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The Potential for Using Ozone to Decrease Pesticide Residues in Honey Bee Comb

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Abstract: Ozone is a strong oxidizer, and we evaluated its potential to eliminate pesticides from honeycomb and empty honey bee hives. Honey bees are exposed to pesticides when foraging for nectar and pollen and when beekeepers use in-hive chemical pest control measures. Persistent pesticides can accumulate in the hive over years, potentially harming the bees. Honeycomb is removed from bee colonies for honey extraction and then placed back on the colonies at a later date, providing a time when combs could be fumigated to eliminate or reduce pesticide residues. We found that ozone gas at a rate $\geq$920 mg O_3/m^3 for 10-20 h lowers coumaphos residues on a glass surface by 93-100% and *tau*-fluvalinate by 75-98%. Ozone was less effective at eliminating pesticides on beeswax, and residues were more effectively eliminated with new combs (comb built by bees within 3 y) than with old combs (combs used by beekeepers for $\geq$10 y). Ozone significantly reduced dimethylphenyl formamide, chlorpyrifos, and fenpyroximate contaminations in comb. When comb is treated with ozone, an off-odor is created, but the volatiles were found to be primarily straight chain aldehydes and carboxylic acids that are probably harmless to bees and humans. Ozone may have some utility for lowering pesticide residues in bee hives, but it would be more effective if a mechanism could be found that provides better penetration into wax, a goal not fully accomplished in our method.

Keywords: bees wax, honey bees, honeycomb, ozone, pesticide residues

Abbreviations: ANOVA: analysis of variance; DMPF: 2,4-dimethylphenyl formamide; HPLC: high-performance liquid chromatography; NIST: National Institute of Standards in Technology; ppb: parts per billion; ppm: parts per million; ppm-v: parts per million by volume

1. Introduction

1.1 Pesticides and Honey Bees

Honey bees (*Apis mellifera*) are managed commercially for both honey production and to provide pollination services to a wide variety of crops. Commercial beekeeping operations typically manage hundreds to tens of thousands of colonies. Unfortunately, various bee health issues have made beekeeping more difficult, especially within the last decade, resulting in fewer colonies now than ever in the U.S. (Ellis et al., 2010; vanEngelsdorp and Meixner, 2010). This problem is not limited to that country, but has affected many other parts of the world as well (Neumann and Carreck, 2010).

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Although the causes for bee declines are diverse and complex, bee exposure to pesticides can have immediate and severe impacts. The use of insecticides has long been evaluated by government agencies throughout the world to minimize bee exposures to the most bee-toxic compounds. Despite such efforts, bees continue to experience both acute and chronic pesticide exposures from a variety of sources. For example, some beekeeping practices directly expose honey bees to pesticides, such as the use of in-hive acaricides to control varroa and tracheal mites. Bees can also become exposed when they collect pollen, nectar, and water from plants that have been sprayed by farmers and gardeners. Mullin et al. (2010) found 121 different pesticides and metabolites in bees wax, bees, and bee-collected pollen in the U.S.. Most colonies contain a combination of pesticides (an average of 8 pesticides per wax sample), most of which occur in the hive at low levels (e.g. less than 1 ppm). However, little is known about the effects of chronic, low-level pesticide exposures on bee health, and less data exists regarding the bee health effects of chronic exposures to mixtures of pesticides. Since bees are insects and susceptible to a wide variety of pesticides (especially insecticides, miticides, and fungicides), methods for reducing the build-up of pesticides in the hive are worthwhile. In addition, hive products such as bees wax are often used for products intended for human consumption and cosmetics, and preventing pesticide residues from occurring in these products is desirable for beekeepers, especially since honey bee hive products are often marketed as being "natural" and "pure."

1.2 Ozone May Provide a Means to Reduce Accumulations of Pesticides in the Hive

Gaseous ozone can be effective against several insect pests and pathogens in stored honey comb (James, 2011). We investigate here the potential for gaseous ozone to eliminate or lower pesticide residues as well, especially coumaphos (an organophosphate) and *tau*-fluvalinate (a pyrethroid). These acaricides are used by honey beekeepers to control varroa mites and are the active ingredients in Checkmite® and Apistan Strips®, respectively. The varroa mite is a parasite of honey bees that is easily the most serious pest in beekeeping, and this pest not only harms bees directly but also transmits at least one honey bee virus. Numerous studies have shown that both coumaphos and *tau*-fluvalinate can persist in the wax and woodenware of beehives for many years (Rice and Gomez-Taylor, 1986; Corta et al., 2000; Tsigouri et al., 2000; Martel and Zeggane, 2002; Bogdanov et al., 2003; Huber et al., 2003; Chauzat et al., 2006; Jasim et al., 2006; Kamel and Al-Ghamdi, 2006) and are the most frequent and abundant pesticide residues found associated with honey bees (Mullin et al., 2010).

As a very strong oxidizer, ozone has been used to eliminate pesticides and other organic contaminants from drinking water for many years (Huber et al., 2003; Jasim et al., 2006; Rice and Gomez-Taylor, 1986). It has also been evaluated for use to breakdown pesticide residues on fruits and vegetables, using either washes of ozone dissolved in water or gaseous ozone (Ikeura et al., 2011; Ong et al., 1996; Walse and Karaca, 2011; Wu et al., 2007). Here, we evaluate the potential for using ozone to decrease pesticide residues on honey comb.

2. Method

2.1 Pesticide Degradation on Glass Surfaces.

We first evaluated the rate that ozone degrades coumaphos and *tau*-fluvalinate using pesticide residues on a glass surface at two concentrations of ozone, 1.07 g O_3/l (500 ppm-v) and 2.14 g O_3/l (1000 ppm-v). Technical grade coumaphos and *tau*-fluvalinate (Sigma, St. Louis, MO) were dissolved in acetonitrile at three concentrations: 10, 25 and 50 mg/l (ppm). Then 400 µl of each solution was placed in a 2.0 ml glass vial, and the acetonitrile was evaporated off, leaving residues of 4.0, 10.0, and 20.0 µg pesticide per vial, respectively. The surface area of the interior of the vials, up to the 400 µl mark, was 222.80 mm^2, thus the residues in the glass vial were 1.8, 4.5, and 9.0

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μg/cm^2. The vials were then treated with ozone for 0, 6, 8, 10, 13, 20 or 24 h. The zero exposure treatment was the control.

These experiments were conducted at 50% relative humidity (RH), and then either 25˚ or 34˚C. Ozone was generated using a prototype water cooled corona discharge generator (O_3 Company, Aberdeen, ID) that produced ~5 g O_3/h. The fumigation chamber was a 0.35 m^3 incubator (NU-4850 CO_2 Incubator, NuAire, Plymouth, MA) with automated controls for both temperature and humidity. Ozone concentration was continuously sampled inside the chamber and monitored using an ozone analyzer (Low Concentration Analyzer, IN USA Inc., Needham, MA) coupled with a HOBO® data logger (Onset, Pocasset, MA). Ozone was continuously supplied into the chamber to maintain the desired concentration. Controls were held for 24 h in an incubator at the same temperature and humidity conditions as for the corresponding ozone treatment, but without ozone. Samples that were treated less than 24 h were then held with the untreated controls for the time remaining. Every treatment was replicated four times using newly mixed pesticide solutions and new fumigation treatments each time in a complete random experimental design.

After the ozone treatments, 400 μl of acetonitrile was added back into each vial to recover and quantify any remaining pesticide. These samples were analyzed with high-performance liquid chromatography (HPLC) using an Agilent 1100 Series HPLC System (Waldbronn, Germany) with an autosampler, an injection size of 15 μl per sample at 29˚C, and a photodiode array detector (DAD) (Martel and Zeggane, 2002). The HPLC column was a LiChrospher RP-18, 250 × 4.6 mm I.D. from Supelco Inc. (Bellefonte, PA). The mobile phase (pH=9) was acetonitrile:water 80:20 (v/v) with a flow rate of 1 ml/min. The coumaphos was monitored at 316 nm (bandwidth 8 nm) and fluvalinate at 254 nm (bandwidth 4 nm), based on their UV_{max} absorbance. Using this system, our detection limit was <0.180 μg/cm^2 of pesticide residue on the glass (10 μg pesticide/ml acetonitrile), for both pesticides.

Regression analysis was used to determine the rate which the pesticides declined under each set of ozone treatment conditions, and to compare these declines among treatments.

2.2 Degradation of Pesticide Residues on Bees Wax (Honeycomb).

2.2.1 Experiment 1

The ability of gaseous ozone to degrade coumaphos and *tau*-fluvalinate on honeycomb (primarily composed of bees wax) was evaluated. Honeycombs were exposed to pesticides using three different methods:

Method 1 Unformulated pesticide applications. A mixture of technical grade coumophos and tau-fluvalinate were evenly sprayed onto frames of honeycomb obtained from a honey bee hive kept by one of the authors (RRJ). The spray applications were made using 10 ml of pesticide solution (10 ppm of each pesticide) per side of each shallow frame. A 200-ml capacity refillable aerosol sprayer was fitted with a test-tube such that the entire 10 ml of solution could be applied, and this was distributed by hand as evenly across the comb as possible.

Method 2 Formulated pesticide applications. tau-Fluvalinate (Mavrik Aquaflow, Wellmark International, Schaumburg, IL) was applied to other combs from the same source, and the pesticide was applied as in Method 1.

Method 3 Pesticide residues found in bee hives. No pesticides were applied, but old comb was obtained from a commercial beekeeper. This comb was dark, indicating that it had been used for several years and had at times been used by the bees for brood rearing. This comb provided us with contamination levels found in a typical beekeeping operation.

For each of the three pesticide methods, three combs were fumigated together by placing the frames in a hive body (wooden boxes designed to hold frames of comb in the Langstroth-style movable-

frame beekeeping system, the predominate system used by beekeepers in the U.S. and Canada). The hive body was then placed in an ozone fumigation chamber for 3 days with 2.14 mg O_3/l (1000 ppm-v). The fumigation chamber was 3.3 × 5 × 3.3 m, and the ozone generator was a water cooled corona discharge system that produced 120 g O_3/h (O_3 Company, Aberdeen, ID). Ozone was supplied continuously to maintain the desired concentration. These treatments were repeated twice more for a total of three replicate fumigation runs and a total of nine frames for each pesticide and each method.

Pesticide residues were sampled from each comb before and after fumigation. For treatments 1 and 2, an 8×8 cm piece of comb was removed after the comb was sprayed and allowed to dry to determine pre-treatment pesticide levels. Similar pre-treatment samples were taken from the old comb in Method 3. After the combs were fumigated, another 8x8 cm piece was sampled from each comb. The pre- and post-treatment samples were sent to the U.S. Department of Agriculture, Agricultural Marketing Service for chemical analysis to determine the coumaphos, coumaphos oxon (an oxidation product of coumaphos), and *tau*-fluvalinate residue levels (see *Chemical Analysis of Pesticides in Wax Samples* below for more details). Residue levels before and after treatments were compared using analysis of variance (ANOVA), with each frame of comb as a subsample and each fumigation ran a replicate block (e.g., a nested random block experimental design).

2.2.2 Experiment 2

Another test was conducted in Florida using honeycomb collected from local commercial beekeepers. As with Method 3 above, no pesticides were applied to these combs. Fumigation treatments were 10.7 mg O_3/l (5000 ppm-v) for 96 h. This experiment was conducted in a metal chamber (2.7 × 3.3 × 3.3 m) using an air cooled corona discharge generator producing 60 g O_3/h (Ozone Solutions TG-40, Hull, IA, supplied with 19 LPM O_2). Three separate fumigations were conducted. For fumigation, six frames of comb were placed in the chamber: three "new combs" (combs that had been used in honey bee colonies for three years or less) and three "old combs" (combs that had been used for at least ten years). Analysis for a wide array of pesticides (see 2.2.3 *Chemical Analysis of Pesticides in Wax Samples* for more details) was conducted on these combs before and after ozone treatment. Pesticide residue levels before and after treatment were compared using ANOVA, but only for those pesticides present in all the combs. A 2×2 ANOVA was used to compare pesticide residues before and after treatments, with the main effects being ozone treatment (with and without ozone) and comb age (new and old). Also, ANOVA was used on the new and old combs separately to test the effect of ozone treatment on pesticide residue concentrations independent of the comb-type. As before, a nested design was used, with fumigation run as the random effect (replicate block), and the frames of comb as subsamples (samples nested within the replicate blocks).

2.2.3 Chemical Analysis of Pesticides in Wax Samples.

Chemical analyses of all wax samples were performed by the USDA-Agricultural Marketing Service (Roger Simons, Gastonia, NC). Pesticide residue extraction was conducted using a modification of the QuEChERS method (Anastassiades et al., 2003; Schenck and Hobbs, 2004). Chemical analysis was performed using liquid chromatography coupled with tandem mass spectrometry detection (LC/MS/MS - Agilent 1100 LC equipped with a Thermo Quantum Discovery Max Triple Quadruple Mass Spectrometer or equivalent), gas chromatography coupled with mass selective detection in electron impact mode (GC/MS-EI - Agilent 6890 GC equipped with a Agilent 5975 Mass Selective Detector in EI mode or equivalent), and gas chromatography coupled with mass selective detection in negative chemical ionization mode (GC/MS-NCI - Agilent 6890 GC equipped with a Agilent 5975 Mass Selective Detector in NCI mode or equivalent). Pesticide residues in samples were quantified using matrix matched calibration standards containing

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multiple pesticides of known concentrations prepared from neat standard reference material. Identification of pesticide residues in the samples was performed by mass spectral comparison of ion ratios with standards of known identity. For the pesticide analysis conducted in the Florida experiment, samples were screened for the presence of 176 pesticides and metabolites, but only those found in the samples are reported. The lowest detection limits varied for each pesticide, but ranged from 1-50 ppb. The lower detection limit for coumophos, coumophos oxon, and tau-fluvalinate was 1 ppb. See Mullen et al. (2010) for more details of the wax sampling, extraction and analytical methods.

2.3 Analysis of Volatile Chemicals Associated with Ozone-treated Comb

James (2011) previously published methods for fumigating comb with ozone to control hive pests, and some beekeepers have asked us about the safety of this comb because its smells from volatiles created by the fumigation. This smell does not appear to inhibit the acceptance of the comb by bees (James 2011), but it is possible that pesticide residues are not completely broken down by the ozone, resulting in hazardous by-produces, so we evaluated these volatiles. Three frames of each of three comb types were collected from independent apiaries: brood comb, wax moth infested comb, and small hive beetle infested comb. Volatile profiles were determined for each frame type before and after ozone fumigation.

Brood frames were fully drawn comb that were empty (no brood, honey or pollen) but had been used for brood rearing at some point. Wax moth frames were obtained from hives heavily infested with this pest, where the bees had previously absconded. These frames had large wax moth larvae still present, and much of the wax had already been consumed, but the moths had not yet formed pupal cases. To create the small hive beetle infested comb, 100 small hive beetle adults and 300 larvae were placed in a sealed box with four deep frames containing pollen and honey for 48 hours before being treated with ozone and sampled. All the combs were exposed to 4.28 mg O_3/m^3 (2000 ppm-v) of ozone for 36 hours under ambient conditions of 23ºC (SD=3ºC) and 41% RH (SD=14% RH).

Volatiles were collected from the comb before and after the fumigation using a system that enclosed the face of the comb. An aluminum frame constructed of 1.2 cm aluminum angle-iron that was pressed into the wax with a glass cover clamped onto the frame. Clean air was forced over the sampled comb section using Teflon tubing (Cole-Parmer, Vernon Hills, IL) and a rate of 41.5 ml/min, regulated by flow meters (Aalborg, Orangeburg, NY, USA), and then was pulled out through a filter containing 80 mg super Q (Alltech Associates, Deerfield, IL, USA) to capture volatile chemicals in the air stream. The vacuum was also regulated by flow meters at a rate of 36.8 ml/min to balance the air in and ensure that volatiles from the frame were collected. Nonyl acetate (400 ng) in 5 µl of methylene chloride (Sigma-Aldrich, St. Louis, MO, USA) was added to the top of the packing material of each filter, and served as an internal standard. The filter was eluted with 200 µl of methylene chloride.

The volatile compounds were identified and quantified using a GC-MS (Hewlett Packard HP6890 with HP5973 MS in EI mode) and a 30 m x 0.25 mm HP1 capillary column with a 0.25 µm film of methyl silicone. The injector inlet temperature was held at 240°C with the carrier gas moving through the column at 30 cm/sec. The oven was held at an initial temperature of 35°C for 1 min followed by a 10°C/min temperature ramp to 230°C and then held for 5 min. The transfer line to the MS was kept at 240°C. All standards were obtained from Sigma-Aldrich (St. Louis, MO). Standards were run for all identified aldehydes with the exception of furfural and heptanal, which were confirmed by comparisons of their mass spectra with the National Institute of Standards and Technology (NIST) library (Stein, 2012), yielding matches of 96% and 87% respectively. For the acids, standards were run for all except pentanoic acid which was identified by its relative position to the other acids and a match of 83% to the NIST library. Both terpenes were confirmed by

running standards, as was 2-methyl 1-butanol, but 3-methyl 1-butanol was confirmed from its chromatography relative to the former and a match of 90% to the NIST library.

3. Results

3.1 Pesticide Degradation on Glass.

Both coumophos and *tau*-fluvalinate degraded faster at 35˚C than at 25˚C, and when the ozone concentration was increased (Figs. 1 and 2). However, for coumaphos, the difference between 25° and 34°C was not great (Fig. 1). For each pesticide, the slopes of the fitted lines, which represent the rate at which the pesticides declined, were very similar among the two ozone concentrations (Figs. 1 and 2, Table 1). An exposure of 10-15 h was sufficient to eliminate low concentrations of coumaphos at either ozone concentration, but a 20 hour exposure at 34°C was required to elimination of the highest pesticide residue levels. Ozone eliminated t*au*- fluvalinate more slowly than coumaphos, especially at 25°C and 2.14 mg O_3/l (Fig. 2, Table 1).

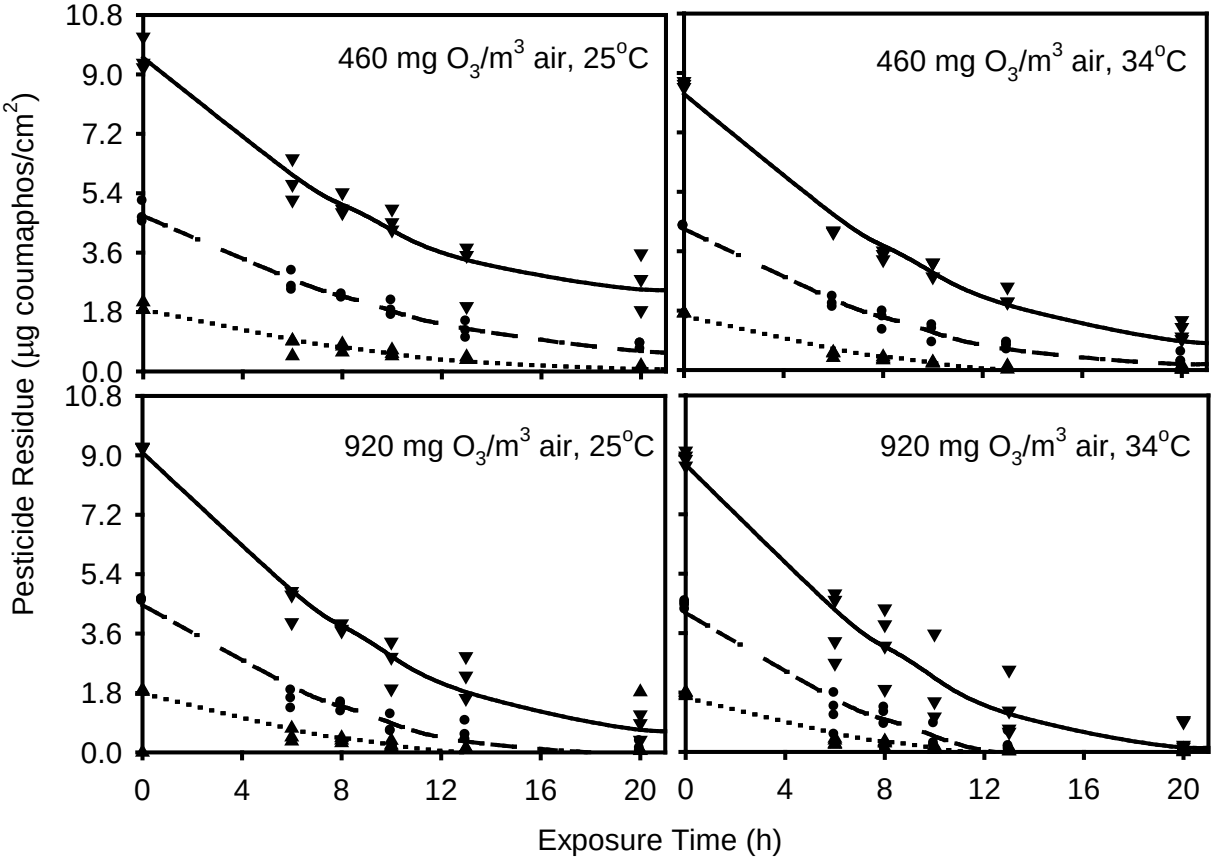

Fig. 1. Rates of coumaphos decline when exposed to gaseous ozone on a glass surface using different ozone concentrations and treatment temperatures. Different lines represent different initial pesticide residue concentrations. See Table 1 for regression statistics.

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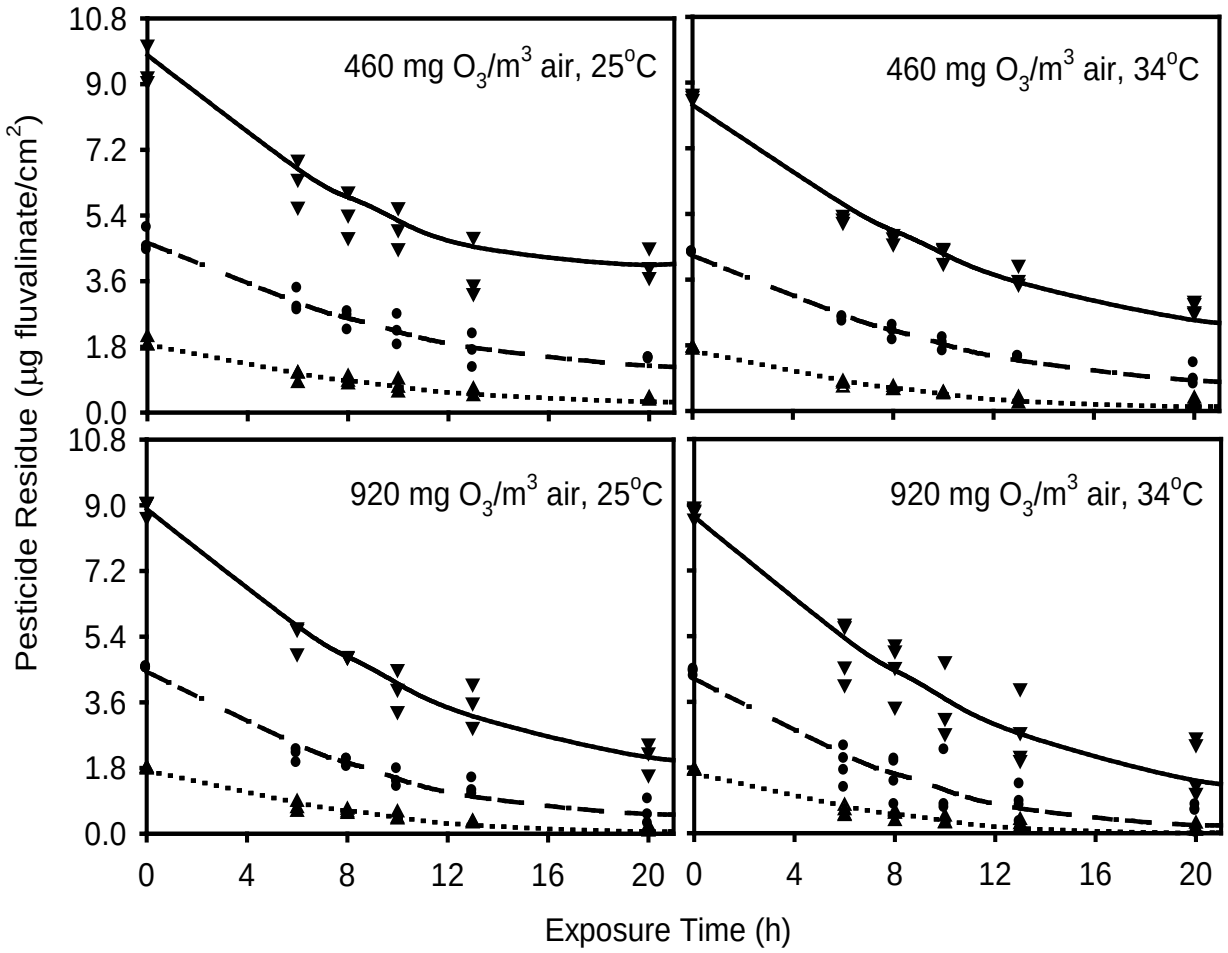

Fig. 2. Rates of *tau*-fluvalinate decline when exposed to gaseous ozone on a glass surface using different ozone concentrations and treatment temperatures. Different lines represent different initial pesticide residue concentrations. See Table 1 for regression statistics.

Table 1. Regression statistics for the rate of pesticide elimination over time after exposure to ozone fumigation under different conditions. All *p*-values were <0.0001. Pesticide residues were on a glass surface.

Pesticide	Temp. (ºC)	Initial Ozone Conc. (g O_3/l)	Initial Pesticide Conc. (ppm)	*F*	Adj. r^2	Linear coefficient	Quadratic coefficient	y-intercept
coumaphos	25	1.07	10	155	0.95	-0.99	0.02	10.4
		1.07	25	372	0.98	-2.06	0.04	26.1
		1.07	50	1644	0.95	-3.86	0.09	52.8
		2.14	10	224	0.95	-1.21	0.03	9.8
		2.14	25	307	0.97	-2.72	0.07	24.7
		2.14	50	383	0.97	-4.52	0.11	50.4
coumaphos	35	1.07	10	214	0.95	-1.11	0.03	9.1
		1.07	25	342	0.97	-2.34	0.06	23.7
		1.07	50	412	0.98	-3.94	0.09	46.5
		2.14	10	146	0.92	-1.21	0.04	9.0
		2.14	25	152	0.92	-2.92	0.08	23.4
		2.14	50	153	0.92	-4.80	0.12.	48.3
tau-fluvalinate	25	1.07	10	186	0.95	-0.85	0.02	10.3
		1.07	25	139	0.93	-1.77	0.04	25.8
		1.07	50	110	0.91	-3.45	0.09	52.4
		2.14	10	336	0.97	-0.97	0.03	9.6
		2.14	25	339	0.97	-2.17	0.05	24.6
		2.14	50	331	0.97	-3.42	0.08	49.4
tau-fluvalinate	35	1.07	10	272	0.96	-0.90	0.02	9.12
		1.07	25	240	0.96	-1.74	0.04	23.6
		1.07	50	324	0.97	-2.89	0.06	46.6
		2.14	10	191	0.94	-0.99	0.03	9.1
		2.14	25	95	0.88	-2.27	0.06	23.5
		2.14	50	143	0.92	-3.54	0.08	48.2

3.2 Pesticide Degradation in Beeswax.

3.2.1 Experiment.

Ozone was more effective at eliminating pesticide residues when unformulated pesticides were sprayed on the surface (Method 1) than when the frames were treated with Mavrik (Method 2), and more effectively than for old frames obtained from a beekeeper (Method 3)(Fig. 3). Ozone decreased coumophos (F=17.54; $d.f.$=1,9; $P\leq$0.002) and fluvalinate (F=6.63; $d.f.$=1,9; $P\leq$0.03) levels significantly in comb that had pesticides applied to the surface (Method 1). Coumaphos oxon increased significantly when ozone was applied (F=21.72; $d.f.$=1,9; $P\leq$0.001; Method 1). Ozone treatments did not significantly affect fluvalinate levels with the formulated product Mavrik (Method 2) (F=3.90; $d.f.$=1,9; $P\leq$0.08). Coumaphos occurred as a contaminant in this comb, as

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indicated by its presence where it had not been applied (i.e. the Mavrik treatments). Residues of both pesticides were present in the beekeeper's old comb (Method 3), and fluvalinate levels were high (Fig. 3). However, in the old comb, these pesticides did not degrade significantly when exposed to ozone ($P \geq 0.1$) (Fig. 3).

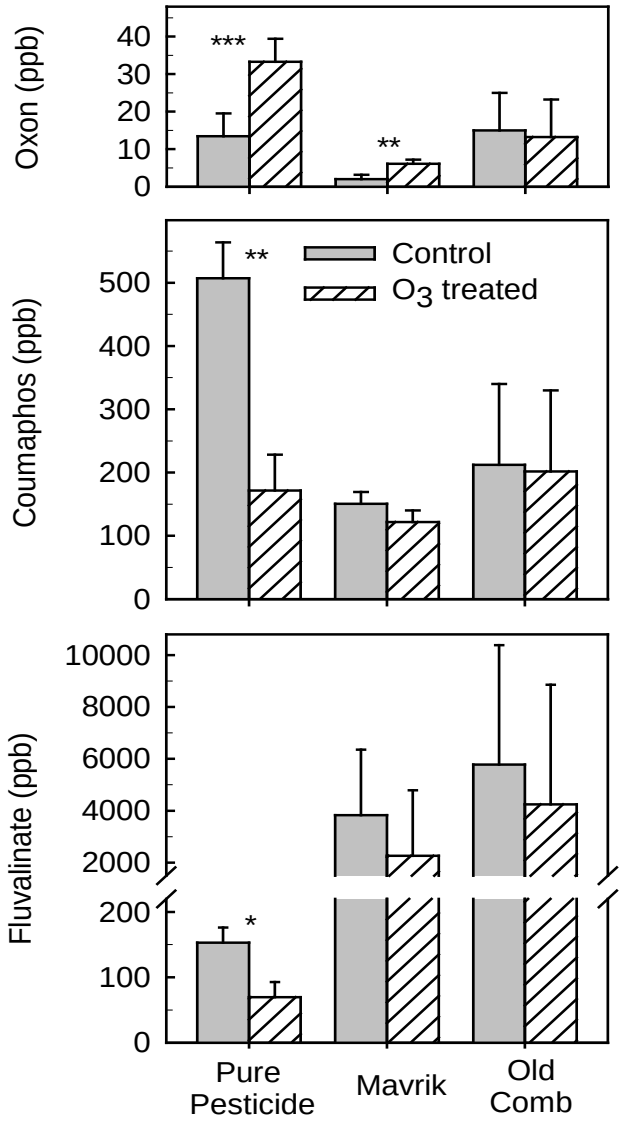

Fig. 3. Pesticide residues in honey comb before and after being fumigated with ozone (2.14 mg O_3/l for 72 h) for three different comb treatments: Pure Pesticide, comb that was sprayed with technical grade pesticide. Mavrik, comb sprayed with formulated *tau*-fluvalinate. Old Comb, comb from a commercial honey bee colony (age was >3 years). Gray bars are mean pesticide residues before ozone fumigation, bars with diagonal stripes are after fumigation. Vertical lines represent standard errors. Asterisks indicate when pre- and post-treatment levels are significantly different (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.2.2 Experiment 2

When a pesticide analysis was conducted on the Florida beekeepers' honeycomb, ten different pesticides were found. Chlorferone was found in six frames of the old comb (Fig. 4). Esfenvalerate was only found in two frames of the old comb (16.7 and 2.6 ppb), and not in the new comb. This pesticide was completely eliminated by ozone treatment in these two samples. Thymol was only found in one old comb (96 ppb), and it was completely eliminated by the ozone treatment. Chlorothalonil was only found in two frames of the new comb (19.4 and 19.6 ppb), and not in any of the old comb. It also was completely eliminated by the ozone treatment. Other pesticides were more common and more stable (Fig. 4). 2,4-Dimethyl formamide (DMPF), chlorpyrifos, coumaphos, and fenpyroximate were significantly degraded by ozone in the new comb, but only chlorpyrifos was degraded in the old comb (Fig. 4). Coumaphos oxon significantly increased in the ozone-treated old comb, but was always found at very low concentrations (Fig. 4). Fluvalinate occurred at much higher concentrations than any other pesticide. Ozone always decreased fluvalinate concentrations, but not significantly, and appears to be more affected in newer combs (for new comb F=3.7; $d.f.$=1,14; P=0.076; for old comb F=0.16; $d.f.$=1, 14; P=0.70).

Wax age had a significant effect on pesticide levels in comb for carbendazim (d.f.=1, 31; F=5.6; P=0.024), chlorferone (d.f. 1, 31; F=13.4; P=0.0009), coumaphos (d.f.=1, 31; F=49.8; $P \leq 0.0001$), coumaphos oxon (d.f.=1, 31; F=9.2; P=0.005), and fluvalinate (d.f.=1, 31; F=9.2; P=0.005)(Fig. 4).

3.3 Volatile Chemicals Associated with Ozone-treated Comb

The volatile profile of ozone-treated comb was dominated by aldehydes and carboxylic acids (Table 2). The former were emitted at an average of 2000 ng/h while the latter were emitted an order of magnitude lower at 200 ng/h. The other major component was another aldehyde, benzaldehyde, which was emitted at about 4000 ng/hr. The overwhelming odor of the treated combs is an aldehyde smell, very similar to nonanal. The odor differences between brood comb and the pest-infested combs were evident in the first volatile collection but after ozone treatment, the oxidation products from wax dominated the volatile profile. The primary peaks from pre-treatment brood comb were 3-carene, α-pinene, nonanal and decanal. Small hive beetle-infested comb was characterized by butanoic acid, benzaldehyde and butanol while wax moth-infested comb was characterized by short chain acids, mainly 2-methyl propanoic, and 3-methyl butyric acid, along with some additional 2-methyl butyric acid, butyric acid, and the aldehyde furfural, which probably provided much of the smell associated with treated combs.

4. Discussion

The degradation of coumaphos and *tau*-fluvalinate on the glass surface demonstrates that ozone does degrade these two compounds. These miticides have previously been reported as prevalent contaminants in honey bee hives (Tsigouri et al., 2000; Korta et al., 2001; Bogdanov, 2006; Chauzat et al., 2006; Johnson et al., 2010; Mullin et al., 2010). Other pesticides have been shown to be degraded by ozone as well (Rice and Gomez-Taylor, 1986; Ikohata and El-Din, 2005a, 2005b; Jasim et al., 2006; Ikeura et al., 2011; Walse and Karaca, 2011), so our results are not surprising. Pesticides applied to the surface of honeycomb were removed more effectively by ozone than when the same compounds occurred in old combs taken from honey bee hives, but less effectively than on the glass substrate. However, a direct comparison to glass needs to be made carefully because the combs where the pesticides were surface applied also had some initial contamination levels that likely were not on the surface, and pesticides embedded in combs from honey bee hives degraded poorly. Unfortunately, nearly all comb in the U.S. contains residues of coumaphos and tau-fluvalinate (Mullen et. al., 2010), so a pesticide-free control was not available. In any case, the beeswax substrate appears to have inhibited pesticide breakdown. In addition, product formulation

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can influenced pesticide degradation by ozone (Walse and Karaca, 2011). When tau-fluvalinate was applied to our combs in a commercially formulated product (Mavrik), degradation was very poor. Veseley et al. (1994) also had difficulty accelerating the degradation rate of tau-fluvalinate when they treated melted wax with ozone.

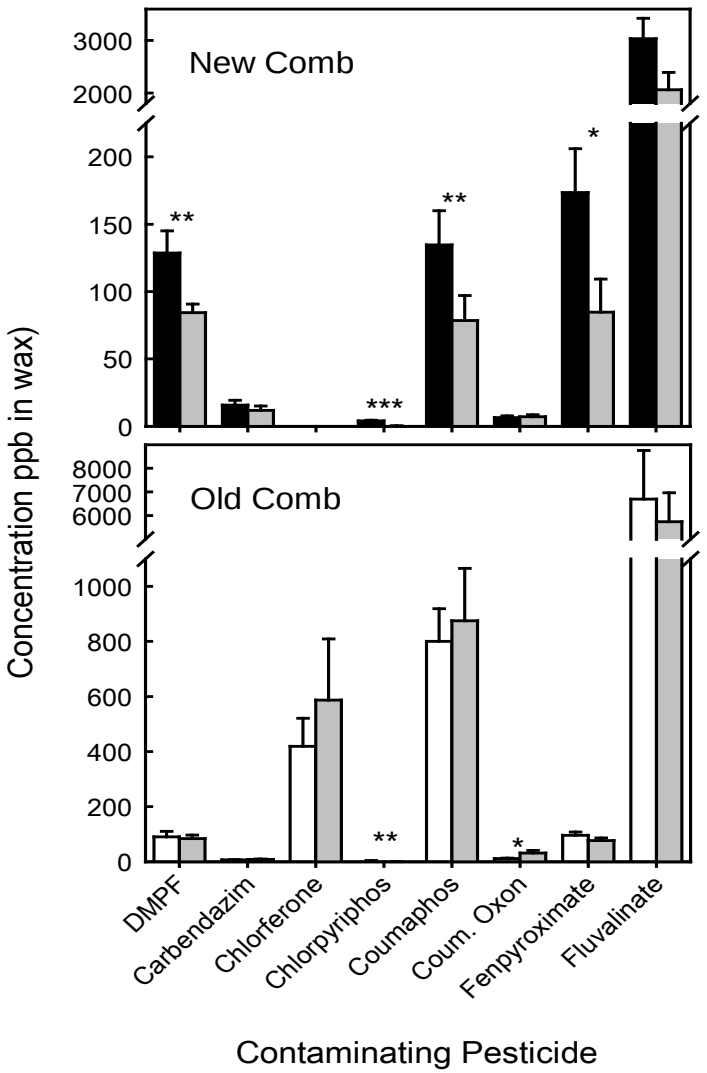

Fig. 4. Pesticide residues in honey comb before and after treatment with gaseous ozone (10.7 g O_3/l for 96 h). Black bars are mean pesticide residues before treatment, gray bars are after treatment. Vertical lines represent standard errors. Comb was collected from commercial beekeepers in central Florida. New comb had been used in beehives for $\leq$ 3 yr, and old comb had been used for >3 yr. Asterisks indicate when pre- and post-treatment levels are significantly different (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Table 2. Profiles of volatile compounds from different kinds of comb before and after ozone treatment. Values are mean (±SE) release rates (ng/h) calculated from GC-MS quantifications with a nonyl acetate internal standard. Missing values denote none detected.

	Before Ozone Treatment			After Ozone Treatment		
Chemical Name	**Brood**	**Small Hive Beetle**	**Wax Moth**	**Brood**	**Small Hive Beetle**	**Wax Moth**
Acids						
Propanoic acid				156±35		
2-Methyl propanoic acid			1082±245			
Butanoic acid		219±59			318±207	434±219
3-Methyl butyric acid		162±9	185±68			
Pentanoic acid				324±213		213±99
Hexanoic acid		127±9		934±759	810±558	1802±261
Heptanoic acid				798±234	665±356	960±165
Octanoic acid				1397±190	717±365	1728±73
Nonanoic acid				2905±833	1200±627	4527±483
Decanoic acid				1008±390	403±185	1432±363
Terpenes						
3-Carene	383±360	507±258	49±9			
α Pinene	81±48	104±56				
Alcohols						
2-Methyl butanol		390±30				
Aldehydes						
Hexanal				2615±475	1927±580	5243±271
Heptanal				1395±440	678±254	2962±335
Octanal		294±139	127±45	4478±977	2480±754	6321±573
Nonanal		381±176	414±100	8486±949	4996±1517	10040±900
Decanal		191±68	158±15	6554±734	4681±2275	6207±1287
Undecanal				372±54	771±307	423±151
Dodecanal				637±81	735±292	518±142
Benzaldehyde		429±158	74±18	6232±1824	3392±646	10473±2700
Furfural			143±24			

In our experiments, ozone was not very effective at eliminating pesticides in old comb, but occurred more readily with new comb. Bees can built a frame of comb in one season, however, when the frame is reused over many years, the bees add wax, creating additional thin layers each year, possibly imbedding pesticide residues from previous years, as well as organic debris from the hive, including frass, old cocoons and caste pupal cases. Old combs generally had a greater number of pesticides present and at greater quantities than new comb (except for fenpyroximate, which was only present in the new combs). Thus, the poor oxidation rate of pesticides in old combs may be due to either the wax substrate being resistant to ozone activity (or impenetrable to ozone), or some sort of inhibitory interaction between the pesticides and the organic debris. Organic matter has previously been shown to decrease the reactivity of ozone in water treatments (Elovitz et al., 2000).

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According to Bogdanov (2006), *tau*-fluvalinate and coumaphos concentrations in beehives are usually below 100 ppb. However, the median *tau*-fluvalinate concentration in U.S. honeycomb reported by Mullin et al. (2010) was 3595 ppb, and coumophos was 1240 ppb. We found concentrations of *tau*-fluvalinate in beekeeper comb as high as 9000 ppb. Based on our results, beekeepers are not going to easily eliminate such high pesticide residues with ozone. Ozone is adsorbed and inactivated by organic substrates such as wood; therefore, it will take a very powerful ozone generator to produce concentrations of ozone significantly greater than we tested here, considering that the chamber will be full of woodenware from beehives. Very large generators may not be cost effective for beekeepers. Furthermore, the fumigation chambers would need to be of sufficiently high quality to prevent ozone leakage, a problem that increases with increasing treatment concentrations. This will also add an expense.

One approach that beekeepers could take to prevent the build-up of pesticide residues in honeycomb is to start with new comb, treat it with ozone annually, and replace their combs more often than is common in the U.S. and Canada. For example, perhaps comb should be replaced every few years rather than reusing it for decades. It is possible that if a beekeeper fumigated every year with ozone, starting with the first year comb, pesticide residues would not accumulate to such high levels.

Temperature affected the degradation rate of coumaphos and fluvalinate, however, the differences between 25° and 34°C were probably not great enough for beekeepers to be too concerned about, but warmer temperatures are more effective than cooler ones. This is an advantage of gaseous ozone as compared to water treatments, where the capacity of water to hold ozone decreases with temperature while the oxidative activity increases (Elovitz et al., 2000). With gaseous ozone, one could take advantage of the increased activity at higher temperatures.

Ozone treated comb has a distinctive aldehyde odor that slowly dissipates in the months after treatment. Beeswax is primarily made up of long chain hydrocarbons, monoesters, diesters and free acids, most of which are at least 16 carbons for the hydroxyl acids and greater than 20 carbons for the diols (Tulloch, 1971). We examined only compounds with relatively high volatility, that is, less than 12 carbons in length, and these are primarily derived from the oxidation of the beeswax itself. Preliminary experiments were conducted to see if the change in odor that occurs when the comb is treated with ozone would make it less attractive to small hive beetles or wax moths, but we found no evidence of any reduction in attraction. Small hive beetles were still attracted to pollen dough treated with ozone, and some of the treated brood frames became infested with wax moths during the three month post-treatment volatile collection period. Thus, the comb was still attractive to the moths.

Additionally, ozone-treated combs from this experiment were returned to the four commercial beekeepers who initially supplied the material. These beekeepers reported that the treated combs were well accepted by the bees. We also worked with a commercial beekeeper for Experiment 1, who found that comb contaminated with wax moths were better accepted by the bees after ozone treatment than wax moth infested comb that had not been treated. He also found the ozone treated comb to be well accepted by the bees (James 2011).

Beekeepers are in great need of some new tools to help them maintain healthy colonies. Colony collapse disorder continues to be a problem, despite continued efforts to identify its cause (vanEngelsdorp et al., 2009, 2010). Pesticides (Johnson et al., 2010) and new or unknown pathogens (Cox-Foster et al., 2007, Johnson et al., 2009) are among the suspected causes of colony collapse disorder, but good basic practices for controlling these factors are not currently available to beekeepers. Honey bee hive components are commonly reused by beekeepers despite the fact that these materials harbor harmful substances and pathogens. Ozone has previously been shown to effectively kill insect pests infesting, and pathogens harbored in, stored comb with little effect on the comb quality and acceptability to bees (James 2011). The research reported here was intended as an initial step to determine whether ozone might also work to decrease pesticide residues. We

demonstrate that it can be used to decrease pesticides, but it will not completely eliminate them, and is most effective on new comb and when contamination levels are moderate to low. The inhibitory effect of the beeswax substrate, or impenetrability of the wax, still needs to be addressed.

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