006). Spores of *Ascosphaera* are less heat resistant (Anderson et al. 1997, James 2005b) but also remain viable for many years (Toumanoff 1951). *Nosema apis* and *Nosema ceranae* are also highly virulent spore-forming pathogens of honey bees, but they were not tested here. These *Nosema* spp. are more temperature sensitive than the other two pathogens mentioned above, and they have a much lower environmental persistence on the comb, <1 mo (Malone et al. 2001), suggestive that ozone conditions adequate to kill *Paenibacillus* and *Ascosphaera* are probably adequate to kill *Nosema* as well. Furthermore, methods for measuring *Nosema* viability were not yet available at the time of this study, making it difficult to determine when ozone was lethal to this pathogen.

Materials and Methods

Ozone Production. A prototype ozone generator (O_3Zone Company, Aberdeen, ID) was used that produces 1–10 g O_3/h (depending on the setting and oxygen input rate). The fumigation chamber was a 0.35 m^3 CO_2 gas incubator (NU-4850 CO_2 Incubator, NuAire, Plymouth, MA). This incubator had a well sealed chamber where both ozone, temperature and humidity could be controlled. Ozone was generated outside the incubator and piped inside, where the concentration was monitored using an ozone analyzer (Low Concentration Analyzer, IN USA Inc., Needham, MA) coupled with a HOBO data logger (Onset, Pocasset, MA). The gas was continuously fed in one side of the chamber and exhausted through an ozone destructor (Ozone Solutions, Hull, IA) on the other side.

Effects of Ozone on Wax. Starting with the first experiment, three strips of wax foundation (6 by 12 cm) were placed in the fumigation chamber and exposed to 460 or 920 mg O_3/m^3 (215 and 430 ppm, respectively) for 2 wk. No discernible effects on the color or flexibility of the foundation were seen, for each ozone concentration. In addition, one frame of drawn comb was left in the fumigation chamber during all the following ozone experiments, again with no discernible effects on the comb quality.

Ozone and Greater Wax Moth Control. Greater wax moths were obtained from in-house colonies. Six life stages were tested for susceptibility to ozone: two ages of eggs, two ages of larvae, pupae, and adults.

Eggs. Adults moths were allowed to mate and lay eggs on wax paper. Eggs were collected daily and placed in the center of small (60- by 15-mm) plastic petri dishes. Two ages of eggs were tested, eggs laid within the previous 24 h, and eggs that were 4 d old. Each set of eggs was treated with ozone for the following durations: 0, 12, 24, 36, or 48 h at 460 or 920 mg O_3/m^3; 25 or 34°C; and 50% RH. All combinations of these treatments were tested (20 different treatment combinations). After treatment, the eggs were incubated at 34°C and checked every 24 h to enumerate hatched larvae until no further emergence was noted for two consecutive days. Hatched larvae were removed as they were counted. Eggs that did not hatch were considered dead. This entire experiment was replicated four different times by using 200–450 eggs per replicate.

Small Larvae. Neonate larvae (≤24 h old) were exposed to ozone for 0, 2, 4, or 6 h at 460 or 920 mg O_3/m^3; 25 or 34°C; and 50% RH. All combinations of these treatments were tested (16 treatment conditions). Larvae were kept at 34°C for 24 h, and then mortality was assessed. This entire experiment was replicated four different times, using 200–450 neonates per replicate.

Large Larvae. Large larvae (4–5 wk old) were placed in large petri dishes (150 by 15 mm) that had the center of the lids removed and covered with chiffon fabric. These were then exposed to ozone for 0, 2, 4, 6, 8, or 10 h at 460 or 920 mg O_3/m^3; 25 or 34°C; and 50% RH. All combinations of these treatments were tested (24 treatment conditions). After treatment, larvae were removed from ozone, incubated at 34°C for 24 h, and then mortality was assessed. This entire experiment was replicated four different times by using 30 larvae per replicate.

Pupae. Pupae were placed in clean glass jars (0.95 liter), 30 pupae per jar. The jar cover consisted of nylon window screen held in place with canning jar bands. Pupae were exposed to ozone for 0, 6, 12, 24, or 36 h at 460 or 920 mg O_3/m^3; 25 or 34°C; and 50% RH. All combinations of these treatments were tested (20 treatment conditions). After being treated with ozone, pupae were incubated for up to 4 wk at 34°C and observed for adult emergence. Pupae from which adults emerged were considered alive, and if development to the adult did not occur, the pupae were considered dead. This entire experiment was replicated four different times by using 30 pupae per replicate.

Adults. Adult moths were placed into clean glass jars (0.95 liter) with screen lids, 20 adults per jar. Adults were exposed to ozone for 0, 2, 4, or 6 h at 460 or 920 mg O_3/m^3; 25 or 34°C; and 50% RH. All combinations of these treatments were tested (16 treatment conditions). After treatment, adults were placed at 34°C for 24 h, and mortality was assessed. This entire experiment was replicated four different times by using 30 adults per replicate.

Statistical Analysis. For each of the life stages tested, multivariate analysis of variance (ANOVA) was conducted using ozone exposure time, ozone concentration, and temperature as the main effects and mortality as the dependent variable, after arcsine square-root transformation to normalize the distribution.

Greater Wax Moth-Infested Comb. When greater wax moths infest comb, they normally occur as a mixture of life stages surrounded by webbing and frass. It is possible that the webbing and comb provide some protection from ozone fumigation, and when several frames are placed together, the webbing might protect the moths. To simulate this effect, two frames of comb heavily infested by greater wax moths were placed together and treated with 1,070 mg O_3/m^3 (500 ppm) for 48 h. The combs sat for 24 h after the treatments were complete, and then they were carefully dissected to recover all the adults, larvae and pupae, both live and dead, and percentage of mortality was recorded. The two frames placed together were fumigated as one replicate, and two untreated frames served as the control. The fumigation was repeated with different pairs of infested comb, at different times, for a total of three replicates. Eggs and very small larvae were too difficult to see and thus were not quantified. Viability of pupae was determined by adult emergence at 30°C, as described above.

ANOVA was used to compare the proportion of live insects on the ozone-treated versus the control comb for each life stage (larvae, pupae, and adults). A square-root transformation on proportion dead was used to normalize the variance and distribution, and the experimental design was a complete block design with date of treatment as the blocking factor.

Honey Bee Pathogens. *Chalkbrood. Ascosphaera apis* spores were obtained by mating strains 7405 and 7406 (obtained from the Agricultural Research Entomopathogen Fungal Collection, Ithaca, NY) on potato dextrose agar at 32°C. Spores were collected 10 d after they first began to appear. Spores were collected from the plates and placed on sterile glass slides and treated using 1,070, 2,140, and 3,200 mg O_3/m^3 (500, 1,000, and 1,500 ppm) for incremental 24-h periods up to 5 d at 25°C and 50% RH. Control samples were kept in an incubator for 5 d at 25°C and 50% RH but without the ozone.

The spores were held for 24 h after fumigation before viability was assessed using a germination test. The methods used were essentially those described by James (2005b). In summary, the spores were incubated at 32°C for 36 h in a nutrient broth before being examined for germination using differential interference contrast microscopy. Spores that had swelled or that had a germ tube were considered viable. In total, 400 spores were examined per sample, except for the 3,200 O_3/m^3 treatment, in which 1,500 spores were evaluated. A greater number of spores was used at this dose to make the test more sensitive (i.e., improved the lower the detection limit of viable spores).

Regression analysis was used to determine the effect of ozone concentration and days of exposure on the proportion of spores surviving. To obtain the best model fit, and the best normalization of the data, an arcsine square-root transformation was used on the survival data, and a $\log_{10}$ transformation was used for the days of exposure.

American Foulbrood. Cadavers of honey bee larvae infected with American foulbrood (referred to here as scales) were obtained from the USDA–ARS Bee Disease Diagnostic Laboratory, Beltsville, MD. For each treatment, a piece of comb with at least 10 scales was selected. Half of each piece was treated, and half reserved as a control. Ozone was applied for 72 h under different conditions of temperature, humidity, and ozone concentration, as reported in Table 2. Controls were kept in an incubator under the same conditions without ozone. After treatment, five foulbrood scales were removed from each piece of comb by using 200 μl of sterile reverse osmosis (RO) water and homogenized in a 1.5-ml microtissue grinder. To ensure that each scale was removed from the honey comb cell in its entirety, the cell was rinsed several times with sterile RO water, and the rinse was added to the microtissue grinder. Final volume for each scale suspension was then brought to 600 μl. A serial dilution was plated on selective agar (bovine heart infusion agar [Difco BHIA, BD Biosciences, Sparks, MD] modified with thiamine hydrochloride [0.1 mg/liter] and nalidixic acid [0.3 μg/liter]) to determine the number of colony forming units (cfu). Plates were incubated at 33°C and 80% RH for 72 h before colonies were counted. The three replicate plates were averaged to determine the number of viable cfu per scale. Foulbrood survival was the difference in cfu between paired control and treated scales. Means in survival were compared among treatments using a Tukey multiple comparisons adjustment (after an arcsine square-root transformation to normalize the data). Each scale pair was the replicate unit.

Results

Ozone and Greater Wax Moth Control. Greater wax moths were very sensitive to ozone, and all life stages were killed by exposure to this gas (Fig. 1). Ozone exposure period, ozone concentration, and treatment temperature all had significant effects on how many insects were killed by the fumigation, except for the neonate larvae, where only exposure time was significant (Table 1). Exposure time had a significant effect on neonate mortality because the controls (zero exposure time to ozone) were significantly different than the treatments (Table 1; Fig. 1C). The other main effects did not have statistically significant effects on neonate mortality because all (or nearly all) the neonate larvae were killed when exposed to ozone at all the concentrations and temperatures tested, thus there was no difference between 25 and 34°C or between 920 and 460 mg O_3/m^3 (Table 1; Fig. 1C). Adult moths were also very sensitive to ozone, with the majority being killed after 6 h of exposure under most treatment conditions (Fig. 1F). Pupae were more resistant to ozone, usually requiring 12–24 h of exposure

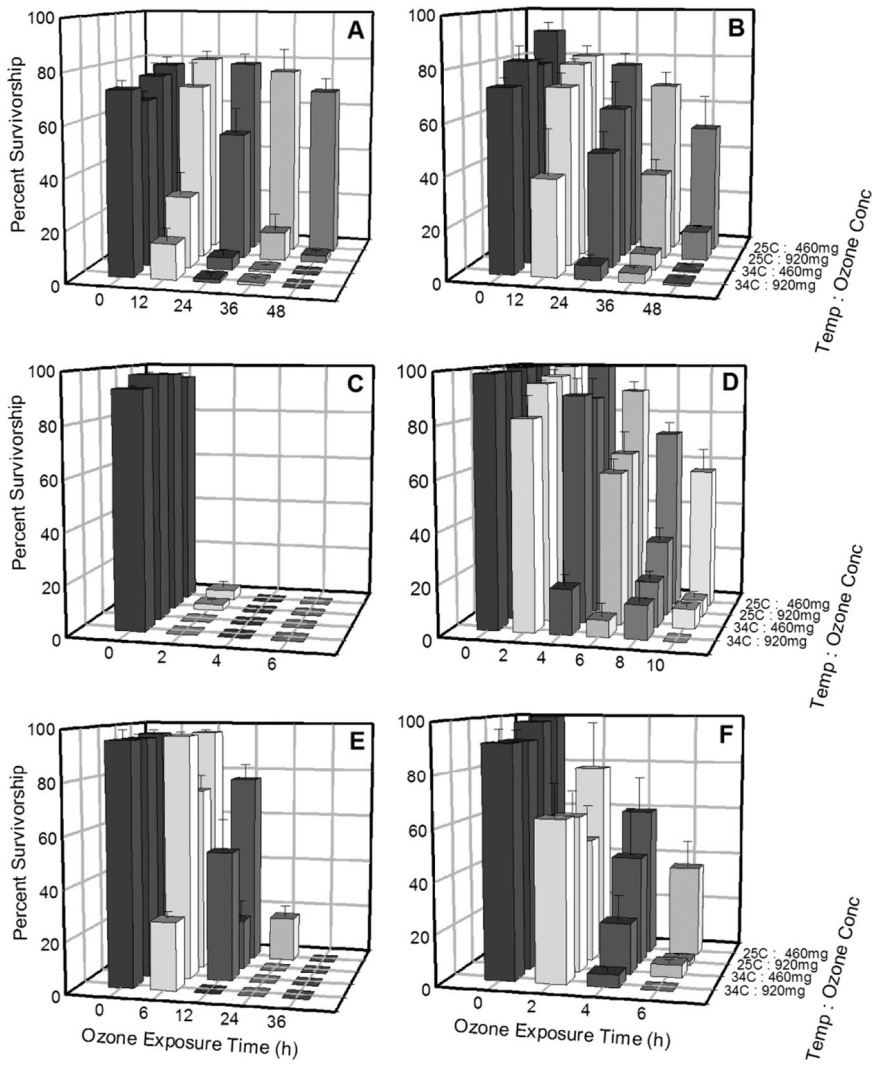

Fig. 1. Effect of ozone exposure on percentage of survival of greater wax moths for different temperatures and ozone concentrations. The life stages tested were 1-d-old eggs (A), 4-d-old eggs (B), neonate larvae (C), large larvae (D), pupae (E), and adults (F). For eggs and pupae, percentage of survivorship is based on percentage of emergence. Note that the x-axis scale differs between graphs. Means with SE bars are presented. See Table 1 for statistical analysis.

(Fig. 1E). The sensitivity of large larvae was intermediate between that of pupae and adults (Fig. 1D). Eggs were the most resistant life stage, especially the older eggs (Fig. 1A and B). Overall, 920 mg O_3/m^3 killed the insects more rapidly than 460 mg O_3/m^3, and ozone was more effective at 34 than 25°C (Table 1; Fig. 1).

Table 1. ANOVA statistics for the effects of ozone exposure time, exposure concentration, and treatment temperature on the mortality of *G. mellonella* in different life stages

Life stage	*F*	df	*P* for main effects		
			Time	Concn	Temp
Eggs (1 d old)	82.58	3,1	0.0001	0.0001	0.0001
Eggs (4 d old)	55.92	3,1	0.0001	0.0004	0.0001
Small larvae (neonates)	25.62	3,1	0.0001	0.9418	0.7168
Large larvae (4–5 wk)	123.76	3,1	0.0001	0.0001	0.0001
Pupae	71.48	3,1	0.0001	0.0003	0.0523
Adults	53.93	3,1	0.0001	0.0110	0.0010

Good control of greater wax moth infestations also was obtained on infested comb (Fig. 2). Combs treated with ozone had significantly fewer live larvae ($F = 65$; df = 1, 1; $P \leq 0.0005$), pupae ($F = 12$; df = 1, 1; $P \leq 0.04$), and adults ($F = 284$; df = 1, 1; $P \leq 0.0001$) than untreated combs. The number of live insects was not significantly greater than zero in any of the life stages ($P \geq 0.09$ for the estimated proportion of live larvae and pupae and $P = 1.0$ for adults).

Honey Bee Pathogens. *Chalkbrood.* Sterilization of *A. apis* spores was obtained only at 3200 mg O_3/m^3, where 99.952% of the spores were killed after 24 h and 100% were killed after 36 h (Fig. 3). When both ozone and days of exposure were included in the regression

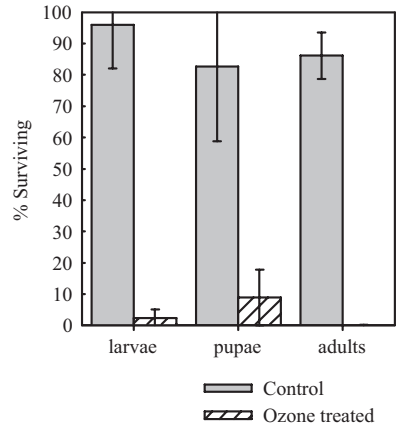

Fig. 2. Mean survival of greater wax moths in naturally infested honey bee comb. Frames were treated 1,070 mg O_3/m^3 for 48 h. Controls are untreated frames. Bars are SEs.

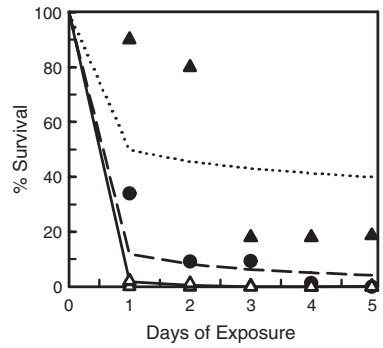

Fig. 3. Percentage of survival of chalkbrood spores (*A. apis*) after exposure to ozone for different time periods, at different concentrations (▲, 1,070 mg/m^3; ●, 2,140 mg/m^3; and △, 3,200 mg/m^3). Experiments were conducted at 25°C and 50% RH. Lines are predictions based on regression analysis; see text for details.

model, the model was significant ($r^2 = 0.90$; $F = 126.39$, $P = 0.0001$):

$$y = -0.213\ (\log_{10}\text{days}) - 0.00056\ \text{ozone concentration} + 0.979,$$

where y is the arcsine square root of the proportion of spores surviving. When separate regression models were evaluated for each ozone concentration, the model was not significant for the lowest dose tested (1070 mg O_3/m^3) ($r^2 = 0.55$; $F = 4.95$, $P < 0.09$), i.e., exposure time ($\log_{10}$ days) did not have a significant effect on mortality). At the intermediate dose (2,140 mg O_3/m^3), the model was highly significant ($r^2 = 0.919$; $F = 45$, $P < 0.0025$). The fitted model was as follows:

$$y = -0.208\ (\log_{10}\text{days}) + 0.352.$$

For the high dose (3200 mg O_3/m^3), the model was also highly significant ($r^2 = 0.996$; $F = 4439$, $P < 0.0001$), and the fitted model was as follows:

$$y = -0.239\ (\log_{10}\text{days}) + 0.135.$$

American Foulbrood. P. larvae was not very susceptible to ozone (Table 2). Under somewhat typical ambient conditions of temperature and relative humidity (33°C and 50–85% RH), some of the bacteria still remained viable even when exposed to very high concentrations of ozone (10,700 mg O_3/m^3) (Table 2). Ozone effectively sterilized this pathogen only when high ozone concentrations were combined with a high temperature (50°C), and the effect of a high temperature was greatest when also combined with near-saturation humidity conditions. Increasing the treatment temperature had a greater effect on sanitation than did increasing the humidity (Table 2).

Discussion

Ozone effectively killed greater wax moths, a serious hive storage pest. The adult and larval stages were more sensitive than were the pupae and eggs, which required longer exposures. A limitation to using ozone fumigation to control storage pests is that once the hive supers and comb are treated, the chemical is gone, and the comb is susceptible to reinvasion of the pest. Thus, if fumigation is to be used as a control method, the supers will either need to be stored in such a manner that the pests are not likely to reinfest, or repeated fumigations will be required. Ozone does break down many volatile compounds, and this may provide an advantage. For example, ozone has been shown to eliminate odors from sewage and waste water (Riva and Sacco 2006) and to play a role in the effect that pollution has on eliminating floral scents attractive to bees (McFrederick et al. 2008). It may be worth further investigation to determine whether ozone reduces compounds that attract greater wax moths to stored comb.

Greater concentrations of ozone were needed to kill chalkbrood spores compared with greater wax moths, and American foulbrood was even more resistant, with complete disinfection only occurring at

Table 2. Ability of ozone to kill *P. larvae* spores under different conditions

Ozone concn (mg/m^3)	% RH	Temp (°C)	Exposure period (d)	*P. larvae* cfu[a] per scale (SE)		% foulbrood survival[b] (95% CI)
				Control	Ozone treated	
6,420	50	33	3	1.79×10^6 (0.477×10^6)	1.52×10^6 (0.375×10^6)	81.4a[c] (24.0–97.2)
8,560	50	50	3	4.44×10^8 (0.665×10^8)	5.82×10^7 (1.63×10^7)	12.5b (5.47–42.7)
8,560	75	50	3	7.47×10^8 (3.31×10^8)	8.01×10^7 (0.644×10^7)	18.0b (2.76–42.3)
8,560	90	50	3	1.24×10^8 (0.207×10^8)	0 0	0.00b
10,700	50	33	3	1.40×10^7 (0.082×10^7)	1.32×10^7 (0.158×10^7)	92.7a (75.4–99.9)
10,700	85	33	3	3.24×10^8 (0.384×10^8)	2.29×10^8 (0.585×10^8)	81.4a (24.0–97.2)

[a] The cfu values are means (with SEs) from five scales.
[b] Transformed back after an arcsine square-root transformation.
[c] Survival data followed by different letter are significantly different from each other ($P \leq 0.05$).

50°C, 90% RH, and 8,560 mg O_3/m^3. Interestingly, higher concentrations of ozone were less effective when the temperature was 33°C. The combination of high ozone concentration, temperature, and humidity are technically feasible to achieve but may not be economical in some situations. A metal fumigation chamber out in the sun during a Florida summer might achieve these conditions without the operator having to add heat or humidity, but treatments made during the fall in more northern areas, or in the arid west, might require the addition of heat or humidity. It is not clear why high temperature and humidity increased the efficacy of ozone, but high temperature and humidity typically have a negative effect on the long-term viability of spore-forming microorganisms. Thus, these conditions may either be stressors, or they may increase the respiration rates, thus making ozone more effective.

Ozone oxidizes both rubber and latex, but it does not seem to break down beeswax. The simple tests conducted here provided a qualitative evaluation of the effect of ozone on wax comb, and even after repeated applications the wax had not become brittle. After fumigation, treated supers and comb did have an off odor, so further studies are needed to determine whether treated comb is acceptable to bees. A beekeeper local to the author's area set up an ozone fumigation chamber of his own. He has treated his hive equipment for 2 yr in a row without noticing any problems with acceptability to the bees (unpublished data).

Honey comb from dead-out colonies, or after honey extraction, sometimes contain pollen stores. Yook et al. (1998) tested the effect of ozone on pollen quality and microbial count. Ozone reduced bacterial and yeast counts by one order of magnitude, and the effect on pollen quality was quite low. However, the quantity of ozone used in these experiments was not well documented and may have been low relative to the rates used in the experiments reported here. I found that pollen provisions collected from alfalfa leafcutting bee, *Megachile rotundata* (F.), nests turned very hard after ozone treatment, at rates I thought would eliminate chalkbrood spore viability (unpublished data).

Beekeepers are in great need of some additional tools to help them maintain healthy colonies. Colony collapse disorder continues to be a problem (vanEngelsdorp et al. 2010), and the cause may be due to pathogens (Cox-Foster et al. 2007, Johnson et al. 2009, vanEngelsdorp et al. 2009, Bromenshenk et al. 2010). Used hive supers and frames with drawn comb are commonly reused by beekeepers despite the fact that these materials harbor pests and pathogens. Comb from honey supers, especially, are moved from one hive to the next across years. Typically, honey supers are removed from beehives during honey harvest, get stored in warehouses after the honey has been extracted, and are then brought out and placed on hives during the next honey production season. Beekeepers do not keep track of which supers came from which hive; they are placed on hives as needed, with a high probability that they get placed on different hives from 1 yr to the next. Although it seems logical that brood comb would be a more likely source of disease transmission for brood diseases (such as chalkbrood, nosema, and foulbrood), *P. larvae* spores can be found in honey and on bees in an infected colony (Pernal and Melathopoulos 2006, Lindström 2008); therefore, honey supers should not be ignored as a possible source of disease. An effective, affordable method of fumigation is sorely needed by the beekeeping community.

However, before any recommendations can be made, field trials are needed to determine whether ozone-treated comb is acceptable to the bees, and if adequate fumigation conditions can be achieved on a large field-use scale, where the woodenware will slow the accumulation of ozone within a chamber. Large ozone generators are already available commercially, with water and sewage sterilization units being the most common. The cost for beekeepers will vary depending on the size of their fumigation chamber. Large chambers will require generators with large production capacity, especially if the chamber is to be filled with a large amount of wood, and the generator cost escalates with generation capacity. Beekeepers will have to determine the optimum size for their operation, weighing the advantages of a large, expensive chamber against the economics of having a smaller chamber that will require more loading and unloading. The price of the ozone generator system is not cheap, currently ≈US$10,000–16,000 for a generator that produces in the range of 40–60 g/h, including the ozone generator and oxygen concentrator, but the price might become cheaper if the market increases, as this would be a new, alternative use for this technology.

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