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An Ozonolysis Based Method and Applications for the Non-Lethal Modification of Insect Cuticular Hydrocarbons

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Abstract

Cuticular hydrocarbons (CHCs) are important, multi-function components of the insect epicuticle. In *Drosophila* spp., CHCs provide protection from desiccation and serve as semiochemicals for both intra- and interspecific communication. We developed a non-lethal method for the modification of *Drosophila* CHCs profiles through the exposure of live insects to a high dose of ozone gas (~45,000 ppm). *Drosophila suzukii* that were treated with ozone showed a 1.63–3.10 fold reduction in unsaturated hydrocarbons with these CHCs shown to regenerate over 108 h. Changes in CHCs were correlated with significantly reduced desiccation resistance in both male and female *D. suzukii* at one h after ozone treatment. Interestingly, individuals treated with ozone showed increased desiccation resistance in comparison to controls at 108 h after ozone treatment. The methodology reported in this paper provides a novel approach to investigate the biosynthesis and functions of CHCs during the lifespan of an insect.

Keywords Cuticular hydrocarbons · Modification · Ozone · *Drosophila suzukii* · Desiccation resistance

Introduction

The *Drosophila* spp. epicuticle contains a wide range of saturated, unsaturated, and branched hydrocarbons broadly defined as cuticular hydrocarbons (CHCs) (Bartelt et al. 1986; Jallon and David 1987; Howard et al. 2003; Howard and Blomquist 2005). *Drosophila* CHCs form a waxy layer on the cuticle that reduces desiccation as well as provides inter- and intra-specific chemical signaling (Chung and Carroll 2015). It has been hypothesized that insect cuticular desiccation resistance decreases as epicuticular lipids change from a solid to liquid state (Ramsay 1935; Hadley 1994; Gibbs 1998). Unsaturated and methyl branched alkanes melt at lower temperatures compared to saturated hydrocarbons (Gibbs and Pomonis 1995). Therefore, saturated hydrocarbons are hypothesized to confer greater cuticular desiccation resistance than unsaturated hydrocarbons at higher temperatures (Gibbs 1998). For example, *D. pseudoobscura* dwelling in arid regions had a greater abundance of long-chained saturated hydrocarbons than laboratory maintained colonies, which correlates to a lower water loss rate (WLR) (Toolson and Kuper-Simbron 1989).

Previously, ozonolysis of CHCs has been used to identify double bond positions of unsaturated hydrocarbons on dipteran and hymenopteran cuticles following extraction using chemical solvents (Bartelt et al. 1982, 1986; Antony et al. 1985). Beroza and Bierl (1967) developed a method that uses ozonolysis to identify insect unsaturated hydrocarbons. This methodology involves extracting CHCs with a non-polar solvent and then treating the extract with ozone prior to analysis by gas chromatography/mass spectrometry (GC/MS) (Beroza and Bierl 1967). Antony et al. (1985) demonstrated that monoenes and dienes extracted from *Drosophila melanogaster* undergo cleavage via ozonolysis to varying extents. For example, the researchers noted a "major" reduction in (*Z*)-7-tricosene after ozonolysis, while (*Z*)-9-tricosene experienced a "minor" reduction (Antony et al. 1985). However, ozonolysis of CHCs on live insects has not been previously reported. If ozonolysis can disrupt CHCs on live insects, this will allow us to investigate how changes to the CHC layer can affect desiccation resistance as well as chemical signaling in many insect species that do not have established molecular methods to disrupt these

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CHCs, opening the doors to further discoveries in basic and applied research. We use *Drosophila suzukii* as a model for our experiment because *D. suzukii* or spotted-wing drosophila is a fruit fly species that has invaded many temperate fruit growing areas, causing hundreds of millions of dollars of damage annually to fruit crops in the United States alone (Bolda et al. 2010; Asplen et al. 2015). The *D. suzukii* CHC profile is largely sexually monomorphic, but small differences in compound abundance have been observed between males and females (Dekker et al. 2015; Snellings et al. 2018). *Drosophila suzukii* has a large variety of CHCs present, including monoenes, dienes, and n-alkanes with (*Z*)-7-tricosene being the most prevalent CHC (Dekker et al. 2015; Snellings et al. 2018). Our study has three objectives. First, we will determine if ozonolysis affects CHCs on living *D. suzukii.* Secondly, we will evaluate the duration of these effects on D. suzukii and whether CHCs will be regenerated after the initial ozonolysis. Thirdly, we will determine whether modifications to CHCs via ozonolysis affects *D. suzukii* desiccation resistance. We hypothesize that ozonolysis of unsaturated hydrocarbons will occur after ozone treatment, that CHC's will recover over time, and that CHC ozonolysis will reduce desiccation resistance.

Materials and Methods

We performed two experiments to address our objectives. In experiment 1, we evaluated the effects of ozone on the CHCs of *D. suzukii* and the duration of these effects. Experiment 2 explored the effect of ozone exposure on the desiccation resistance of *D. suzukii*.

Colony Details & Maintenance

Drosophila Suzukii were sourced from a colony reared out of tart cherries (*Prunus cerasus*) collected from the Trevor-Nichols Research Center located in Fennville, Michigan in 2015. Flies were maintained on 5 mL of solid diet (Dalton et al. 2011) in 50 mL polystyrene vials (Lab Express; Ann Arbor, MI, USA; Cat. # 8002-cs) that were capped with plugs (Genesee Scientific; El Cajon, CA, USA; Cat. # 49–101). The colony chamber was set on an 8-h dark period to a 16-h photoperiod while maintaining an average relative humidity of 77% and temperature of 23 °C.

Drosophila Handling

Newly emerged flies were collected, separated by sex, and placed into vials with 5 mL of solid diet (Dalton et al. 2011) where they were allowed to age (3–5 d or 9–11 d) prior to experiments. Thirty to sixty aged flies of a single sex were transferred using a small paintbrush and forceps into grade 304 stainless steel cages for ozone exposures. Steel cages were fabricated from half of a 5.33 cm diameter spherical tea infuser (Fu Store; ShenZhen, GuangDong, China;Model # 8,541,896,633) that was folded back onto itself to create a "clamshell". Stainless steel was used due to its low reactivity with ozone. All tools and surfaces were cleaned with 70% ethanol between each experimental run/treatment to minimize the chance of contamination.

Flies in experiment 1 were anesthetized with carbon dioxide before being placed into stainless steel cages for treatment and, after treatment, into new vials with diet. Flies in experiment 2 were aspirated from vials containing diet into stainless steel cages for treatment and into new vials with diet after treatment application. The difference in handling procedures between experiments 1 & experiment 2 was due to the extremely low humidity of the carbon dioxide anesthetizing gas, which could directly affect the outcome of the desiccation trial (experiment 2). After ozone exposure, flies from both experiments were maintained in the colony chamber described above.

Ozone Treatment Application

An ozone generator (Absolute Ozone; Edmonton, AB, Canada; NANO) applied 45,000 ppm of ozone by using 99.5% oxygen as a feed gas at 14 psi and a flow rate of 5.1×10^{-5} m^3/s (51 mL/min) in experiment 1 & 2. Ozone was delivered in polytetrafluoroethylene tubing (Ozone Solution; Hull, IA, USA; PTFE) and humidified by passing it through 100 mL distilled water in a 250 mL glass gas washing bottle (bubbler flask) before entering the treatment flask (Fig. 1). Flies were exposed to humidified ozone for 5 s in a specially modified, glass 250 mL Erlenmeyer flask with a dorsal 29/42 ASTM ground glass joint and a lateral 34/45 ASTM ground glass joint which acted as a port to allow quick insertion or removal of stainless-steel cages (described above). Control flies were treated with humidified oxygen (99.5% purity) for 5 s and handled in the same fashion as the ozone treated flies, while untreated flies were not exposed to ozone nor oxygen. Humidified gaseous ozone was added to the treatment flask before treatment application to allow concentrations to equilibrate. Ozone concentrations were measured throughout applications using an ozone monitor (2B Technologies, Model 106-H) sampling gas exiting the treatment flask. Relative humidity and temperature of oxygen treatments were monitored using an electronic hygrometer sensor (Sensirion; Stäfa, Switzerland; EK-H4) in a 250 mL flask attached to the end of the treatment apparatus.

CHC Collection and Analysis

Five flies of the same treatment and sex were placed into a ½ dram glass vial (Kimble Chase, Vineland, NJ, USA;

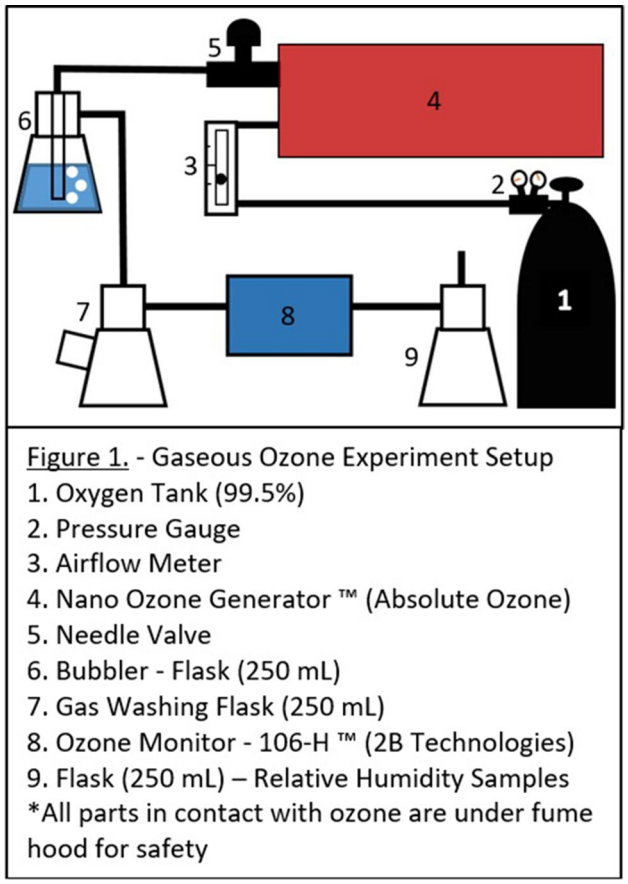

Fig. 1 Ozone generation, concentration/calibration, and treatment apparatus set-up

Art. No. 60910L 12) along with 200 µL of a hexane wash. The hexane wash contained an internal standard of 25 ng/µL hexacosane (Sigma-Aldrich; St.Louis, MO, USA; #241,687-5G). The hexane wash was added to the glass vials with 100 µL calibrated glass pipets (VWR International; Radnor, PA, USA; Cat. No. 53432–921) and an aspirator (VWR International; Radnor, PA, USA; Cat. No. 53432–921). Flies were left in hexane for 10–15 min. Samples were then placed on a mini-vortex (Fisher Scientific; Waltham, MA, USA; Cat. No. 12–810-1) for 30 s at a vortex rate of 5 (~ 1,800 rpm). The hexane solution was transferred into a 0.25 mL glass insert (Supelco; Bellefonte, PA, USA; Cat. No. 24717) inside a 2 mL glass vial (Supelco Cat. No. 27330) for GC/MS analysis. The 2 mL glass vials were capped with 9 mm Blue S/T Caps (Supelco; Bellefonte, PA, USA; 29,044-U). Samples were stored in a -20 °C freezer (Fisher Scientific; Waltham, MA, USA; Cat. No. 13986149) prior to and after GC/MS analysis.

Samples were eluted through a DB-17HT column (Agilent Technologies; Santa Clara, CA, USA; 122–1831) that had a length of 30 m, a diameter of 0.25 mm, and a 0.15 µm thickness. Helium was used as a carrier gas at a rate of 1 mL/min through a GC (Agilent Technologies; Santa Clara, CA, USA; 7890A) / MS (Agilent Technologies Inc., Santa Clara, CA, USA, 5975C). Samples injected into the GC/MS were eluted after a four-minute solvent delay and a starting oven temperature of 50 ° C that increased by 4 ° C/min until a final temperature of 300 ° C was attained. The temperature remained constant for 10 min once 300 ° C was reached. Electron ionization (70 eV) mass spectra were collected in total ion mode.

Integration and quantitation of peak areas were determined by using the QuanLynx program of the MassLynx MS Software version 4.2 to evaluate total ion chromatograms (Waters 2020). After determining peak areas, the total area of each peak per sample was divided by the total number of flies (5) from each sample to give a mean estimate of cuticular hydrocarbon mass per fly. The mass of each compound was calculated by referencing the peak area of hexacosane (5,000 ng), the internal standard (IS).

Experiment 1: Ozonolysis of Hydrocarbons

Comparison of Unsaturated Hydrocarbons, Aldehydes and Saturated Hydrocarbons 1 h After Ozonolysis

The CHC profile of 3–5 and 9–11 d old male and female flies were compared (4 groups total). Treatments consisted of an untreated control, oxygen treatment (99.5% purity), and an ozone treatment (45,000 ppm) (Table 1). The total amount, in nanograms (ng), of unsaturated hydrocarbons ((*Z*)-5-tricosene, (*Z*)-7-tricosene, (*Z*)-9-tricosene/tricosane, (*Z*)-5-pentacosene, (*Z*)-7-pentacosene, (*Z*)-9-pentacosene/pentacosane), aldehydes (heptanal, nonanal, tetradecanal, pentadecanal, hexadecanal, octadecanal) and saturated hydrocarbons (heneicosane, heptacosane, 2-methyl octacosane, nonacosane) peaks were quantified from CHC extracts from living flies 1 h after treatment application.

The masses of unsaturated hydrocarbons, aldehydes, and saturated hydrocarbons were collected from five cuticular hydrocarbon extraction samples (5 flies/sample) for each treatment within a group. As the data were not normally distributed, a Kruskal–Wallis rank sum test was used for all three responses, with a separate analysis run for each sex and age for each of the three responses (12 models total). Kruskal–Wallis rank sum tests were performed by using the 'kruskal. test' function in R version 3.5.1 (R Core Team 2015). Wilcoxon rank sum tests were performed for post-hoc analyses by using the 'pairwise.wilcox.test' function in R version 3.5.1, which adjusted *p*-values by using the 'Holm' method (R Core Team 2015).

Table 1 Experiment 1 and 2 data collection times, treatments, fly age and replication. An '*' in the 'Replication (Male, Female)' column indicates the same number of replications at every data collection time in the 'Data Collection Hour after Treatment Application' column and a ';' indicates a separation of replication numbers consistent with the data collection times in the 'Data Collection Hour after Treatment Application' column

EXPERIMENT SET-UP				
Experiment	CHC Extraction Hour after Treatment Application	Treatment	Fly Age (days) at Treatment application	Replication (Male, Female)
Experiment 1	1	Oxygen	3–5	25, 25
	1, 12, 36, 108	Untreated	3–5	*25, *25
	1, 12, 36, 108	Ozone	3–5	*25, *25
	1	Oxygen	9–11	25, 25
	1, 12, 36	Untreated	9–11	*25, *25
	1, 12, 36	Ozone	9–11	*25, *25
Experiment 2	1	Oxygen	3–5	140, 141
	1; 108	Untreated	3–5	138, 140; 100, 99
	1; 108	Ozone	3–5	141, 140; 101, 99

Experiment 1: Hydrocarbon Regeneration

Comparison of Unsaturated Hydrocarbons, Aldehydes and Saturated Hydrocarbons 1, 12, 36, and 108 H After Ozonolysis

We compared the CHC profiles of ozone treated and untreated 3–5 d and 9–11 d old male and female *D. suzukii* at 1, 12, 36, and 108 h after ozone exposure in 12 separate models (Table 1). A 2×4 factorial ANOVA model was used to analyze unsaturated and saturated CHC concentrations based on experimental treatment (ozone and untreated) and CHC extraction time after treatment (1, 12, 36, 108 h) as fixed factors. Aldehyde masses were fit to a linearized model, 'lm' function in R version 3.5.1 prior to ANOVA analysis and, subsequently, a post-hoc Tukey test (R Core Team 2105). ANOVAs and Tukey tests were performed by using the 'aov' and 'TukeyHSD' functions, respectively, in R version 3.5.1 (R Core Team 2015).

Experiment 2: Desiccation Resistance Assessment

Two desiccation resistance trials were performed on male and female *D. suzukii* (3–5 d). In the first trial, flies were evaluated 1 h after ozonolysis and in the second, 108 h after ozonolysis. Methods were modified from Folk et al. (2001). Experimental arenas consisted of 50 mL polystyrene vials (Lab Express; Ann Arbor, MI; Cat. # 8002-cs) containing 4.5 g of desiccant (W. A. Hammond Drierite Co. Ltd., Stock No: 11001) at their base with a permeable plastic barrier placed above the desiccant. Nine to 11 single sex adult flies were placed into each arena and vials were capped with plastic wrap (Gordon Food Services, Item: 115,193). Relative humidity and temperature were measured inside an experimental vial without flies during each trial using an electronic hygrometer sensor (Sensirion, EK-H4). Fly survival was assessed at 30 min intervals for 10 h or until all flies had died. Fly mortality was determined by lightly shaking a vial and recording the number of individuals that reoriented to a standing position; those who did not re-orient were recorded as dead. No censored data were included in Kaplan–Meier analyses because trials were completed when all flies had died.

Data for both desiccation trials were also analyzed by Mantel–Haenszel log-rank tests and Cox proportional hazard models with separate analyses performed for male and female flies using the R 'survival' package (Therneau 2021). Trial one compared the survival of male or female *D. suzukii* 1 h after ozonolysis with flies treated with oxygen or an untreated control. Optimized Cox Proportional Hazard comparisons were made by first evaluating differences between the oxygen and untreated controls; if they were found to be similar, the combined oxygen and untreated controls were compared to the ozone treated flies (Crawley 2007; Rich et al. 2010). Trial two compared the survival of male or female *D. suzukii* 108 h after ozonolysis with an untreated control of the same age, so a pairwise comparison of Cox Proportional Hazards was made. Hazard ratios (HR) were determined from Cox Proportional Hazard models. The hazard ratio, or instantaneous rate of death, represents the rate of death in comparison to control survival at any point of time.

Results

Ozonolysis Affects the Composition of CHCS on Live D. Suzukii

Chromatograms of *D. suzukii* CHCs for untreated controls and ozonated flies show differences 1 h following ozonolysis but not at 108 h after ozonolysis (Figs. 2 and 3). In the former, the total amount of (*Z*)-7-tricosene and other unsaturated hydrocarbons were reduced by approximately half and new

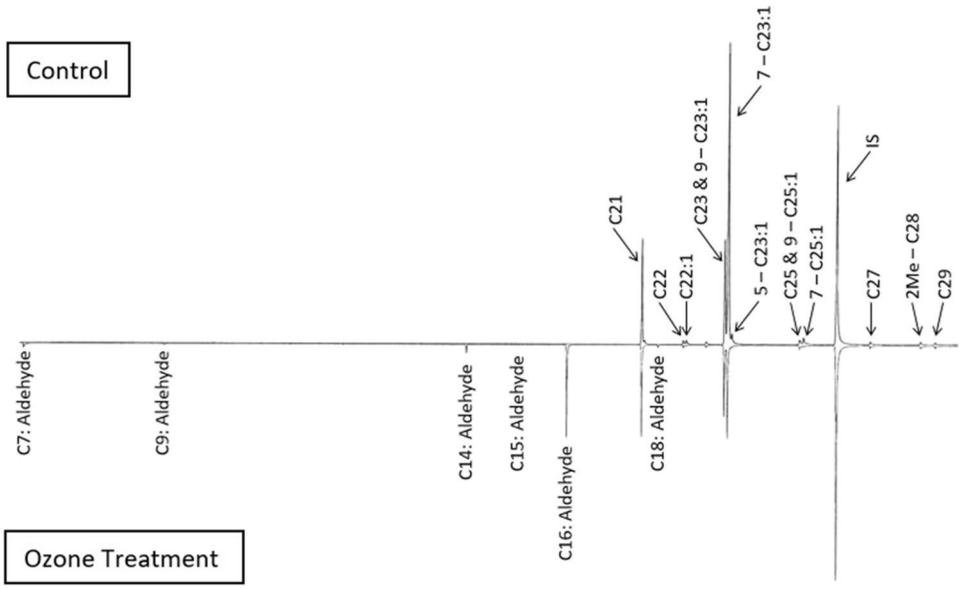

Fig. 2 Comparison of a gas chromatogram of a control fly (top) versus an ozone fly (bottom) cuticular hydrocarbon profile at 1 h after ozonolysis. Hexacosane (25 ng/μL) was used as an internal standard (IS). Peaks labelled with corresponding compound name

aldehyde peaks (heptanal, nonanal, tetradecanal, pentadecanal, hexadecanal, octadecanal) were detected in the ozone treatment (Fig. 2). In the latter, peak heights for (*Z*)-7-tricosene and unsaturated CHCs were similar to those observed for the untreated flies, and aldehydes were not observed for either group (Fig. 3). The mass chromatograms of the aldehydes are presented in the supplementary data file (Fig. S1 – S6).

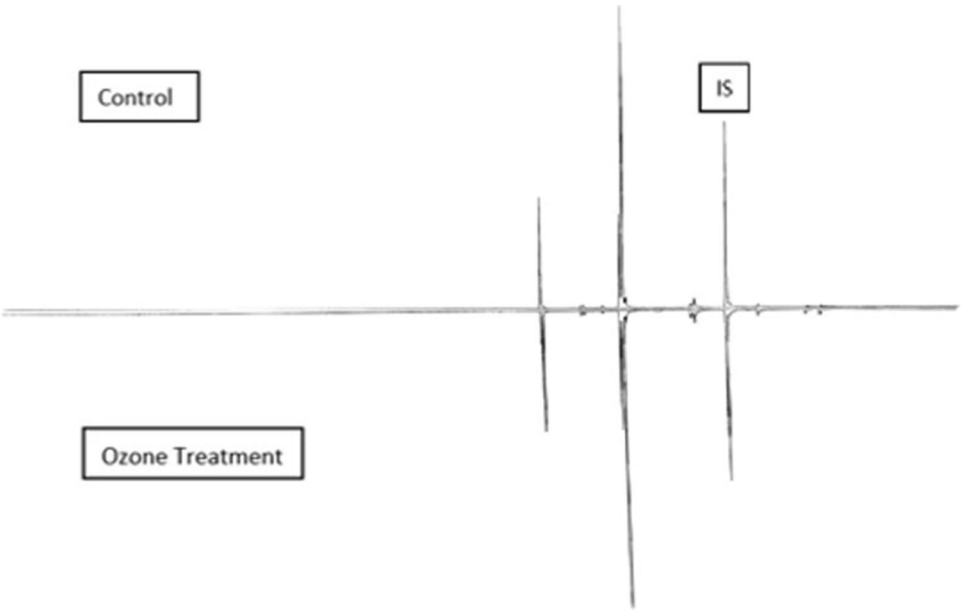

Fig. 3 Comparison of a gas chromatogram of a control fly (top) versus an ozone fly (bottom) cuticular hydrocarbon profile at 108 h after ozonolysis. Hexacosane (25 ng/μL) was used as an internal standard (IS). Refer to the peak labels in Fig. 2 for peak identities

Table 2 Mean (ng) and standard error of mean (SEM) of unsaturated hydrocarbons, aldehydes and saturated hydrocarbons extracted from female and male flies at 1 h after treatment application (3–5 & 9–11 d old). Disparate letters signify differences within a population-based Wilcoxon rank sum test with Holm p-adjustment

CUTICULAR COMPOUND MASS (NG) OF *D. SUZUKII* AT 1 H AFTER TREATMENT						
Unsaturated hydrocarbons						
Females						
	3–5 d			**9–11 d**		
Treatment	**Mean**	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	721	52	b	1017	85	b
Oxygen	1462	101	a	2832	78	a
Untreated	1366	45	a	2664	108	a
Males						
	3–5 d			**9–11 d**		
	Mean	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	586	5	b	894	110	c
Oxygen	1674	32	a	1453	132	b
Untreated	1817	76	a	2036	64	a
Aldehydes						
Females						
	3–5 d			**9–11 d**		
Treatment	**Mean**	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	574	18	a	715	28	a
Oxygen	0	0	b	0	0	b
Untreated	0	0	b	0	0	b
Males						
	3–5 d			**9–11 d**		
	Mean	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	382	11	a	443	32	a
Oxygen	0	0	b	0	0	b
Untreated	0	0	b	0	0	b
Saturated hydrocarbons						
Females						
	3–5 d			**9–11 d**		
Treatment	**Mean**	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	495	12	a	716	45	a
Oxygen	333	24	b	668	35	a
Untreated	319	9	b	648	32	a

Table 2 (continued)

CUTICULAR COMPOUND MASS (NG) OF *D. SUZUKII* AT 1 H AFTER TREATMENT

Males						
	3–5 d			**9–11 d**		
	Mean	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	344	7	a	503	37	a
Oxygen	350	10	a	378	38	a
Untreated	397	18	b	485	20	a

Experiment 1: Ozonolysis Decreases the Total Amount of Unsaturated Hydrocarbons, Producing Aldehydes, But Shows Mixed Effects On the Total Amounts of Saturated Hydrocarbons

The masses of unsaturated hydrocarbons, aldehydes, and saturated hydrocarbons at 1 h after treatment for 3–5 d and 9–11 d flies are presented in Table 2. The amounts of unsaturated hydrocarbons of 3–5 d old females were significantly reduced by ozonolysis with 2.03- and 1.89-fold reductions compared to that for the oxygen treated and untreated flies, respectively ($\chi^2 = 9.62$, $df = 2$, $p = 0.0081$), whereas the masses were similar between the oxygen treated and untreated flies ($df = 1$, $p = 0.548$). A similar pattern was observed for 9–11 d old females with 2.79- and 2.63-fold reductions in unsaturated hydrocarbons from ozone treated flies compared to oxygen treated and untreated flies, respectively ($\chi^2 = 10.22$, $df = 2$, $p = 0.006$), and no difference was detected between oxygen and untreated flies ($df = 1$, $p = 0.222$). Likewise, 3–5 d old ozone treated males demonstrated 2.85- and 3.10-fold reductions in unsaturated hydrocarbons compared to oxygen treated and untreated 3–5 d old males, respectively ($\chi^2 = 11.18$, $df = 2$, $p = 0.0037$), and similar unsaturated hydrocarbon amount between oxygen and untreated flies ($df = 1$, $p = 0.056$). Nine-11 d old males presented a slightly different pattern, with 1.63- and 2.28-fold reductions in unsaturated hydrocarbons in ozone treated flies compared to oxygen treated and untreated flies ($\chi^2 = 11.52$, $df = 2$, $p = 0.0031$), and a 1.4 fold reduction in levels observed from the oxygen to the untreated control flies ($df = 1$, $p = 0.032$).

The masses of aldehydes extracted from 3–5 d old females, 9–11 d old females, 3–5 d old males and 9–11 d old males were significantly increased by ozonolysis compared to oxygen treated and untreated flies ($\chi^2 = 13.29$, $df = 2$, $p = 0.0013$, $\chi^2 = 13.29$, $df = 2$, $p = 0.0013$, $\chi^2 = 13.29$, $df = 2$,$p = 0.0013$, $\chi^2 = 13.29$, $df = 2$, $p = 0.0013$, respectively). No aldehydes were extracted from oxygen or untreated flies in 3–5 d old females, 9–11 d old females, 3–5 d old males, and 9–11 d old males.

The masses of saturated hydrocarbons extracted from 3–5 d old females were significantly increased after ozone treatment compared to oxygen treated and untreated flies ($\chi^2 = 9.42$, $df = 2$, $p = 0.0090$), while the masses were similar between oxygen treated and untreated flies ($df = 1$, $p = 0.841$). There was no difference in the masses of saturated hydrocarbons extracted from 9–11 d old females between ozone treated, oxygen treated, and untreated flies ($\chi^2 = 1.46$, $df = 2$, $p = 0.4819$). The masses of saturated hydrocarbons extracted from 3–5 d old males treated with ozone and oxygen were significantly reduced in comparison to that found in untreated flies ($\chi^2 = 8.96$, $df = 2$, $p = 0.0113$),

while saturated hydrocarbon amounts were similar between ozone treated and oxygen treated flies ($df = 1$, $p = 0.548$). There was no significant difference in the mass of the saturated hydrocarbons extracted from 9–11 d old male untreated flies with those from flies treated with either ozone or oxygen ($\chi^2 = 4.58$, $df = 2$, $p = 0.1013$).

Experiment 1: Hydrocarbons Regenerate within 108 Hours After Ozonolysis

The masses of unsaturated hydrocarbons, aldehydes, and saturated hydrocarbons at 1, 12, 36, and 108 h after treatment for 3–5 d and 9–11 d flies are presented in Table S1 and Figs. 5, 6 and 7. Statistical output for ANOVA models is presented in Table S2.

Ozone exposure significantly reduced the mass of unsaturated hydrocarbons for male and female flies of both ages at 1 h with masses returning to levels comparable to untreated flies within 108 h (Fig. 4). For female flies of both ages, regeneration was comparable to untreated flies within 36 h with 3–5 day old ozone treated flies producing significantly more unsaturated hydrocarbons than the untreated flies, with mean ± SEM values of 2145 ± 79 and 1489 ± 112 ng, respectively (Fig. 5a). In contrast, ozone treated male flies of either age did not produce comparable masses of unsaturated hydrocarbons to their respective controls until 108 h after treatment (Table S2a).

Ozone exposure resulted in the formation of aldehydes from both treated sexes at both ages, but virtually no aldehydes were recovered from the untreated flies of either sex

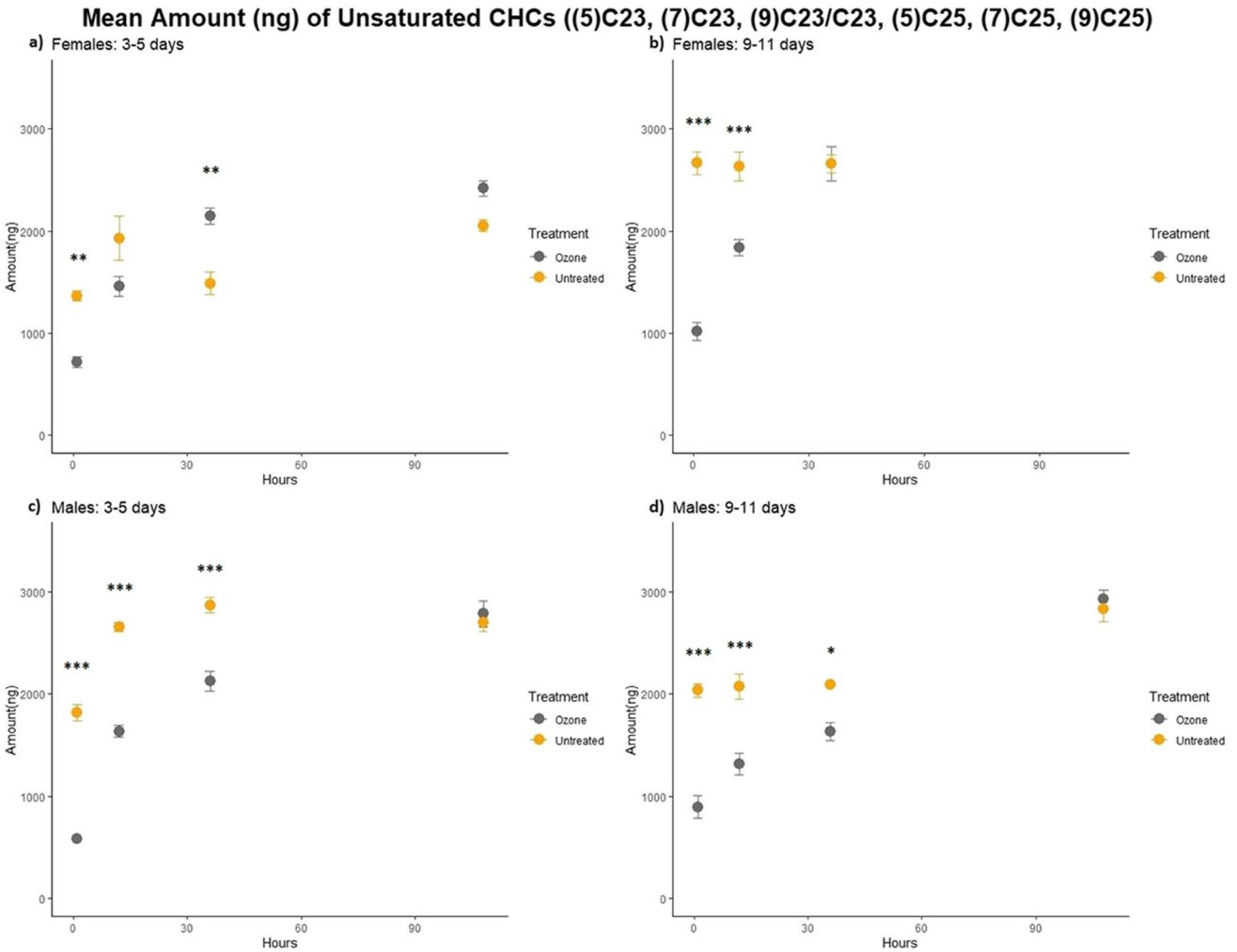

Fig. 4 The amount (ng) of unsaturated hydrocarbons (gas chromatogram peaks: (Z)-5-tricosene, (Z)-7-tricosene, (Z)-9-tricosene/tricosane, (Z)-5-pentacosene, (Z)-7-pentacosene, (Z)-9- pentacosene/pentacosane) collected from ozone treated and untreated flies over time (1, 12, 36 & 108 h). Graphs separated by sex and fly age (3–5 d, 9–11 d). A significant difference between ozone treated and untreated flies within a CHC extraction hour are marked with a '*' (*: 0.05, **: 0.01, ***: 0.001). **a** Females: 3-5 days. **b** Females: 9-11 days. **c** Males: 3-5 days. **d** Males: 9-11 days

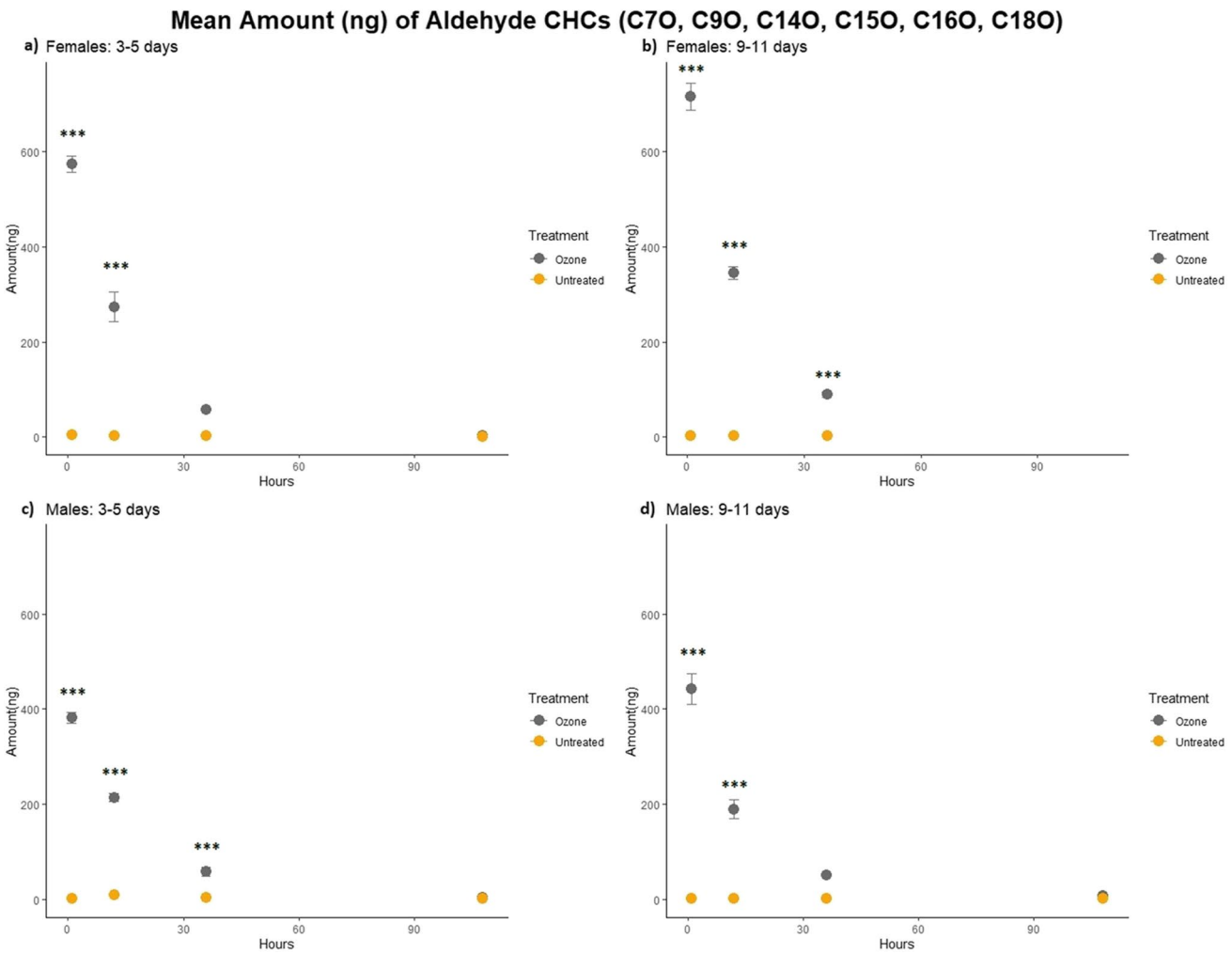

Fig. 5 The amount (ng) of aldehydes (gas chromatogram peaks: heptanal, nonanal, tetradecanal, pentadecanal, hexadecanal, octadecanal) collected from ozone treated and untreated flies over time (1, 12, 36 & 108 h). Graphs separated by sex and fly age (3–5 d, 9–11 d). A significant difference between ozone treated and untreated flies within a CHC extraction hour was marked with a '*' (*: 0.05, **: 0.01, ***: 0.001). **a** Females: 3-5 days. **b** Females: 9-11 days. **c** Males: 3-5 days. **d** Males: 9-11 days

or age at any time point (Fig. 5). Extractions of ozone treated flies contained aldehydes immediately after treatment with similar patterns of aldehyde reduction over subsequent time periods observed for both sexes and age groups (Table S2b). The aldehyde content approached 0 ng for the 3–5 d and 9–11 d old males and for the 3–5 d old females at 108 h after exposure. The data for 9–11 d old females at 108 h after treatment are not presented due to contamination issues. However, the mass of aldehydes for this group was reduced from 715 ± 28 ng at 1 h to 90 ± 6 ng at 36 h after ozone treatment.

Saturated hydrocarbon measurements provided the least consistent trends across sex and age groups compared to unsaturated hydrocarbons and aldehydes (Table S2c). Significantly higher masses of unsaturated hydrocarbons were detected for ozone treated 3–5 d old females at 1, 12, and 36 h after treatment (Fig. 6a). Likewise, higher masses of unsaturated hydrocarbons were detected for ozone treated 9–11 d old females at 1 and 12 h with a significant difference detected at 36 h (Fig. 6b). In contrast, no significant differences between ozone treated and untreated males of either age group were detected at any time point (Fig. 6c,d).

Experiment 2: Ozonolysis Decreases Desiccation Resistance in D. Suzukii

Kaplan–Meier survival curves for desiccation trials conducted 1 h and 108 h after ozonolysis are presented in Table 3 and Fig. 7. The mean (± SEM) relative humidity and temperature of desiccation trials at 1 h after treatment application were 18.13% (± 1.54%) and 28.89 °C (± 0.20), respectively. The mean (± SEM) relative

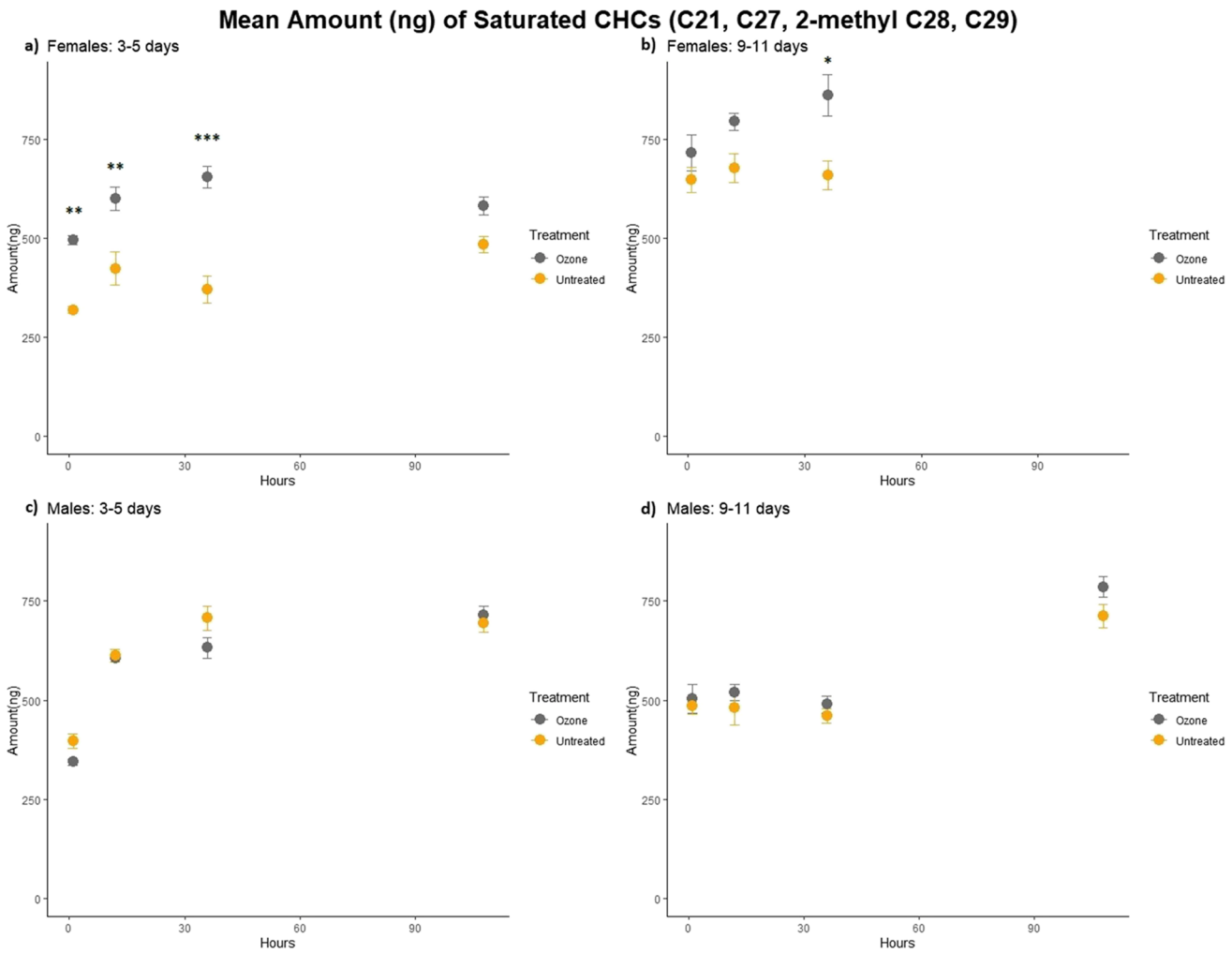

Fig. 6 The amount (ng) of saturated hydrocarbons (gas chromatogram peaks: heneicosane, heptacosane, 2-methyl octacosane, nonacosane) collected from ozone treated and untreated flies over time (1, 12, 36 & 108 h). Graphs separated by sex and fly age (3–5 d, 9–11 d). A significant difference between ozone treated and untreated flies within a CHC extraction hour was marked with a '*' (*: 0.05, **: 0.01, ***: 0.001). **a** Females: 3-5 days. **b** Females: 9-11 days. **c** Males: 3-5 days. **d** Males: 9-11 days

Table 3 Kaplan–Meier survival analyses of desiccation resistance from 3–5 d old flies. Flies (9–11) were placed into a plastic vial with a desiccant, drierite, and sealed. Flies were considered living until they failed to re-orient to a standing position after a shake of the vial. Flies that did not re-orient were marked as deceased. No censored data were included in the analyses

KAPLAN–MEIER SURVIVAL ANALYSIS					
Sex	Time (h)	Treatment	Sample Size	$\tilde{x}$ (Median h)	95% CL (Lower–Upper)
Female	1	Untreated	141	5	4.5–5.5
		Oxygen	140	4	4–5
		Ozone	140	2.5	2.5–5
		Untreated/Oxygen	281	4.5	4.5–5
	108	Untreated	99	4	3.5–4
		Ozone	99	4.5	4–5
Male	1	Untreated	140	3	3–3.5
		Oxygen	138	3	2.5–3.5
		Ozone	141	2	2–2
		Untreated/Oxygen	278	3	3–3.5
	108	Untreated	100	2	2–2.5
		Ozone	101	2.5	2.5–3

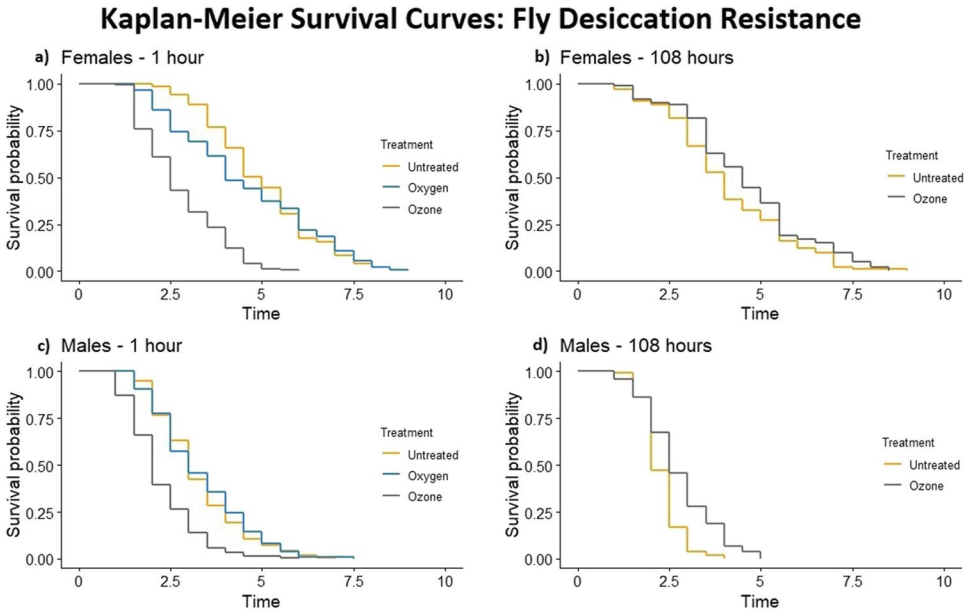

Fig. 7 Kaplan–Meier Survival Curves of 3-5 d old male and female flies at 1 h (RH = 18.13% (± 1.54%), Temp = 28.89 °C (± 0.20)) and 108 h (RH = 15.93% (± 2.12), Temp = 28.32 °C (± 0.34 °C)) after treatment application. Half-hour sampling periods until 10 h or all flies died. **a** Females -1 hour. **b** Females -108 hours. **c** Males - 1 hour. **d** Males - 108 hours

Table 4 Cox proportional hazard analysis models performed on fly survival within a sex and hour after treatment application. Model parameters (Baseline:Comparison) compared the control fly survival to ozone treated fly survival. The 'β' is the parameter estimate. The 'SE' is the standard error of the 'β'. The 'HR' is the hazard ratio. The 'CL' is the confidence level

SURVIVAL MODELS: COX PROPORTIONAL HAZARD REGRESSION ANALYSIS

Sex	h	Parameters	*β*	*SE*	*df*	*z*	*P*	*HR*	*95% CL (Lower–Upper)*
Female	1	Untreated + Oxygen:Ozone	1.451	0.119	1	12.150	< 0.001	4.267	3.377–5.392
	108	Untreated:Ozone	–0.284	0.144	1	–1.972	0.049	0.753	0.567–0.998
Male	1	Untreated + Oxygen:Ozone	0.903	0.107	1	8.411	< 0.001	2.467	1.999–3.045
	108	Untreated:Ozone	–0.756	0.153	1	–4.942	< 0.001	0.470	0.348–0.634

humidity and temperature of desiccation trials at 108 h after treatment application were 15.93% (± 2.12) and 28.32 °C (± 0.34 °C), respectively. Optimized Cox Proportional Hazard models for male and female flies 1 h after ozonolysis compared oxygen and untreated controls to ozone treated flies (Crawley 2007; Rich et al. 2010). Output from Cox Proportional Hazard models for the two trials (1 h and 108 h) is presented in Table 4.

Mantel–Haenszel log-rank tests of female and male survival 1 h after ozonolysis showed significant differences between ozone treated and control flies ($\chi^2 = 158$, $df = 2$, $p < 0.001$, $\chi^2 = 75$, $df = 2$, $p < 0.001$, respectively). Cox Proportional Hazard models of female and male survival 1 h after ozonolysis showed significantly reduced survival times of ozone treated flies to the combined control flies 1 h after ozonolysis ($z = 12.15$, $df = 1$, $p < 0.001$; $z = 8.411$, $df = 1$, $p < 0.001$, respectively). A Hazard Ratio of 4.267 was observed when comparing controls to ozonated female flies with median times of death of 4.5 h and 2.5 h, respectively (Tables 3 and 4). Similarly, ozonated males had a Hazard Ratio of 2.467 when compared to control flies with median times of death of 2 h and 3 h, respectively (Tables 3 and 4).

Mantel–Haenszel log-rank tests of female and male survival 108 h after ozonolysis showed significant differences between ozone treated and control flies ($\chi^2 = 4.2$, $df = 1$, $p = 0.04$, $\chi^2 = 22.4$, $df = 1$, $p < 0.001$, respectively). Cox Proportional Hazard models of female and male survival 108 h after ozonolysis showed that ozone treated flies had significantly increased survival times compared to control flies 1 h after ozonolysis ($z = -1.972$, $df = 1$, $p = 0.0486$, $z = -4.942$, $df = 1$, $p < 0.001$, respectively). Ozonated females had a Hazard Ratio of 0.753 when compared to control flies with median times of death of 4.5 h and 4 h, respectively (Tables 3 and 4). Similarly, ozonated males had a Hazard Ratio of 0.47 when compared to control flies with median times of death of 2.5 h and 2 h, respectively (Tables 3 and 4).

Discussion

Our results demonstrate that ozonolysis of live *D. suzukii* significantly reduces the total amount of unsaturated hydrocarbons, increases the mass of aldehydes, and has variable effects on the levels of saturated hydrocarbons in hexane

extracted CHCs (Figs. 4, 5 and 6) (Table S1). Furthermore, the levels of CHCs from flies treated with ozone return to untreated CHC levels within 108 h after exposure (Figs. 4, 5 and 6 and Table S1). However, flies regenerated unsaturated hydrocarbons at different rates depending on their sex and age (Fig. 4 and Table S1). Desiccation resistance was correlated to changes in CHC abundance, with an immediate decrease followed by recovery over the same time. (Fig. 7 and Table 3). Surprisingly, desiccation resistance significantly increased in comparison to untreated controls at 108 h after treatment application, although a concomitant significant increase in unsaturated hydrocarbons was only observed for 3–5 d females at 36 h after treatment application (Figs. 4 and 7 and Table S1 & S2).

Our study is the first to quantify ozonolysis of CHCs on living insect specimens and may provide an important new method for exploring the biosynthesis, structure, and function of these important constituents of the insect epicuticle. Current methodology for the modification of living insect CHCs includes genetic modification and direct CHC application via perfuming (Ferveur 1997) (Billeter et al. 2009). While these methodologies are useful for determining the function of CHCs, they are expensive and/or time-intensive, requiring the genetic modification of individual species/lineages. Our method could be used to modify the unsaturated hydrocarbons of any insect and allow the measurement of CHC generation time, potential for regeneration as well as how they modify behavior and survival.

While CHC generation in *Drosophila* species has been described in previous work (Bartelt et al. 1986; Jallon and David 1987; Toolson and Kuper-Simbron 1989; Dekker et al. 2015; Snellings et al. 2018), ours is a novel study providing data on the regeneration of unsaturated hydrocarbons following their removal from living subjects. The regeneration of CHCs to untreated levels suggests that maintaining CHCs is of great importance to *D. suzkuii*. Insect CHCs have been found to function as (1) pheromones, (2) to increase desiccation resistance, and (3) to protect from entomo-pathogens (Quinlan and Hadley 1993; Gibbs 1998; Howard and Blomquist 2005; Blomquist and Bagnères 2010; Ortiz-Urquiza and Keyhani 2013; Chung and Carroll 2015).

Our results also provide evidence that *D. suzukii* of different sexes and ages regenerate CHCs to untreated levels, albeit point estimates of CHC's vary across these groups (Fig. 4 and Table S1). For example, 3–5 d old and 9–11 d old females regenerated unsaturated hydrocarbons to levels observed in untreated flies by 12 and 36 h after treatment application, respectively (Fig. 4 and Table S1). However, 3–5 d and 9–11 d males did not regenerate unsaturated hydrocarbons to untreated fly levels until 108 h after treatment application (Fig. 4 and Table S1). This suggests that females either have a greater capacity to generate CHCs or prioritize the regeneration of CHCs after ozonolysis.

The mass of saturated hydrocarbons increased over time after ozone exposure in female flies. We propose that the CHC compensation mechanism for the regeneration of unsaturated hydrocarbons also affects saturated hydrocarbon biosynthesis. This hypothesis is supported by Qiu et al. (2012) that shows both alkane and alkene biosynthesis is catalyzed by a P450 decarbonylase, *CYP4G1* in *Drosophila.*

The over-expression of the P450 decarbonylase would allow the regeneration of unsaturated CHCs to normal levels, as well as the biosynthesis of saturated CHCs to elevated levels (Chung and Carroll 2015).

Our data provide strong correlative evidence for the importance of unsaturated CHC's for desiccation resistance in *D. suzukii*. Gibbs (2002) hypothesizes that alkenes and alkanes form layers on the epicuticle dependently on lipid melting points. Alkenes may form liquid layers on the cuticle and allow greater permeability of water due to their lower melting temperatures (Gibbs 2002). This layered packing of alkanes and alkenes could help to explain the decreased survival rate of ozone treated flies as well as the uniform reduction of all unsaturated hydrocarbons. Furthermore, SEM images of a tick cuticle, *Rhipicephalus sanguineus* (Latreille) (Ixodida: Ixodidae), after ozone exposure qualitatively shows the damaging impact of ozone to the epicuticle layer (Moreira et al. 2018). This provides additional evidence to support the correlation of decreased desiccation resistance resulting from ozone mediated damage to the epicuticle.

While our data strongly suggest that desiccation resistance is directly linked to unsaturated hydrocarbon quantity, it is possible that observed differences are due to other ozone induced effects. The ozonolysis performed in this experiment, while largely non-lethal, did result in some mortality. Dose response curves developed in Savage (2020) predict that a CT product, the product of concentration (ppm) and time (min), of 3,750 ppm-min of gaseous ozone would result in 11% and 25% mortality immediately following ozonolysis for males and females, respectively (Savage 2020). One potential, non-desiccation, source of mortality could be tracheal damage resulting from ozone exposure. However, Sousa et al. (2008) concluded that there was no association between respiration rate and ozone susceptibility after developing and analyzing ozone time-mortality curves of *T. castaneum, R. dominica,* and *O. surinamensis* (Sousa et al. 2008). Our study did not directly measure the respiration rate of specimens before or after ozone exposure.

Potential future applications of ozonolysis of CHCs include characterizing arthropod physiology and behavior in regards to desiccation, chemical communication, and entomopathogen resistance (Quinlan and Hadley 1993; Gibbs 1998; Howard and Blomquist 2005; Blomquist and Bagnères 2010; Ortiz-Urquiza and Keyhani 2013; Chung and Carroll 2015). Unsaturated hydrocarbons are important

in *Drosophila spp.* for identification of conspecifics and courtship/mating behaviors (Antony et al. 1985; Jallon and David 1987; Ferveur 1997, 2005; Howard and Blomquist 2005). For example, 7,11-heptacosadiene has been shown to be an aphrodisiac for male *D. melanogaster* (Antony et al. 1985). Cleavage of the double bonds of 7,11-heptacosadiene at the 7th and 11th carbon positions would occur after an ozone treatment using our methodology. This method could be combined with mating assays to determine how courtship and copulation are affected after ozonolysis of unsaturated hydrocarbons.

Ozonolysis of CHCs could also be combined with genetic modification of CHCs or CHC perfuming (Ferveur 1997, 2005). For example, ozonolysis of the genetically modified oenocyte-less (oe-) fly lineage of *D. melanogaster,* that produces no CHCs (Billeter et al. 2009) could be used to further elucidate whether ozonolysis affects desiccation resistance in the absence of CHCs. Additionally, courtship and copulation are shown to be mediated by unsaturated hydrocarbons, such as the anti-aphrodisiac (*Z*)-7-tricosene, in both *D. suzukii* flies and *D. melanogaster* males (Ferveur 1997; Snellings et al. 2018). Post ozonolysis "perfuming" of insects could be used to evaluate the relative importance of specific semiochemicals in courtship and mate selection. Ozonolysis of unsaturated hydrocarbons on female *D. suzukii* was correlated to reduced courtship and/or copulation by untreated males (Savage and Grieshop, unpublished data). This is counter-intuitive to the reduction of the (*Z*)-7-tricosene (an anti-aphrodisiac) after ozonolysis but may be explained by the interaction of ozone with other insect tissues. For example, dominant lethal chemicals are produced after ozone exposure, which cause mutagenicity and reduced reproductive potential in *D. virilis* (Erdman and Hernandez 1982). Alternatively, newly formed aldehydes formed after ozonolysis on the female epicuticle may become bioactive and confound a normal behavioral response.

In conclusion, the masses of *D. suzukii* unsaturated hydrocarbons are significantly reduced by a factor of 2–3 after ozone treatment due to the process of ozonolysis. This creates aldehydes that remain on the cuticle for between 36 and 108 h. The saturated CHC amount on flies is largely unaffected by ozone treatment, except for female flies at 3–5 d where a significant increase in saturated hydrocarbons was found. Additionally, flies demonstrated differential CHC regeneration based on sex and age. Females regenerated CHCs more quickly than males, as well as having an increased CHC regeneration rate at 3–5 d than 9–11 d. Finally, the reduction and recovery of desiccation resistance in ozone treated flies was correlated to the reduction and regeneration of unsaturated hydrocarbons. However, the desiccation resistance of ozone treated flies was elevated above untreated flies after unsaturated CHC regeneration. These findings provide a novel methodology for insect CHC reduction/modification, evidence for CHC regeneration after reduction/modification, and evidence supporting the contribution of unsaturated hydrocarbons to desiccation resistance.

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Authors' Contributions Benjamin Savage designed experiments, executed experiments, collected data, performed statistical analysis, wrote code for statistical analysis, developed figures, and wrote the article.

Zinan Wang provided methodological support for cuticular extractions, GC/MS set-up, and developed figures.

Susan Masten provided ozone concentration measurement equipment and scientific advice.

Henry Chung provided experimental design support.

Matthew Grieshop provided scientific leadership, equipment, and funding.

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Data Availability Available upon request.

Code Availability Available upon request.

Declarations

Conflicts of interest/Competing interests NA

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