EXPLORING THE LETHAL AND SUB-LETHAL INSECTICIDAL PROPERTIES OF OZONE USING SPOTTED WING DROSOPHILA, *DROSOPHILA SUZUKII* (MATSUMURA) (DIPTERA: DROSOPHILIDAE) AS A MODEL ORGANISM.

By

Benjamin Alexander Savage

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Entomology – Master of Science

2020

ABSTRACT

EXPLORING THE LETHAL AND SUB-LETHAL INSECTICIDAL PROPERTIES OF OZONE USING SPOTTED WING DROSOPHILA, *DROSOPHILA SUZUKII* (MATSUMURA) (DIPTERA: DROSOPHILIDAE) AS A MODEL ORGANISM.

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Ozone is a highly unstable allotropic form of oxygen that oxidizes organic compounds. In entomology, ozone has been evaluated as an alternative insecticide of stored product pests and to determine double/triple bond positions in hydrocarbons of insect pheromones. My research sought to explore the insecticidal characteristics and sub-lethal interactions of ozone on the model organism, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). Laboratory evaluation of gaseous ozone concentration-time (CT) response curves found male and female flies experienced similar LCT 99 products of approximately 1.13 x 10^5 ppm-min and 1.55 x 10^5ppm-min, respectively. The LCT 50 of males and females were similar when exposed to 14,600 ppm ozone treatments, but males showed elevated mortality in comparison to females at 30,100 ppm ozone treatments. Aqueous ozone (~18.52 ppm) exposure demonstrated no difference in toxicity on aqueous ozone treated flies from controls. Thus, gaseous ozone shows insecticidal potential of *D. suzukii*, while aqueous ozone does not. Sub-lethal ozone exposure on flies reduced unsaturated cuticular hydrocarbons (CHCs), which correlated to a reduction in desiccation resistance within one hour of ozonolysis. Unsaturated CHCs recovered over 108 hours along with desiccation resistance. The ozonolysis methodology presented in this thesis may be adopted to modify CHC profiles, characterize CHC regeneration and further describe the physiological function/s of unsaturated CHCs.

Keywords: Ozone, insecticide, sub-lethal, gaseous, aqueous, unsaturated cuticular hydrocarbons, desiccation resistance

ACKNOWLEDGEMENTS

Experiments were made possible with the help of Allison Fischer, Charlotte Schuttler, Elizabeth Szcepanski, Kyle Akred, Keith Koonter, Henry Chung, Ph.D., Juan Huang, Ph.D., Phil Fanning, Ph.D., Susan Masten, Ph.D. and Zinan Wang. Experiments were performed at the Organic Pest Management lab at Michigan State University directed by Matthew Grieshop, Ph.D. An opinionated, frank, encouraging, loyal, critical, cream de la cream scientist to whom I'm proud to call an advisor and friend. Funding for this research was provided by the Michigan Apple Committee. My assistantship was funded by the United States Department of Agriculture Specialty Crop Research Initiative where I assisted in the research and development of Solid Set Canopy Delivery Systems.

A sincere thanks and appreciation goes out to all my colleagues and coworkers for your guidance and assistance. I would have never reached this point without your help.

Family and friends deserve a major thanks for your encouragement as well as taking the time to listen to my struggles. "A graduate degree is dark and full of terrors".

A million thanks, much respect and hugs for everyone,

Benjamin Alexander Savage

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CHAPTER 1. LITERATURE REVIEW OF OZONE CUTICULAR HYDROCARBONS AND *DROSOPHILA SUZUKII*

1. INTRODUCTION TO OZONE

Gaseous ozone exists naturally in the stratosphere where it reflects harmful ultraviolet emissions from the sun (Hartley 1881). Ozone is naturally replenished by ionization in the atmosphere via lightning strikes and energetic solar ultraviolet (UV) radiation, which splits and combines oxygen molecules, O_2, into ozone molecules, O_3 (Rowland 2009). Ozone protects organic life from harmful UV radiation, which may harm organic life in the future if stratospheric ozone depletion increases (Longstreth, J D et al. 1995).

Ozone is triatomic oxygen. It is an unstable gas that is toxic and has a strong and sweet-smelling odor. As ozone can cause respiratory issues, OSHA (Occupational Safety and Health Administration) has set a maximum time weighted average (TWA) of 0.1 ppm of ozone exposure during an 8-hour work period and a 0.3 ppm short-term exposure limit (STEL). Although ozone is toxic, it readily degrades overtime into harmless by-products. Ozone is about thirteen times more soluble in water than oxygen. At 25 °C, it has a solubility of ~109 mg/L. The half-life of aqueous ozone depends on temperature, pH (aqueous), and the concentration of reactive species including natural organic matter (NOM), carbonate/bicarbonate, and reduced metals such as Fe(II) and Mn(II) (Hewes and Davison 1971; B. Langlais et al. 1991). Gaseous ozone decomposes within 12 hours at atmospheric pressure and aqueous ozone decays in 37.5 minutes at a pH of 6 in buffered water (1 M potassium phosphate and 1 N sodium hydroxide) (Hewes and Davison 1971). Aqueous ozone has a few disadvantages when compared to gaseous ozone; including low solubility and low stability (Kasprzyk-Hordern et al. 2003). These differences greatly affect the potential applications of gaseous and aqueous ozone.

2. APPLICATIONS OF OZONE

Ozone can be produced electrolytically, by corona discharge, or by ultraviolet light from either air or pure oxygen. Ozone is highly unstable and has a high oxidative potential (2.07 V). This has led to the use of ozone in a variety of scientific and industrial applications. These include: drinking/waste water sanitation, medical treatments, food processing sanitation, insecticides and in analytical chemistry (Kim, Yousef, and Dave 1999; Rico et al. 2007; Kells et al. 2001; Beroza and Bierl 1967; Ikehata and El-Din 2005; Siedler et al. 2008). Numerous companies and waste water treatment facilities in Europe currently use ozone to disinfect water of harmful microbes and react with organic compounds (e.g., Bryant, Fulton, and Budd 1992; Stover and Jarnis 1981; Kasprzyk-Hordern, Ziółek, and Nawrocki 2003). In the case of alternative medicine, ozone is applied to patients whom have bacterial infections in preparation for medical treatment, but many contradictory articles do not agree with its effectiveness (Siedler et al. 2008). Ozone is used in food processing to remove bacteria from the surfaces of fruit, which improves the shelf-life of fruit without affecting the flavor of the product (Kim, Yousef, and Dave 1999; Rico et al. 2007). As an insecticide, ozone has been evaluated to control stored product pests, aphids and bed bugs (Kells et al. 2001; Ebihara et al. 2013; Feston 2015). And finally, analytical chemists use ozone's ability to oxidize organic molecules, such as alkenes, for identification of double or triple bond positioning (Beroza and Bierl 1967).

2.1 Ozone as an Insecticide

Ozone has been applied in a variety of different fields due to its ability to oxidize and/or sanitize organic compounds. One such application of ozone is as an insect pest management tool. In particular, stored product pest researchers have evaluated ozone to control pests in grain bins because of the contained area within a grain storage container. This allows much greater ozone concentration control than in open environments.

Ozone has been evaluated as a fumigant treatment for a variety of stored product insect pests. Stored product pest researchers sought a new insect pest fumigant to be used alongside phosphine, where researchers had been reporting resistance to phosphine fumigation (Chaudhry 2000; Pimentel et al. 2007). A study from Brazil in 2008 experimented with using ozone on stored product pests, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) and *Oryzaephilus surinamensis* (Linneaus) (Coleoptera: Curculionidae), and determined that no cross-resistance occurred from phosphine resistant populations to ozone exposure (Sousa et al. 2008). The insect respiratory system is known to be the primary path for fumigation toxicity of phosphine (Cotton 1932; Pimentel et al. 2007), however, this study showed that respiration rates and ozone toxicity didn't correlate (Sousa et al. 2008).

The fumigant insecticidal function of gaseous ozone complicates dose response experiments because both ozone concentration and exposure time contribute to mortality. Thus a concentration*time (CT) product (ppm-min) is sometimes used to quantify dose responses and organismal response to ozone over time (M. X. McDonough et al. 2010; Marissa X. McDonough, Mason, and Woloshuk 2011; Feston 2015). For example, *T. castaneum* experienced 100% mortality after 1800 ppm gaseous ozone for 120 min in laboratory conditions (216,000 ppm-min) and 47,000 ppm gaseous ozone for 6 min in field conditions (282,000 ppm-min) (M. X. McDonough et al. 2010). McDonough et al. (2011) reported that an ozone concentration of 1800 ppm significantly reduced the time to reach 100% mortality in *Tribolium castaneum and Plodia interpunctella, Sitophilus zeamais*, and *S. oryzae*, than at 50 ppm (Marissa X. McDonough, Mason, and Woloshuk 2011). This suggests that the CT product equation works properly and is a viable method for determining mortality at differing ozone concentrations and times.

Ozone has varied toxicity in different insect species. The adult red flour beetle, *T. castaneum*, adult maize weevil, *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae), and larval Indian meal

moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) experienced mortality (>75% & >90%) after days (3 d & 5 d) of ozone (25 ppm & 50 ppm) exposure on approximately 9 tons of maize in a galvanized steel grain bin (Kells et al. 2001). This study demonstrated that the adult maize weevil had greater susceptibility to ozone than the adult red flour beetle or the larval Indian meal moth illustrating the importance of species specific dose response models (Kells et al. 2001).

Ozone's efficacy as an insecticide has varied across field and laboratory experiments. *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) and *T. confusum* (du Val) (Coleoptera: Tenebrionidae) experienced higher levels of mortality from 13.9 mg/L of aqueous ozone in empty vessels compared to vessels containing 2 kg of wheat, providing evidence that ozone will oxidize insects less readily when in the presence of ozone susceptible organic molecules (Işikber and Öztekin 2009). However, a study using ozone fumigation in field conditions using a modified stainless steel screw conveyor (22.7 kg grain capacity) reported no significant change in 100% CT product mortality of *Sitophilus zeamais* and *T. castaneum* when compared to laboratory conditions, 286,920 ppm-min and 216,000 ppm-min respectively (M. X. McDonough et al. 2010).

Life stage has also been shown to affect ozone toxicity (Feston 2015; Işikber and Öztekin 2009; McDonough et al. 2011). Feston (2015) found that eggs of bed bugs (Cimex lectularius) (Linnaeus) (Hemiptera: Cimicidae) experienced 100% mortality at 2,040,000 ppm-min while larva/adults died at 270,000 ppm-min (Feston, 2015). *Tribolium confusum* was found to vary in mortality dramatically between life stages after ozone exposure, with larva being the most susceptible to ozone (Leesch, J. G 2002; Işikber and Öztekin 2009). The cause of mortality from ozone exposure in insects, or mode of action, has not been fully described and is hypothesized to be affected by many different environmental variables including, points of potential ozone interaction, temperature, and humidity (Isikber and Athanassiou 2015)

Ozone can also be applied as a solution containing the dissolved gas. Insect mortality in response to aqueous ozone has not been as extensively studied as that due to gaseous ozone exposure. The lack of published research on aqueous ozone mortality is most likely due to the low attainable concentrations of ozone in aqueous solutions. Ebihara et al. developed an ozone-mist sprayer and reported >90% mortality of the red aphid, *Uroleucon nigrotuberculatum* (Olive) (Hemiptera: Aphididae) under greenhouse conditions (Ebihara et al. 2013). In this study, aphids were sprayed from a distance of 2-5 cm with gaseous ozone (8.4-3200 ppm) and water droplets combined at the nozzle, effectively making the experiment a gaseous ozone application. A second paper evaluated a radial airblast sprayer with an ozone generator unit attachment that has been marketed to control plant diseases and insects (Grieshop et al. 2019). This study reported a lack of appreciable insect, bacterial and fungal pest management from the ozone treated areas after spraying an apple orchard at <1 ppm of ozone. However, neither of these studies evaluated ozone induced mortality under laboratory conditions.

2.2 Sub-Lethal Effects of Ozone

Ozone has been studied for lethal and sub-lethal interactions on insect pests. Sub-lethal interactions can range from a variety of different areas, including mutagenicity, variable fecundity, analytical hydrocarbon identification via ozonolysis and cuticular damage. As sub-lethal interactions have yet to be as extensively studied as lethal interactions, there is a large knowledge gap.

Ozone has been shown to cause delayed mitotic division and mutagenicity. *Chortophaga viridifasciata* (De Geer) (Orthoptera-Acrididae) embryos at anaphase, telophase, interphase and early prophase stages were delayed from entering into later stages of mitotic division (mid and late prophase) when exposed to 3.5-4.5 ppm ozone (Fetner 1963). Mitotic inhibition was hypothesized to be caused by an increase in free radicals (OH^-). Mutagenicity was indirectly measured by Erdman and Hernandez (1982) by calculating 'dominant lethals' from the percentage of pupae that did not develop from eggs after newly eclosed adult male *Drosophila virils* (Sturtevant) (Diptera: Drosophilidae) were exposed to 30

± 2 ppm ozone for 3 hour increments over 25 days and mated with virgin females (Erdman and Hernandez 1982). Dominant lethals in ozone treatments were found to be significantly higher than controls when mating males were at the sperm bundle (5-9 d old), spermatid (8-15 d old) and spermatogonia (18-25 d old) stages of spermatogenesis (Erdman and Hernandez 1982).

Insect fecundity is also affected by ozone exposure. *Musca domestica* (Linnaeus) (Diptera: Muscidae) oviposition was measured by recording 'soiling of papers' in experimental arenas in two week intervals over 18 generations (Beard 1965). Oviposition significantly increased when exposed to ozone enriched air (0.1 ppm ozone) in comparison to the control (air) and elevated ozone (>0.1 ppm) conditions (Beard 1965). Fly oviposition in elevated ozone (>0.1 ppm) conditions reduced to zero, but the precise experimental ozone concentration was not reported in the article. In the Erdman and Hernandez study (1982), oviposition was significantly decreased in unexposed female *D. virilis* after mating with ozone (30 ± 2 ppm) exposed male flies (Erdman and Hernandez 1982). These experiments show that marginal differences in ozone concentration and exposure can significantly affect fecundity of flies.

The insect integument represents a major point of exposure to ozone and cuticular hydrocarbons (CHC) in the epicuticle may be modified by exposure to ozone via ozonolysis. In past CHC studies, ozonolysis has been used to identify double bond positions of unsaturated hydrocarbons and phermones in dipteran, hymenopteran and lepidopteran following extraction using chemical solvents (Antony et al. 1985; R. J. Bartelt, Jones, and Kulman 1982; Kochansky et al. 1975; Robert J. Bartelt et al. 1986). Alkenes, an unsaturated hydrocarbon, undergo oxidative splitting at the site of the carbon-carbon double bond by ozone and produces ketone, aldehyde and peroxide by-products (Criegee 1975). Methodology developed by Beroza and Beirl (1967) involves extracting cuticular hydrocarbons with a non-polar solvent and then treating the extract with ozone prior to GC-MS analysis (Beroza and Bierl 1967). Antony et al. (1985) demonstrated that cuticlular monoenes and dienes extracted from

Drosophila melanogaster (Meigen) (Diptera: Drosophilidae) underwent cleavage via ozonolysis to varied extents. For example, 7-tricosene experienced a "large" reduction after ozonolysis, while 9-tricosene did not (Antony et al. 1985).

In addition to cuticular hydrocarbon ozonolysis after extraction, cuticular damage has been qualitatively imaged. An ozone toxicity study on a tick, *Rhipicephalus sanguineus* (Latreille) (Ixodida: Ixodidae), imaged cuticular damage from ozone using a scanning electron microscope (SEM), but didn't perform any CHC extractions to quantify damage (Moreira et al. 2018). No studies to date have explored the ozonolysis of unsaturated hydrocarbons on living insects.

2.3 Questions

Lethal and sub-lethal effects by ozone on arthropods is a research field that has potential for growth, despite published research articles beginning in 1965 (Beard 1965). Fundamental questions still exist regarding ozone to insect interactions, post-exposure effects and lethality of different species. Questions that we found to be of interest include:

a. Does ozone have similar insecticidal characteristics on other insect pests?
b. Are there unidentified sub-lethal effects that occur after ozone exposure on insects?

3. CUTICULAR HYDROCARBONS

Insect cuticular hydrocarbons exist on the outermost layer of the insect exoskeleton, commonly referred to as the wax layer or epicuticle lipids (Gibbs 1998; Nicolson 2008; Blomquist and Bagnères 2010). Hydrocarbons function to prevent water loss and serve as important chemical communication cues (Quinlan and Hadley 1993; Gibbs 1998; Howard and Blomquist 2005; Blomquist and Bagnères 2010; Chung and Carroll 2015; Ginzel and Blomquist 2016).

3.1 Cuticular Hydrocabon Structure

Insects create a variety of cuticular hydrocarbons (CHCs) on the wax layer of the epicuticle. Creation of hydrocarbons begins with lipids inside of the insect, specifically, by splitting long chain

unsaturated fatty acids(Reed et al. 1995). Cuticular hydrocarbons can be biosynthesized after desaturation of unsaturated fatty acids and are transported to the oenocytes of the exoskeleton (Pennanec'h et al. 1991). Oenocytes located at the basal dermal layer of the exoskeleton emit hydrocarbons onto the outermost surface of the exoskeleton, but the mode of transportation has yet to be described (Blomquist and Bagnères 2010).

The majority of insects produce hydrocarbons that are 21-40 carbons in length (Ginzel and Blomquist 2016). *Drosophila* spp. cuticles are characterized by a wide range of saturated, unsaturated and methyl-branched hydrocarbons (Jallon and David 1987; Howard et al. 2003; Robert J. Bartelt et al. 1986). The blend of cuticular hydrocarbons and lipids can be species and sex specific (Jallon and David 1987). For example, the CHC profile of *D. melanogaster* is sexually dimorphic where the female produces the unsaturated diene, 7,11 heptacosadiene, and males produce larger amounts of unsaturated monoene, 7-tricosene (Jallon and David 1987). The length and composition of CHC profiles has been directly correlated to desiccation resistance and definitively linked to chemical communication cues.

3.2 Cuticular Hydrocarbon Function

Water loss in insects can occur through respiration, excretion, and the cuticle (Nicolson 2008). Cuticular hydrocarbons play a large role in determining desiccation resistance of an insect (Ramsay 1935; Gibbs 1998). For example, overall permeability of water through the cuticle of the grasshopper, *Romalea guttata* (Palisot de Beauvois) (Orthoptera: Romaleidae), has been shown to increase dramatically as temperature increases, where water loss was 4.62 mg per h at 15° C and 13.28 mg per h at 30° C (Quinlan and Hadley 1993; Nicolson 2008). Hadley and Quinlan (1986) found that physically disrupting the pronotum epicuticle of the *Periplaneta americana* (Linnaeus) (Blattodea-Blattidae) with lipid solvents (hexane and KOH/Cl) and swabs removes surface lipids, resulting in an increase of water loss rate through the cuticle (N. F. Hadley and Quinlan 1987). It has been hypothesized that as lipids

change from solid to liquid, as determined by a critical temperature, the ability for water to diffuse out of an insect increases (Ramsay 1935; Neil F. Hadley 1994; Gibbs 1998). Long chain saturated hydrocarbons have higher melting points than methyl-branched/unsaturated hydrocarbons and may impart greater desiccation resistance due to this (Gibbs 1998).

Current experimental methodologies restrict the direct measure of cuticle permeability in regards to specific hydrocarbons, but correlative evidence supports the hypothesis of greater desiccation resistance coming from saturated hydrocarbons. In *D. pseudoobscura* (Frolova), a greater ratio of saturated to unsaturated hydrocarbons has also been linked to desiccation resistant insects living in arid regions rather than more humid laboratory environments (Toolson and Kuper-Simbron 1989). A similar trend was found in *Tibicen dealbatus* (Davis) (Homoptera: Cicadidae) where a greater abundance of long chained saturated CHCs correlated to a reduction of water loss rate (Toolson 1984). Desiccation resistance has yet to fully described in regards to cuticular permeability because CHCs exhibit complex interactions based on chemical and physical properties (Gibbs 1998). Interactions between CHCs and individual CHC amounts are important to desiccation resistance as well as communication.

Insect CHCs function as contact chemical communication cues intra- and inter-specifically (Howard and Blomquist 2005). Documentation of chemical communication via CHCs has been well documented in social insects such as bee and ant species (Meer et al. 2019). Furthermore, solitary insects such as members of the family Drosophilidae identify conspecifics and sex using volatile pheromones, visual cues, acoustic cues and contact pheromones (Markow and O'Grady 2005; Tauber and Eberl 2003; Benton 2007; Greenspan 1995). The CHC 7,11-heptacosadiene has been shown to be an aphrodisiac for male *D. melanogaster* because it is sexually specific to females (Antony et al. 1985). Conversely, 7-tricosene on female *D. melanogaster* has been shown to be an anti-aphrodisiac for males when evaluating courtship and copulation success (Ferveur 1997). Empirical evidence highly supports

the importance of CHCs as contact pheromones and future directions of CHC research may focus on multiple CHC interactions on behaviors.

3.3 Questions

Cuticular hydrocarbons are generally 21-40 carbon chains that may be saturated, methyl-branched or unsaturated (Ginzel and Blomquist 2016). Their functions range from reducing cuticular water permeability to contact pheromones. A few questions present themselves based on the review of the ozone and cuticular hydrocarbons sections. In particular, questions pertaining to ozonolysis and cuticular damage caused by ozone exposure:

a. How does ozone react with CHCs on living specimens?

b. If ozone does react with CHCs, how do they affect CHC function?

4. MODEL ORGANISM: SPOTTED WING DROSOPHILA (*D. SUZUKII*)

4.1 Biology and Life History

Drososphila suzukii (Matsumura) (Diptera: Drosophilidae), spotted wing drosophila, is an invasive vinegar fly from Asia, first detected in California in 2008 (Bolda, Goodhue, and Zalom 2010). *Drosophila suzukii* goes through a holometabolus life cycle, which includes 4 life stages: an egg, larva, pupa, and adult. This fly has rapidly become a key pest of soft fruit due to the adult female's ability to pierce the skin of ripening fruit with a specialized, serrated ovipositor (Kanzawa 1939; Mitsui, Takahashi, and Kimura 2006; Bolda, Goodhue, and Zalom 2010; Walsh et al. 2011). Spotted wing Drosophila readily reproduces in raspberries, blackberries, blueberries, strawberries, grapes and cherries and US estimated fruit crop losses in California, Oregon and Washington estimated at $511 million annually (Bolda, Goodhue, and Zalom 2010).

Spotted wing Drosophila develop from egg to an ovipositing adult fly in 8-24 d depending on environmental conditions (Kanzawa 1939; Walsh et al. 2011). Eggs normally hatch 24-48 hours after oviposition (Kanzawa 1939; Walsh et al. 2011). A first instar measuring less than a millimeter in length

emerges and grows after successive molts to approximately four mm in length over the course of a one to two weeks (Kanzawa 1939; Walsh et al. 2011). The pupa has little to no mobility, but is encased in a hardened, protective puparium. The puparium provides protection from the elements and is camouflaged with a light to dark brown tint to reduce detection by predators or parasitoids. The pupal stage lasts 7 days and the metamorphosis results in an adult fly (Kanzawa 1939; Walsh et al. 2011). Adults measure 2-3 mm in length can live a few months depending on environmental conditions and females lay up to 380 over the course of her lifespan (Walsh et al. 2011). In the Western United States flies can complete 3-9 generations in a year according to degree day models based on research from Kanzawa (1939) and Sasaki and Sato (1995) (Kanzawa 1939; Sasaki and Sato 1995; Cooper, L. 2010). Long adult life, coupled with short generation time and synovigenic egg production leads to a rapid breakdown in generational cohort structure further complicating management of this pest (Wiman et al. 2016).

4.2 Management

Current management of *D. suzukii* is accomplished using chemical, cultural and biological tactics (Simberloff and Rejmanek 2011). Chemical control allows rapid response to control pest populations, which is important to reduce crop losses from pest damage. Cultural control is important as a preventative measure to manage pest populations so pest populations do not accumulate to potentially damaging levels. Biological control is the most passive, and sometimes, most effective long term strategy to control pest populations. Integrated pest management seeks to combine all aspects of insect pest management to reduce damage to crops and protect farmer crop harvest (Simberloff and Rejmanek 2011). It is important to combine management strategies to reduce reliance on any specific strategy and to provide the most effective population management possible.

The majority of *D. suzukii* management programs rely on insecticides directed at the adult stage (Walsh et al. 2011). Current management of this pest relies on repeated applications of broad spectrum,

contact insecticides from the pyrethroid, spinosyn and organophosphate chemical classes (Bruck et al. 2011; Van Timmeren and Isaacs 2013). Certified organic producers rely almost entirely on organic formulations of Spinosyns (Van Timmeren and Isaacs 2013) to control fly populations, so the development of pesticide resistance is a serious concern.

While pesticides can provide economic control of *D. suzukii* there are a number of disadvantages associated with them including the development of resistance and mortality of non-target insects. A study by Gress and Zalom (2019) detailed rising levels of resistance in the populations of *D. suzukii* in Watsonville, California to Entrust, the primary insecticide used by certified organic growers (Gress and Zalom 2019). Spinosyn, organophosphate and pyrethroid insecticides have shown considerable off-target lethal and sub-lethal consequences to honey bees, bumblebees, and native bees (Kevan 1975; Mayes et al. 2003; Gill, Ramos-Rodriguez, and Raine 2012). The importance of pesticides to organic and conventional farmers is indisputable in regards to controlling pest populations at low costs, but the disadvantages of pesticides is well documented and broad in scope. The creation of novel pesticides is ongoing and is necessary if current control rates are to continue.

Cultural management practices consist of manipulating the environmental area in and around a crop to reduce pest damage. Cultural controls can exist as physical barriers (i.e. mesh netting) or the physical removal/burial/composting of infected culture (Audsley, N., Tonina, L., and Mori, N. 2019). Physical barriers can prevent insect pests from obtaining direct contact, artificially reducing the number of food sources and oviposition sites. The removal of infected cultures can help to reduce food sources as well as reduce fly populations the following season.

For *D. suzukii*, covering fruit crops with exclusion netting have been effective at reducing fly populations, while also maintaining fruit yield and quality (Leach, Van Timmeren, and Isaacs 2016). While cultural controls, such as exclusion netting, are effective at reducing *D. suzukii* populations, they become even more effective when implemented in conjunction with insecticides (Leach, Van Timmeren,

and Isaacs 2016). Another cultural control method includes removing, composting and burying post-harvest fruit waste (Isaacs et al. 2013; Hooper and Grieshop 2020). Composting of post-harvest fruit waste seeks to reduce late season feeding and oviposition sites, which may help reduce the following season's fly population (Hooper and Grieshop 2020). Another method for controlling *D. suzukii* populations on raspberries (Rubus idaeus cv. 'Himbo Top') is by harvesting fruit in 2 day intervals for optimal fruit yield and pest reduction (Leach et al. 2018). Additionally, fruit waste that is heavily infested with *D. suzukii* can be placed in plastic bags and laid in direct sunlight for 5 days to ensure 99% mortality of *D. suzukii* (Leach et al. 2018).

Biological control seeks to control populations by utilizing natural predators or parasitoids of a specific target species. Three forms of biocontrol have been used in the past; classical, augmentative and conservation biocontrol. Classical biocontrol identifies native or non-native predators or parasitoids and introduces them to control invasive species from dominating ecosystems. Augmentative biocontrol utilizes mass rearing techniques to grow massive populations of a predator or parasitoid to be released and then to control target pest populations. Conservation biocontrol is an environmentally conscientious way of increasing native predator/parasitoid populations by improving native habitats and by providing more natural habitat refuge, which is meant to provide greater pest control as well.

Larval and pupal parasitoids native to Japan have been identified as potential classical biological control agents of *D. suzukii* (Duyck et al. 2009; Mitsui and Kimura 2010; Kasuya et al. 2013).These include *Ganaspis xanthopoda* (Ashmead) (Hymenoptera: Figitidae) and *Asobara japonica* (Belokobylskij) (Hymenoptera: Braconidae), which both target the larval stage of *D. suzukii* and have broad host ranges (Mitsui and Kimura 2010). A study from Kayusa et al (2013) found additional evidence of a D. *suzukii*-associated type of *G. xanthopoda* that preferentially parasitized pupa that would serve as a better host-specific classical biocontrol option (Kasuya et al. 2013).

Biocontrol agents of *D. suzukii* in Spain, *Pachycrepoideus vindemmiae* (Rondani) (Hymenoptera: Pteromalidae) and *Trichopria cf. drosophilae* (Perkins) (Hymenoptera: Diapriidae), have been found as well, outlining the potential of augmentative biocontrol of *D. suzukii* in Europe (Gabarra et al. 2015). Researchers in the California, USA evaluated *Ganaspis brasiliensis* (Ihering) and *Leptopilina japonica* (Novković & Kimura) (Hymenoptera: Figitidae) under quarantine as biocontrol agents of *D. suzukii* larva (Wang et al. 2019). An application for a permit to release *Ganaspis brasiliensis* as a larval parasitoid is currently under review by the USDA as of August, 2019. This could be the beginning of a long-term plan to control fly populations in North America.

4.3 Cuticular Hydrocarbons of *Drosophila suzukii*

The cumulative abundance of CHCs on *D. suzukii* cuticles are heavily dependent on age after eclosion (Snellings et al. 2018). *Drosophila suzukii* has a large variety of cuticular hydrocarbons present, including monoenes, dienes, a triene, methyl branched alkanes and n-alkanes (Snellings et al. 2018). In particular, 7(Z)-tricosene is the most prevalent hydrocarbon on the *D. suzukii* cuticle (Revadi et al. 2015; Snellings et al. 2018). *Drosophila suzukii* cuticular hydrocarbon profile is largely sexually monomorphic with only small quantitative differences in compound abundance (Revadi et al. 2015; Snellings et al. 2018). Socially experienced males that were aged in the presence of the opposite sex demonstrated elevated amounts (ng) of unsaturated (9-C21:1, 6,9-C22:2, 5-C24:1,7-C28:1, 9-C28:1, 9-C30:1, 7-C30:1, 11-C30:1, 7-C31:1), methyl branched (2-MeC27, 2-MeC29, 13-MeC29) and alkane (C14, C34) hydrocarbon abundances at 4 d old in comparison to socially experienced females (Snellings et al. 2018). Snellings et al. (2018) found that while CHCs are quantitatively significantly different, the relative amount (ng) of *D. suzukii* CHCs are sexually monomorphic. Furthermore, naive male flies (1 d old) that were reared separately from the opposite sex showed a significant elevation of the amount (ng) of 7-tricosene in comparison to naive females, while no other differences in CHC amounts were noted (Snellings et al. 2018).

Adult fly CHCs have been shown to mediate courtship and copulation of *D. suzukii* but have never been evaluated in the context of desiccation resistance (Revadi et al. 2015; Dekker et al. 2015; Snellings et al. 2018). A positive correlation was found between elevated CHC abundance, mating and age (Revadi et al. 2015), which could hint at the importance of CHCs at different life stages of an adult *D. suzukii*. Perfuming females with pure 7-tricosene, 9-tricosene and tricosane reduced courtship and mating with subsequent perfuming with natural ratios of 7-tricosene, 9-tricosene and tricosane blends resulting in no difference in courtship and mating from controls (Snellings et al. 2018).

5. OBJECTIVES

Insect mortality from ozone has been recorded in studies evaluating ozone's potential to control stored product pest populations. Ozone lethality has been sparsely evaluated on dipteran species, which are widely used as model organisms in studies ranging from genomics, ecology, behavior, physiology, population dynamics, waste management, medicine and alternative food options. Insect CHCs have been previously reported to be reduced by ozone exposure, but only when exposing CHC extractions to ozone and never on live specimens. Fly CHCs may be reduced on living specimens after ozone exposure, and if so, how would this affect the physiology of the fly directly after application and overtime?
The objectives of this thesis are:

1. **Determine lethality of gaseous and aqueous ozone by producing dose response curves on the model organism, *D. suzukii*.**
    a. Hypothesis: Flies will experience variable mortality during differing exposure durations and concentrations.
2. **Explore the sub-lethal impacts of ozone on cuticular hydrocarbons, evaluate the duration of these effects and determine whether modifications to cuticular hydrocarbons affect desiccation resistance on the model organism, *D. suzukii***

a. Hypothesis: The fly CHC profile will undergo ozonolysis after ozone exposure and desiccation resistance will decrease due to ozonolysis of desiccation relevant hydrocarbons.

CHAPTER 2. EXPLORING THE INSECTICIDAL POTENTIAL OF AQUEOUS AND GASEOUS OZONE USING SPOTTED WING DROSOPHILA, *DROSOPHILA SUZUKII* (MATSUMURA) (DIPTERA: DROSOPHILIDAE) AS A MODEL ORGANISM.

ABSTRACT

Gaseous and aqueous ozone was evaluated as a potential insecticide using the invasive fruit pest, spotted wing *Drosophila* (*Drosophila suzukii*) as a model species. Dose response curves for CT, concentration-time product (ppm-min), for gaseous ozone at 14,600 ppm and 30,100 ppm shows potential. The lethal concentration-time (LCT) 50 ± SEM estimates at 72 h after 14,600 ppm ozone exposure for females and males were (1.47 ± 0.09) x 10^4 and (1.37 ± 0.08) x 10^4, respectively. LCT 50 ± SEM estimates at 72 h after 30,100 ppm ozone exposure for females and males were (1 $\pm$ 0.07) x 10^4 and (0.66 $\pm$ 0.06) x 10^4, respectively. LCT 99 ± SEM estimates at 72 h after 14,600 ppm ozone exposure for females and males were (1.59 ± 0.33) x 10^5 and (1.17, ± 0.22) x 10^5, respectively. LCT 99 ± SEM estimates at 72 h after 30,100 ppm ozone exposure for females and males were (1.51 ± 0.36) x 10^5 and (1.08 ± 0.28) x 10^5, respectively. In contrast, ozone dissolved in distilled water at 18.5 ppm did not provide any mortality after total immersion of subjects for 30 seconds. I found that gaseous ozone primarily causes mortality immediately following exposure, with slight increases 72 h following ozone treatments. Gaseous ozone could therefore have some utility as a post-harvest fumigant for *D. suzukii* in closed vessels where concentrations could be maintained. However, ozone dissolved in aqueous solution was not observed to have insecticidal potential.

Keywords: Ozone, Gaseous, Aqueous, *Drosophila suzukii*, spotted wing Drosophila, dose response curves, insecticide

1. INTRODUCTION

Ozone is triatomic oxygen and has been used for microbial sanitation, food processing, odor reduction, water and wastewater treatment, pesticide degradation, and stored product pest management (Masten and Davies, 1994; Kim, Yousef, and Dave 1999; Kells et al. 2001; Rico et al. 2007). Ozone is a versatile oxidizing agent with biocidal potential with a short residual in aqueous solution due to its spontaneous degradation into nontoxic constituents at neutral pH within minutes at room temperature (~30 minutes) (Kim, Yousef, and Dave 1999). Depending on its application, ozone is applied in the form of gas or dissolved in water or other liquids.

Ozone has been evaluated as a fumigant treatment for a variety of stored product insect pests. Stored product pest researchers sought another insect pest fumigant to be used to replace or use in conjunction with phosphine, due to insect resistance to phosphine fumigation (Chaudhry 2000; Pimentel et al. 2007). A study from Brazil in 2008 experimented with using ozone on stored product pests, *T. castaneum*, the lesser grain borer, *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) and adult rice weevil, *Oryzaephilus surinamensis* (Linneaus) (Coleoptera: Curculionidae), and determined that no cross-resistance occurred between phosphine resistant populations to ozone susceptible flies (Sousa et al. 2008). The insect respiratory system is known to be the primary path for fumigation toxicity (Cotton 1932; Pimentel et al. 2007), however, Sousa et al (2008) showed that respiration rates and ozone toxicity didn't correlate (Sousa et al. 2008). Currently, ozone's mode of action, has not been fully described and is hypothesized to be multifaceted and dependent on multiple environmental variables including: temperature, humidity, and surface characteristics of surrounding materials (Isikber and Athanassiou 2015).

The fumigant insecticidal function of gaseous ozone complicates dose response experiments because both ozone concentration and exposure time contribute to mortality. Thus a

concentration*time (CT) product (ppm-min) is often used to quantify dose responses and how organisms react to gaseous ozone concentrations over time (M. X. McDonough et al. 2010; Marissa X. McDonough, Mason, and Woloshuk 2011; Feston 2015). For example, the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), experienced 100% mortality after 1800 ppm gaseous ozone for 120 min in laboratory conditions (216,000 ppm-min) and 47,000 ppm gaseous ozone for 6 min in field conditions (282,000 ppm-min) (M. X. McDonough et al. 2010). McDonough et al. (2011) reported that an ozone concentration of 1800 ppm significantly reduced the time to reach 100% mortality in *Tribolium castaneum and Plodia interpunctella, Sitophilus zeamais*, and *S. oryzae*, as compared to that observed at 50 ppm (McDonough et al. 2011). This suggests that the CT is a viable method for determining mortality at differing ozone concentrations and exposure times.

Studies have found variable ozone toxicity to insects, dependent on species and environmental conditions. The adult red flour beetle, *T. castaneum*, adult maize weevil*, Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae), and larval Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) experienced mortality (>75% & >90%) after days (3 d & 5 d) of ozone (25 ppm and 50 ppm) exposure in approximately 9 tons of maize in a galvanized steel grain bin (Kells et al. 2001). Kells et al. (2001) showed that the adult maize weevil had greater mortality susceptibility to ozone than the adult red flour beetle or the larval Indian meal moth (Kells et al. 2001). In another study, *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) and *T. confusum* (du Val) (Coleoptera: Tenebrionidae) experienced mortality from 13.9 ppm of ozone more easily alone in a container than when placed in 2 kg of wheat, providing evidence that ozone will oxidize insects less readily when in the presence of ozone susceptible organic molecules (Işikber and Öztekin 2009). However, a study using ozone fumigation in field conditions, a modified stainless steel screw conveyor (22.7 kg grain capacity), reported no significant change in 100% CT product mortality of *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionida) and *T. castaneum* by ozone when compared to laboratory conditions, 216,000 ppm-min

and 286,920 ppm-min respectively (McDonough et al. 2010). These studies highlight the variable effect of environmental conditions on ozone toxicity to insects.

Another potential mode of ozone application in pest management is dissolving ozone into aqueous solution. Insect mortality in response to aqueous ozone has not been as extensively studied as gaseous ozone exposure. Ebihara et al. developed an ozone-mist sprayer and reported >90% mortality of the red aphid, *Uroleucon nigrotuberculatum* (Olive) (Hemiptera: Aphididae) under greenhouse conditions (Ebihara et al. 2013). In this study, aphids were sprayed from a distance of 2-5 cm with gaseous ozone (8.4-3200 ppm) and water droplets combined at the nozzle, effectively making the experiment a gaseous ozone application. A second paper evaluated an axial fan radial airblast sprayer equipped an ozone generator unit attachment that was marketed to control plant diseases and insects (Grieshop et al. 2019). This study reported no control of bacterial, fungal and insect pests after a full season of applications at 0.75 ppm of aqueous ozone. However, neither of these studies evaluated aqueous ozone induced insect mortality under laboratory conditions.

Drososphila suzukii (Matsumura) (Diptera-Drosophilidae), spotted wing drosophila, is an invasive vinegar fly from Asia, first detected in California in 2008 (Bolda, Goodhue, and Zalom 2010). Drosophila suzukii quickly became a destructive invasive pest of soft fruits, due to its bladed ovipositor, short life cycle (~3 weeks) and broad host range. The fly prefers laying eggs in ripening fruit (Mitsui, Takahashi, and Kimura 2006), depositing its eggs using a serrated ovipositor. Spotted wing Drosophila completes an entire life cycle, from egg to adult, in 9-11 d at 25°C allowing dozens of generations per year (Kanzawa 1939). Spotted wing Drosophila readily reproduces in raspberries, blackberries, blueberries, strawberries, grapes and cherries and US estimated fruit crop losses in California, Oregon and Washington estimated at $511 annually (Walsh et al. 2011). Current management of this pest relies on repeated applications of broad-spectrum, contact insecticides from the pyrethroid, spinosyn and

organophosphate chemical classes (Bruck et al. 2011; Van Timmeren and Isaacs 2013). Certified organic producers rely almost entirely on organic formulations of Spinosyns (Van Timmeren and Isaacs 2013) to control fly populations, so the development of pesticide resistance is a serious concern. In 2019, the potential for resistance was identified for the Spinosyn class of insecticides for flies near Watsonville, CA (Gress and Zalom 2019). The development of novel insecticides to control *D. suzukii* populations is of growing concern to fruit growers.

The objectives of my study were to evaluate the potential of ozone as a pest management tool as either a 1) gas or 2) dissolved in water, using *D. suzukii* as a model organism. As reviewed above, gaseous ozone has known potential as a pest management tool based on the stored product pest literature; however, aqueous ozone has not been as extensively evaluated.

2. MATERIALS AND METHODS

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

2.1 Colony Details & Maintenance

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

2.2 Drosophila Handling

Flies were transferred between colony rearing vials and aging vials after anesthetization with CO_2 delivered via a Fly Stuff Fly Pad (Genesee Scientific, San Diego, CA) and fly handling with forceps (BioQuip Products Inc., Rancho Dominguez, CA). Flies were held in 50 ml vials containing fresh solid diet (Dalton et al. 2011) during the aging period in the colony chamber (see 2.1). Aged flies (~10 female and ~10 male) were anesthetized with CO_2 gas and then placed into 5.33 cm diameter spherical exposure cages (Olive Oil Marketplace, Staunton, IL) made of 304 stainless steel for aqueous ozone experiments. Gaseous ozone experiments used the same exposure cage, but were modified to 2.16 cm diameter.

Flies in cages from a single treatment and time exposure were placed into 0.34 m x 0.34 m x 0.6 m mesh insect arenas (BioQuip Products Inc., Rancho Dominguez, CA) following treatment application. Flies were then collected via aspiration from an arena and placed into a new vial, which was then stored in the colony chamber for observation (see 2.1).

2.3 Ozone Generation and Handling

Ozone was generated using a corona-discharge Nano Ozone Generator (Absolute Ozone, Edmonton, Canada) fed with 99.5% oxygen under a fume hood (Figure 2.1.). Gaseous ozone products were delivered to experimental arenas using PTFE tubing and 316 stainless steel fittings (Figure 2.1). Stainless steel and PTFE were used due to their extremely low reactivity coefficient with ozone. Gaseous ozone concentrations were monitored using a Model 106-H Ozone Monitor (2B Technologies, Boulder, CO) (Figure 2.2).

The two gaseous ozone concentrations evaluated (14,600 and 30,100 ppm) were generated by feeding oxygen gas at 2.5 SCFH and 4 psi through an ozone generator (Figure 2.2). An independent feed leading from the oxygen tank to the application chamber allowed for the dilution of ozone concentration within the experimental arena. The 14,600 and 30,100 ppm ozone treatments were generated by diluting the

ozone carrying gas with oxygen at 10 SCFH and 4 SCFH, respectively. Aqueous ozone was generated by bubbling gaseous ozone, approximately 65,000 ppm ozone, into 300 mL of double-distilled water (Dean Foods, El Paso, TX) inside a 473 mL glass jar (Ball Corporation, Broomfield, CO) for one hour (Figure 2.2.).

2.4 Experiment 1: Gaseous Ozone Mortality Response

Gaseous ozone dose response experiments were conducted on 4-8 d old male and female *D. suzukii* adults. Treatments were replicated 10 times over the course of 4 trials and a total of 2,381 male and female flies were sampled (Table 2.1). Treatments included an untreated control, oxygen (99.5%) treated control, 14,600 ppm gaseous ozone treatment and 30,100 gaseous ppm ozone treatment. (Table 2.1). Fly mortality was evaluated for 14,600 ppm and 30,100 ppm ozone treated flies at different doses, concentration-time products (CT), of: 5,000, 10,000, 20,000, 40,000 and 80,000 ppm-min (Table 2.1). For comparison, flies were exposed in the test chamber to 99.5% oxygen for 5.48 min, while untreated control flies were left untouched.

The gaseous ozone treatment apparatus (Figure 2.2) was created by attaching a 250 mL glass bubbler (150 mL of distilled water), a 250 mL 2-neck glass application chamber, an ozone monitor (106-H Ozone Monitor) and a 250 mL glass flask at the end for obtaining relative humidity values from exiting gas during oxygen treatments. Cages were attached to a hooked glass stopper and introduced to the application chamber via a separate neck on the side of the chamber once the proper concentration was maintained for at least two minute. Cages remained in the application chamber until the desired CT product was obtained. This process was repeated for all treatment applications in gaseous ozone. Gaseous ozone concentrations were measured during treatment applications for verification and consistency using the ozone monitor. Relative humidity and temperature of oxygen treatments of experiment 1 were measured inside a 250 mL glass flask during each trial using an electronic hygrometer sensor (Sensirion, Zurich, Switzerland) (Figure 2.2.). Relative humidity and temperature were not

monitored during ozone treatments due the possibility of sensor oxidation. Cages and forceps were hand washed before and after each replicate with detergent (Alconox, White Plains, NY) in order to reduce possible contamination.

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

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

Lethal concentration-time (LCT) values of 50 and 99 were compared between males and females within a model by using the 'EDcomp' function in R version 3.5.1 (Ritz and Strebig 2016). Differences between LCT values were calculated using standard errors derived by the delta method (Ritz et al. 2015). Functions were weighted based upon the number of individuals treated during each application (8-11

male, 9-11 female). Reported values were rounded to three significant figures to represent the relative accuracy of LCT estimates.

2.5 Experiment 2: Aqueous Ozone Concentration-Time Response

The aqueous ozone experiment was conducted by exposing 10 male and 10 female *D. suzukii* adults (5-8 d old) to dissolved ozone in distilled water. Experimental treatments included an untreated control, distilled water/0 ppm ozone control, distilled water/18.5 ppm ozone treatment, which was as high an aqueous concentration that could be developed using distilled water and a pure oxygen feed. The experiment was replicated 8 times during two trials and a total of 420 male and female flies were sampled (80 flies of a sex per treatment) (Table 2.1). Untreated control flies were grouped into vials without receiving treatment. The water control and ozonated water flies were caged and exposed to 300 ml of distilled water or 300 mL of ozonated distilled water, respectively, for 30 seconds after which they were dried on a paper towel. Aqueous ozone concentrations were measured at the beginning and end of each trial using the I-2019 ozone measuring kit (Chemetrics, Midland, VA). A 1:10 dilution (ozonated water:water) was performed prior to analysis and ozone concentration measurements were determined following manufacturer recommendations. Solution temperature and pH were measured using the 9107BNMD pH/ATC electrode (ThermoFisher Scientific, Waltham, MA) and Star A221 pH meter (ThermoFisher Scientific, Waltham, MA) before treatment application. Experimental apparatus were cleaned before and after each replicate with detergent (Alconox, White Plains, NY) in order to reduce possible contamination.

Experimental subjects of a single treatment and replication were transferred to separate vials (see 2.2) and mortality was measured every 24 hours over a 72-hour interval starting at 0 h after treatment application. Flies were considered dead if they were unable to return to a standing position after the vial was hand agitated. Mortality data were non-parametric and were analyzed using a one-

way Kruskall-Wallis test to determine the effect of treatment on female and male fly mortality at 0 h, 24 h, 48 h and 72 h. The 'kruskal.test' function was used in R version 3.5.1 (R Core Team 2015).

3. RESULTS

Female and male flies experienced high mortality during treatment application of 14,600 ppm and 30,100 ppm of gaseous ozone during experiment 1 (Figure 2.3). Conversely, aqueous ozone caused very little to no mortality after treatment application during experiment 2 (Figure 2.4.). Both experiments 1 and 2 demonstrated increased mortality rates at 24 h, 48 h and 72 h, where mortality rates were greatest at 72 hours.

3.1 Experiment 1: Gaseous Ozone Concentration-Time Response

Gaseous ozone treatments of 14,600 ppm and 30,100 ppm were applied to female and male *D. suzukii* to develop dose response curves and to obtain LCT 50 and 99 estimates. Control, untreated and oxygen treated flies, mortality was measured alongside ozone treatments. Ozone and control treatment flies were observed for a mortality at 0 h, 24 h, 48 h and 72 h. The mean ±SEM temperature and relative humidity of oxygen treatments across trials were 23.64 ±0.08 °C and 75.72 ±1.25%.

Kruskal-Wallis Rank Sum Tests determined no differences in mortality between untreated and oxygen treated flies at 0 h, 24 h, 48 h and 72 h (Chisq=NA, df=1, p=NA, Chisq=0.7647, df=1, p=0.3819, Chisq=0.1688, df=1, p=0.6812, Chisq=0.6064, df=1, p=0.435, respectively). The mean ± SEM mortality proportions of control flies (untreated and oxygen treated) at 0 h, 24 h, 48 h and 72 h were 0 ± 0, 0.015 ± 0.0057, 0.0175 ±0.0061 and 0.02 ±0.0064, respectively. Thus, the *c* parameter in the following dose response models was set to zero.

Female and male fly mortality was modelled after 14,600 ppm ozone treatments at different CT products. In general, LCT 50 and 99 values decreased overtime after ozone treatments (Figure 2.3.)

(Table 2.2 and 2.3). The LCT 50 (±SEM) estimates for females at 0 h, 24 h, 48 h and 72 h were (1.92 ± 0.11) x 10^4, (1.66 ± 0.1) x 10^4, (1.54 ± 0.1) x 10^4 and (1.47 ± 0.09) x 10^4, respectively (t=16.932, p<0.0001, t=16.05, p<0.0001, t=16.227, p<0.0001, t=15.969, p<0.0001, respectively) (Figure 2.3.) (Table 2.2.). The LCT 50 (±SEM) estimates for males at 0 h, 24 h, 48 h and 72 h were (1.86 ± 0.11) x 10^4, (1.51 ± 0.09) x 10^4, (1.48 ± 0.09) x 10^4 and (1.37 ± 0.08) x 10^4, respectively (t=17.196, p<0.0001, t=17.294, p<0.0001, t=16.949, p<0.0001, t=16.87, p<0.0001, respectively) (Figure 2.3.) (Table 2.2.). No differences in female and male LCT 50 estimates were observed upon 14,600 ppm ozone treatments (Table 2.4). Furthermore, no differences in female and male LCT 99 estimates were observed either (Table 2.4). The LCT 99 estimate for female and male flies at 72 hours after 14,600 ppm ozone treatments were (1.59 ± 0.33) x 10^5 and (1.17 ± 0.22) x 10^5, respectively (Table 2.3.).

Female and male fly mortality was modelled after 30,100 ppm ozone treatments at different CT products. The LCT 50 (±SEM) estimates for females at 0 h, 24 h, 48 h and 72 h were (1.48 ± 0.11) x 10^4, (1.19 ± 0.09) x 10^4, (1.05 ± 0.08) x 10^4 and (1 ± 0.07) x 10^4, respectively (t=13.6296, p<0.0001, t=13.7915, p<0.0001, t=13.333, p<0.0001, t=13.7348, p<0.0001, respectively) (Figure 2.3.) (Table 2.2.). The LCT 50 (±SEM) estimates for males at 0 h, 24 h, 48 h and 72 h were (0.84 ± 0.08) x 10^4 (0.72 ± 0.06) x 10^4, (0.69 ± 0.06) x 10^4 and (0.66 ± 0.06) x 10^4, respectively (t=10.9205, p<0.0001, t=12.5251, p<0.0001, t=11.1422, p<0.0001, t=11.1054, p<0.0001, respectively) (Figure 2.3.) (Table 2.2.). Significant differences were observed in female and male LCT 50 estimates after the 30,100 ppm ozone treatments at 0h, 24 h, 48 h and 72 h (Table 2.4). Ozone at a concentration of 30,100 ppm ozone resulted in a higher rate of mortality upon males than females at varying durations. For example, the estimated ratio, or relative potency, of 30,100 ppm on male flies was 1.8 times greater than female flies at 0 hours after treatment (Table 2.4.). Overtime, the relative potencies from males to females at 24 h, 48 h and 72 h were 1.7, 1.5 and 1.5, respectively (Table 2.4.). Finally, no differences were observed in female and male LCT 99 estimates (Table 2.4).

3.2 Experiment 2: Aqueous Ozone Mortality Response

Flies were exposed to aqueous ozone to determine mortality. The mean ± SEM ozone concentration of the ozonated distilled water treatment was 18.52 ± 0.76 ppm. The mean ± SEM temperature and pH of the distilled water and ozonated distilled water treatments across trials were 23.45 ± 0.25 °C and 6.38 ± 0.21, respectively. A Kruskal-Wallis rank sum test found no significant interaction of the main effect, treatment, for female or male flies at 0 h (Chisq=NA, df=2, p=NA, Chisq=2, df=2, p=0.3679, respectively), 24 h (Chisq=1.9368, df=2, p=0.3797, Chisq=2.1905, df=2, p=0.3345, respectively), 48 h (Chisq=2.5156, df=2, p=0.2843, Chisq=0.4842, df=2, p=0.785, respectively) and 72 h (Chisq=4.6, df=2, p=0.1003, Chisq=0.0261, df=2, p=0.987, respectively).

A single male fly died and no female flies died at 0 h after treatment application. The mean proportion of mortality for flies (females and males) observed in the untreated, control and ozone treatments at 72 hours were 0.05, 0.05 and 0.08, respectively. Mean mortality at 72 hours remained at or below 12.5% in all treatments of both sexes (Figure 2.4).

4. DISCUSSION

The goal of my research was to evaluate ozone, as a potential insecticide using the invasive fly species, *D. suzukii* as a model species. My data suggests that gaseous ozone at 14,600 ppm will instantly (0 h) cause 50% mortality of female and male flies after 1.16-1.46 min and 1.13-1.42 min, respectively (Figure 2.3.) (Table 2.2. and 2.3.). Additionally, gaseous ozone at 30,100 ppm rapidly (0 h) causes 50% mortality of females and males after 0.42-0.56 min and 0.23-0.33 min, respectively (Figure 2.3.) (Table 2.2. and 2.3.). Ozone concentrations of 14,600 ppm and 30,100 ppm demonstrated overlapping LCT 99 lower and upper 95% confidence levels, which indicates that both ozone concentrations attained 99 % mortality of flies at similar CT products (Table 2.3.). While gaseous ozone shows potential as an

insecticide, ozone dissolved in water is highly unlikely to have a lethal impact on *D. suzukii* under field conditions (Figure 2.4.).

Across all ozone treatments, the majority of mortality was observed at 0 hours after treatment with only a slight increase at 72 h after treatment. For example, male flies treated with 20,000 ppm-min of 14,600 ppm ozone observed mortality was 43.9% and 59.2% at 0 and 72 hours after treatment (Fig 3a, d). Thus, gaseous ozone was observed to have a good capacity for "knock-down" activity at the tested concentrations with flies succumbing in a matter of minutes to 14,600 ppm ozone and in mere seconds to 30,100 ppm ozone. It has been reported that fast knock-down activity is a common characteristic of currently recommended insecticides for *D. suzukii* control (Isaacs, Tritten, et al. 2013). My results show that ozone has limited residual/latent insecticidal properties after initial exposure, but may be used as an effective fast knock-down insecticide to control *D. suzukii* populations when applied as a fumigant within a closed environment.

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

The exposure of flies to ozone dissolved in water did not provide a detectable increase in mortality compared to a water or untreated control. The mean aqueous ozone concentration, 18.52

ppm, tested in this study is considerably higher than the 1-10 ppm produced by most commercial ozonation units used for sanitizing drinking water and wastewater (Oxidation Technologies, LLC 2017). Thus we feel it is safe to conclude that aqueous ozone has very little potential to develop lethal activity in insects using current application methodologies in agricultural pest management. Grieshop et al. (2019), evaluated a commercial airblast sprayer that delivered <1 ppm dissolved ozone concentration, and concluded that it did not effectively manage insect or disease pests of apples when used in a replicated, season-long experiment.

My experiments provide definitive evidence that while gaseous ozone has insecticidal potential, aqueous ozone does not. Thus, future applications of this technology should focus on systems where gaseous ozone concentrations can be maintained at suitable levels, for example on post-harvest material stored in a closed vessel or perhaps in greenhouse production. The ozone treatments were generated by using the same oxygen flow rate, but had differing overall flow rates from a separate oxygen dilution feed into the treatment chamber that allowed ozone concentration dilution. This factor may play a role in why we saw differing LCT 50 values between treatments. Additionally, application of ozone concentrations outside of contained areas proves difficult because the ability to accumulate toxic concentrations of ozone is diminished, while also presenting a significant public health/occupational hazard. For example, *D. suzukii* adults are free-living outdoor flies that infests fruits in orchards and vineyards, so applying gaseous ozone at 14,600 ppm or 30,100 ppm would be impossible without confining a treatment area.

In conclusion, this study produced the first ozone dose response curves on a *Dipteran* spp., *D. suzukii*, and found that ozone primarily causes mortality during direct ozone exposure, while causing reduced mortality rates over 72 h after ozone applications. The 14,600 ppm ozone treatment demonstrated similar LCT 50 estimates across fly sex, but the 30,100 ppm ozone treatment significantly

increased mortality of males in comparison to females when comparing LCT 50 estimates. Overall, both the 14,600 ppm and 30,100 ppm ozone treatments produced similar LCT 99 values, which supports the validity of the CT product quantification model for *D. suzukii*. Finally, aqueous ozone was not found to possess insecticidal characteristics and did not cause increased mortality of flies in comparison to controls.

APPENDIX

Figure 2.1. Ozone generation process

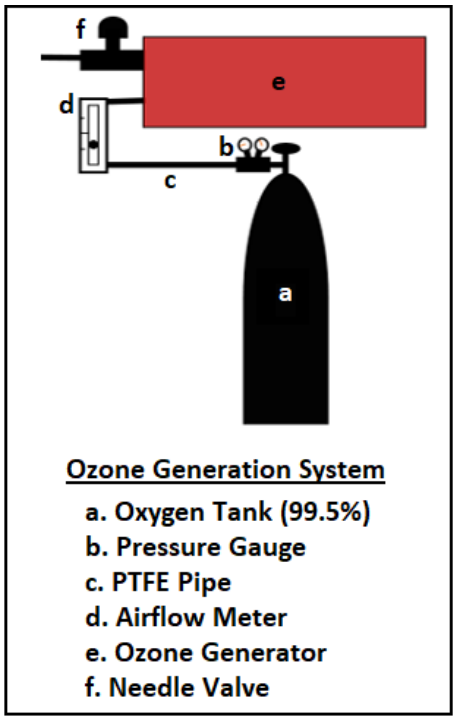

Figure 2.2. Aqueous & gaseous ozone treatment apparatus

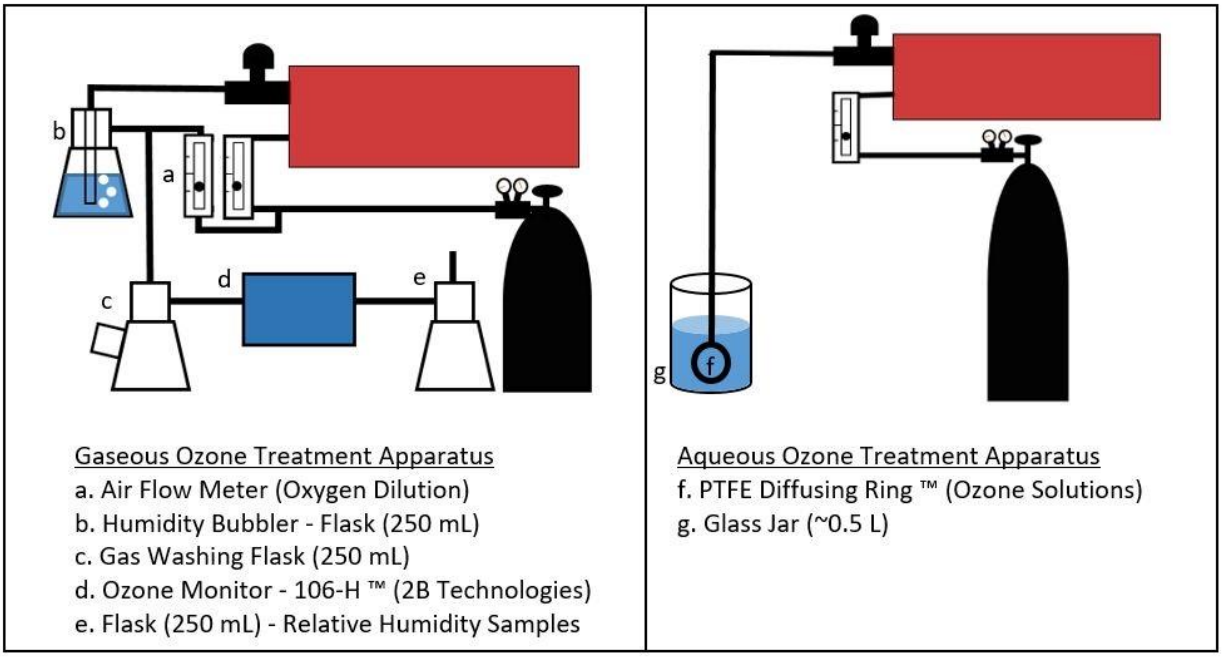

Figure 2.3. Gaseous ozone concentration-time product response curve at 0, 24, 48 & 72 hours for male and females.

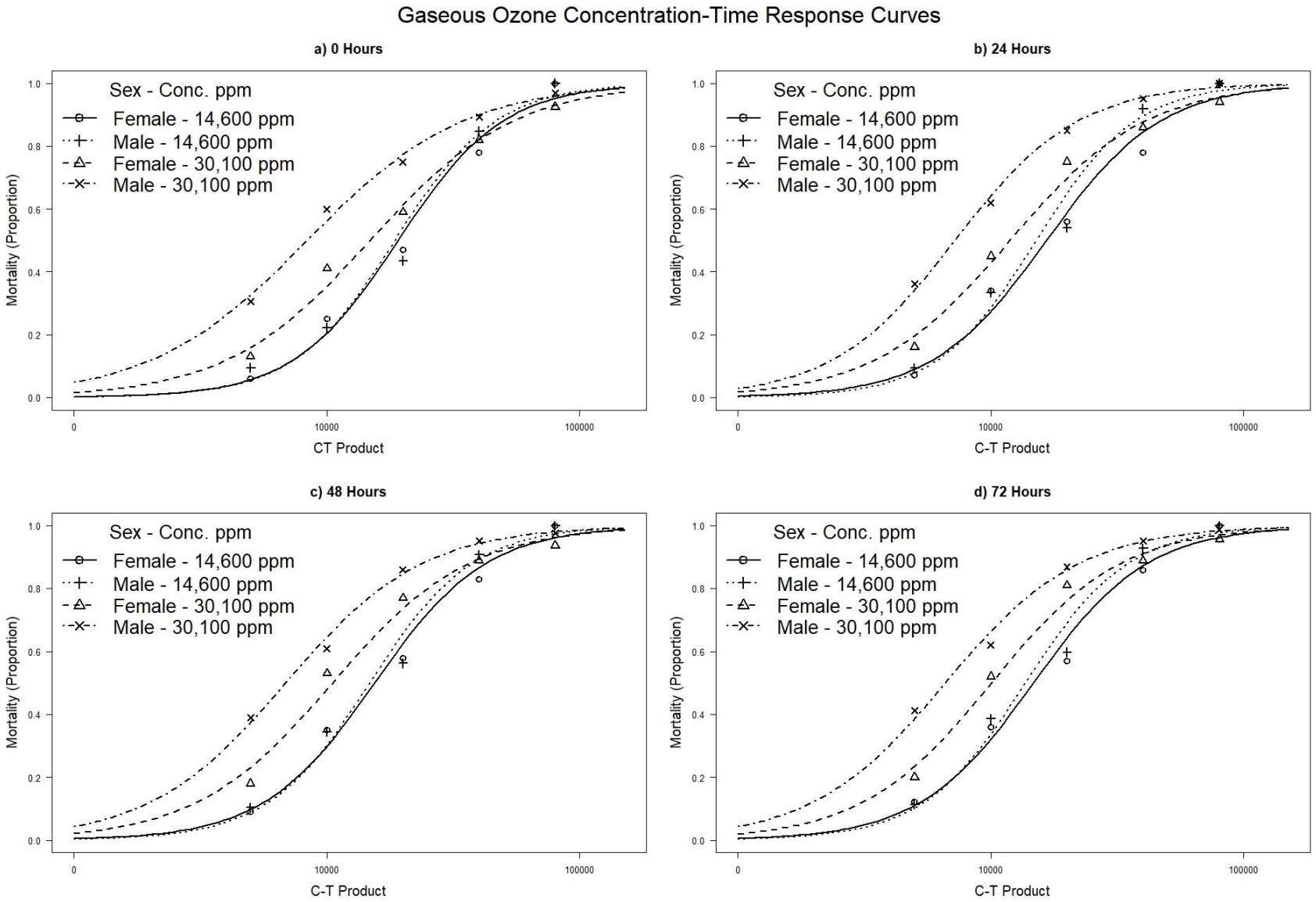

Figure 2.4. Boxplots of aqueous ozone adult fly mortality at 0, 24, 48 & 72 Hours of males and females.

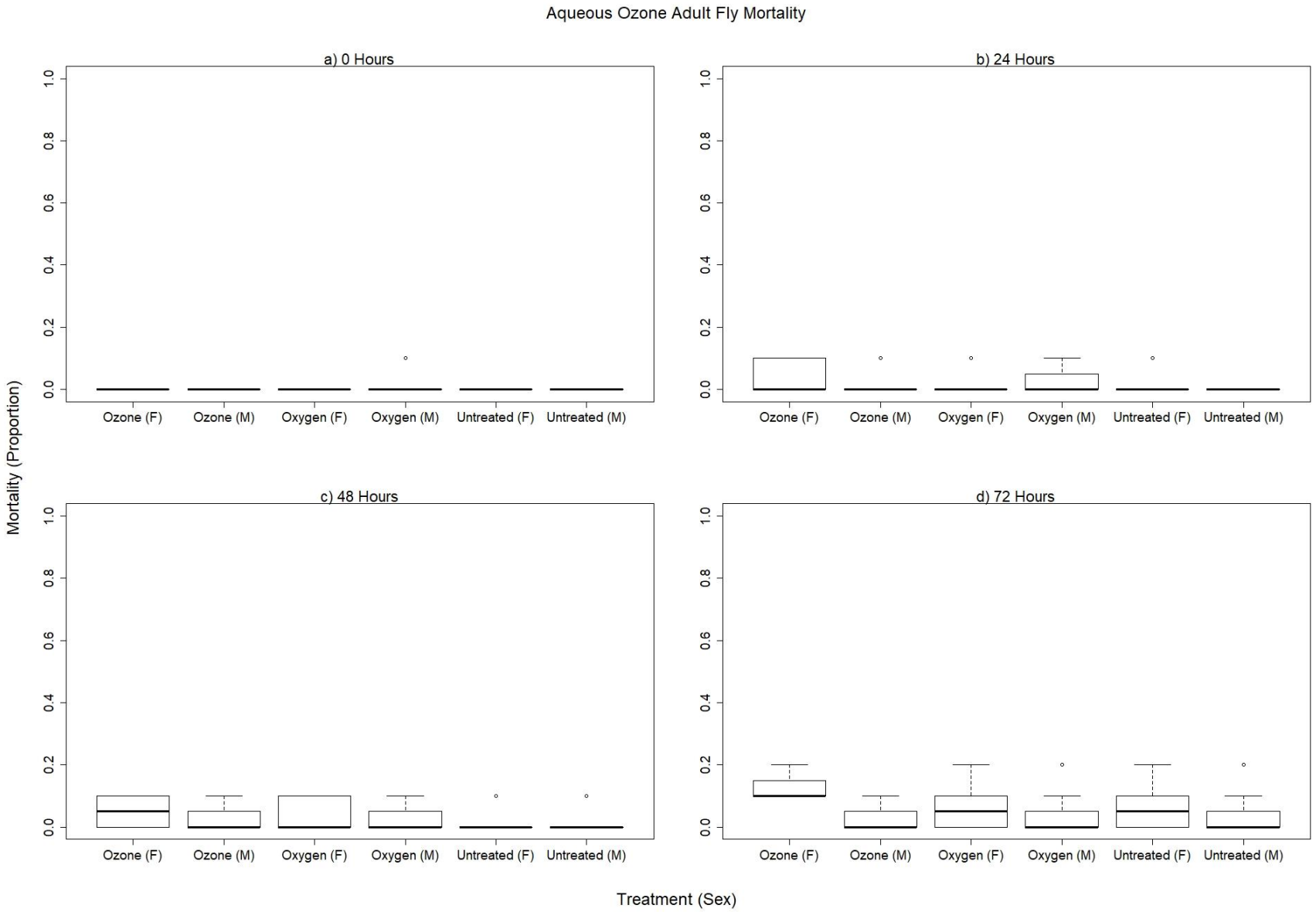

Table 2.1. Experiment 1 and 2 treatment parameters.

Experiment 1: Gaseous Ozone Mortality					
Treatments	**Concentration (ppm) 'Ozone : Oxygen'**	**Fly Age (Days)**	**Sample Size (M,F)**	**CT Product (ppm-min)**	**Exposure Time (Sec. ; Min.)**
14,600 ppm Ozone	14,600 : 980,400	4-8	98,99	5,000	20.55 ; 0.34
			99,100	10,000	41.10 ; 0.68
			98,100	20,000	82.19 ; 1.37
			99,100	40,000	164.38 ; 2.74
			100,100	80,000	328.77 ; 5.48
30,100 ppm Ozone	30,100 : 964,900	4-8	97,100	5,000	9.97 ; 0.17
			99,100	10,000	19.93 ; 0.33
			99,100	20,000	39.87 ; 0.66
			101,99	40,000	79.73 ; 1.33
			97,98	80,000	159.47 ; 2.66
Oxygen	0 : 995,000	4-8	99,99	0	328.77 ; 5.48
Untreated	-	4-8	100,100	-	-

Experiment 2: Aqueous Ozone Mortality					
Treatment	**Constituents**	**Age (Days)**	**Sample Size (M,F)**	**CT Product**	**Exposure Time (Sec., Min.)**
Ozone	Distilled Water/18.52 ppm Ozone	5-8	80,80	9.25	30, 0.5
Water	Distilled Water		80,80	0	30, 0.5
Untreated	-		80,80	-	-

Table 2.2. The LCT 50 (SEM) estimates and 95% confidence levels (lower-upper) of female and male flies from the 14,600 ppm and 30,100 ppm ozone treatments. Values were derived from the 0 h, 24 h, 48 h and 72 h observation time points.

LCT 50 Gaseous Ozone Data : 0 h, 24 h, 48 h and 72 h

Treatment	Sex	Sample Size	0 hours		24 hours		48 hours		72 hours	
			LCT 50 x 10^4	95% CL x 10^4	LCT 50 x 10^4	95% CL x 10^4	LCT 50 x 10^4	95% CL x 10^4	LCT 50 x 10^4	95% CL x 10^4
14,600 ppm	Female	499	1.92 (0.11)	1.69-2.14	1.66 (0.1)	1.46-1.86	1.54 (0.1)	1.36-1.73	1.47 (0.09)	1.29-1.65
	Male	494	1.86 (0.11)	1.65-2.07	1.51 (0.09)	1.34-1.68	1.48 (0.09)	1.31-1.65	1.37 (0.08)	1.21-1.53
30,100 ppm	Female	497	1.48 (0.11)	1.27-1.7	1.19 (0.09)	1.02-1.36	1.05 (0.08)	0.9-1.21	1 (0.07)	0.86-1.15
	Male	493	0.84 (0.08)	0.69-0.99	0.72 (0.06)	0.61-0.83	0.69 (0.06)	0.57-0.81	0.66 (0.06)	0.54-0.78

Table 2.3. The LCT 99 (SEM) estimates and 95% confidence levels (lower-upper) of female and male flies from the 14,600 ppm and 30,100 ppm ozone treatments. Values were derived from the 0 h, 24 h, 48 h and 72 h observation time points.

LCT 99 Gaseous Ozone Data : 0 h, 24 h, 48 h and 72 h

			0 hours		24 hours		48 hours		72 hours	
Treatment	**Sex**	**Sample Size**	**LCT 99 x 10^5**	**95% CL x 10^5**	**LCT 99 x 10^5**	**95% CL x 10^5**	**LCT 99 x 10^5**	**95% CL x 10^5**	**LCT 99 x 10^5**	**95% CL x 10^5**
14,600 ppm	Female	499	1.72 (0.33)	1.08-2.37	1.81 (0.37)	1.08-2.53	1.59 (0.32)	0.97-2.21	1.59 (0.33)	0.95-2.23
	Male	494	1.54 (0.28)	0.99-2.1	1.21 (0.22)	0.78-1.63	1.26 (0.23)	0.80-1.71	1.17 (0.22)	0.74-1.59
30,100 ppm	Female	497	3 (0.81)	1.41-4.58	2.03 (0.51)	1.03-3.02	1.83 (0.47)	0.92-2.74	1.51 (0.36)	0.8-2.22
	Male	493	2.16 (0.66)	0.88-3.45	0.95 (0.23)	0.51-1.39	1.19 (0.32)	0.57-1.81	1.08 (0.28)	0.53-1.63

Table 2.4. Delta method comparison of LCT 50 & 99 values between males and female flies at 14,600 ppm and 30,100 ppm ozone concentrations. A significant was marked with a '*' (* : 0.05, ** : 0.01, *** : 0.001).

Female and Male Ozone LCT Estimate Comparisons

LCT 50 Comparisons: 0 h, 24 h, 48 h and 72 h

Treatment	Time (Hour)	Estimate (Ratio)	Std. Error	T	P
14,600 ppm	0	1.0303	0.0854	0.3553	0.7224
	24	1.0974	0.0933	1.0440	0.2965
	48	1.0442	0.0891	0.4966	0.6195
	72	1.0744	0.0926	0.8035	0.4217
30,100 ppm	0	1.7747	0.2082	3.7203	0.0002 (***)
	24	1.6508	0.1780	3.6553	0.0003 (***)
	48	1.5315	0.1791	2.9673	0.0030 (**)
	72	1.5206	0.1761	2.9565	0.0031 (**)

LCT 99 Comparisons: 0, 24,48 and 72 Hours

Treatment	Time (Hour)	Estimate (Ratio)	Std. Error	T	P
14,600 ppm	0	1.1187	0.2950	0.4023	0.6875
	24	1.4993	0.4087	1.2216	0.2219
	48	1.2635	0.3441	0.7657	0.4439
	72	1.3645	0.3764	0.9683	0.3329
30,100 ppm	0	1.3851	0.5619	0.6854	0.4931
	24	2.1375	0.7383	1.5406	0.1234
	48	1.5382	0.5660	0.9509	0.3416
	72	1.4000	0.4973	0.8043	0.4212

CHAPTER 3. AN OZONOLYSIS BASED METHOD AND APPLICATIONS FOR THE NON-LETHAL MODIFICATION OF INSECT CUTICULAR HYDROCARBONS

ABSTRACT

Cuticular hydrocarbons (CHCs) are important constituents of the insect epicuticle that provide multiple functions. In *Drosophila* spp., CHCs provide desiccation resistance 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 (~45,000 ppm). *Drosophila suzukii* that were treated with ozone showed a 1.63-3.10 fold reduction in unsaturated CHCs with CHCs shown to regenerate over 108 h. Reductions/changes in CHCs were correlated with significantly reduced desiccation resistance in both male and female *D. suzukii* at one h after ozone treatment. Males and females 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 characterizing the creation of CHCs during a fly's lifespan as well as their various functions.

Keywords: Cuticular Hydrocarbons, Ozone, Drosophila, *Drosophila suzukii*, Desiccation Resistance, Unsaturated, Saturated

1. INTRODUCTION

The *Drosophila* spp. epicuticle contains a wide range of saturated and unsaturated hydrocarbons broadly identified as cuticular hydrocarbons (CHCs) (Jallon and David 1987; Howard et al. 2003; Robert J. Bartelt et al. 1986). *Drosophila* CHCs are known to prevent desiccation and provide semiochemical communication cues (Chung and Carroll 2015). CHC profiles have been identified for numerous *Drosophila* spp. and other insect species as described in extensive cuticular hydrocarbon books and reviews (Howard and Blomquist 2005; Blomquist and Bagnères 2010; Ginzel and Blomquist 2016).

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae) or spotted wing *Drosophila* is an Asian fruit fly that has invaded many temperate fruit growing areas, causing billions of dollars of annual damage to fruit crops in the United States (Bolda et al. 2010). The *Drosophila suzukii* cuticular hydrocarbon 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 cuticular hydrocarbons present, including monoenes, dienes (unsaturated hydrocarbons) and n-alkanes (saturated hydrocarbons) (Snellings et al. 2018). In particular, 7(Z)-tricosene is the most prevalent hydrocarbon on the fly's cuticle (Dekker et al. 2015; Snellings et al. 2018).

Alkenes, an unsaturated hydrocarbon, undergo oxidative splitting at the site of the carbon-carbon double bond by ozone and produces ketone, aldehyde and peroxide by-products (Criegee 1975). The mechanism controlling this process is called ozonolysis. Ozone is used for microbe sterilization, drinking/waste water/hazardous waste treatment, pest management and in analytical chemistry to identify organic constituents (Beroza and Bierl 1967; Stover and Jarnis 1981; Bryant et al. 1992; Masten and Davies 1994; Kim et al. 1999; McDonough et al. 2010). In past CHC studies, ozonolysis has been used to identify double bond positions of unsaturated hydrocarbons with dipteran and hymenopteran species

following extraction using chemical solvents (Antony et al. 1985; Bartelt et al. 1982; Bartelt et al. 1986). Studies using ozonolysis to identify insect unsaturated hydrocarbons have employed the ozone exposure methodology developed by Beroza and Bierl (1967). This methodology involves extracting cuticular hydrocarbons with a non-polar solvent and then treating the extract with ozone prior to GC-MS analysis (Beroza and Bierl 1967). Antony et al. (1985) demonstrated that cuticlular monoenes and dienes extracted from *Drosophila melanogaster* underwent cleavage via ozonolysis to varied extents. For example, researchers noted a "major" reduction in 7-tricosene after ozonolysis, while 9-tricosene experienced a "minor" reduction (Antony et al. 1985). According to Moreira et al. (2018), cuticular damage was imaged using scanning electron microscopy (SEM) after an ozone treatment (68 mg/L) of 150 minutes. They hypothesized that the outer most layer of the cuticle, the epicuticle, was experiencing damage from the ozone exposure. We believe that by performing an ozone treatment on *D. suzukii*, CHCs will be damaged and, thus can be quantified. No studies to date have explored the ozonolysis of unsaturated hydrocarbons on living insects.

CHCs play an important role in regulating insect resistance to desiccation (Ramsay 1935; A. G. Gibbs 2007).It has been hypothesized that as lipids change from solid to liquid, as determined by a critical temperature, the ability for water to diffuse out of an insect increases (Ramsay 1935; Hadley 1994; A. G. Gibbs 1998). Unsaturated and methyl branched alkanes were found to melt at lower temperatures than saturated hydrocarbons and provides evidence for greater cuticular permeability at elevated temperatures (A. Gibbs and Pomonis 1995). Saturated hydrocarbons are hypothesized to impart greater desiccation resistance than unsaturated hydrocarbons due to their higher melting points (Gibbs 1998).For example, *D. pseudoobscura* dwelling in arid regions had a greater abundance of long-chained saturated hydrocarbons than laboratory maintained colonies, which correlated to a reduction in water loss rate (WLR) (Toolson and Kuper-Simbron 1989).

Currently, we know that *in vitro* unsaturated cuticular hydrocarbons undergo ozonolysis. Additionally, while it is broadly understood that cuticular hydrocarbons play a role in desiccation resistance, this role has yet to be fully described. Our objectives were to determine how ozonolysis would affect the CHCs on living *D. suzukii,* evaluate the duration of these effects, and determine whether modifications to CHCs affected desiccation resistance.

2. MATERIALS AND METHODS

I performed three experiments to address these objectives. In experiment 1, analysis 1, I evaluated the effects of ozone on the CHCs of *D. suzukii* and in experiment 1, analysis 2, I focused on determining the duration of these effects. Experiment 2 explored the effect of ozone exposure on the desiccation resistance of *D. suzukii*.

2.1 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, Cat. # 8002-cs). 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.

2.2 Drosophila Handling

Drosopihla suzukii flies were removed from vials after emergence by using carbon dioxide to anesthetize them. *Drosophila suzukii* were separated by sex when anesthetized and placed into vials with 5 mL of solid diet (Dalton et al. 2011) and allowed to age (3-5 d or 9-11 d). Aged flies (30-60) of a single sex were placed with forceps and brushes into 316 stainless steel cages, then placed into the gas-washing flask for a 5 second treatment (Figure 3.2). Male and female flies in a single treatment were separated into groups of 30 to 60 flies due to the size of the stainless steel cage. Cages were fabricated

from 304 stainless steel spherical tea infusers that were 5.33 cm in diameter (Fu Store, 8541896633). Stainless steel cages were used due to their extremely low reactivity with ozone.

Flies were placed into new vials containing 5 mL solid diet and then the colony chamber within 5 minutes of treatment application. Flies in experiment 1 and 1.1 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. Aspiration was used in conjunction with using a 0.4 m x 0.4 m x 0.6 m mesh insect arena (Amazon, ASIN B01LN8ETBS) to collect living flies from the stainless steel cages after a 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 in experiment 2.

While *D. suzukii* cuticular hydrocarbons aren't considerably sexually dimorphic, they do show small differences in abundance of cuticular hydrocarbons between sexes (Dekker et al., 2015). Thus, to minimize the chance of contamination, all tools and surfaces coming into contact with flies outside of vials were cleaned with 70% ethanol between treatments and trials.

2.3 Ozone Treatment Application

An ozone generator (Absolute Ozone, Item: NANO, Edmonton, Canada) applied 45,000 ppm of humidified ozone by using 99.5% oxygen as a feed gas at 14 psi and a flow rate of 6.5 square cubic feet per h (SCFH) in a 250 mL glass gas-washing flask for 5 seconds (Figure 3.1.). Ozone was humidified in a 250 mL glass bubbler flask and 100 mL distilled water (Figure 3.1.). Control flies were treated with humidified 99.5% oxygen for 5 seconds and handled in the same fashion as the ozone treated flies, while untreated flies were not exposed to ozone nor pure oxygen. Gaseous ozone concentrations were measured during treatment applications using the Model 106-H Ozone Monitor (2B Technologies,

Boulder, CO). Relative humidity and temperature of oxygen treatments were measured inside a 250 mL glass flask (Figure 3.1.) during each trial using an electronic hygrometer sensor (Sensirion, EK-H4).

2.4 Cuticular Hydrocarbon Collection and Analysis

After treatment, flies in vials containing diet were anesthetized with carbon dioxide for CHC extraction. Flies (five) of the same treatment and sex were placed by forceps into a ½ dram glass vial (Kimble Glass Incorporated, Art. No. 60910L 12) along with 200 µL of a hexane wash (Figure 3.2). The hexane wash contained an internal standard of 25 ng/µL hexacosane (Sigma-Aldrich, #241687-5G). The hexane wash was added to the glass vials by using 100 µL calibrated glass pipets (VWR International, Cat. No. 53432-921) and an aspirator (VWR International, Cat. No. 53432-921). Flies were left to wash in the hexane for 10-15 minutes. Samples were then placed on a mini-vortex (Fisher Scientific, Cat. No. 12 810 1) for 30 seconds at a vortex rate of 5. The hexane solution was transferred into a 0.25 mL glass insert (Supelco, Cat. No. 24717) inside a 2 mL glass vial (Supelco, Cat. No. 27330) for GCMS analysis. The 2 mL glass vials were capped with 9 mm Blue S/T Caps (Supelco, 29044-U).

Samples were run through a DB-17HT column (Agilent Technologies, Part No. 122-1831) that had a length of 30 m, a diameter of 0.25 mm and a 0.15 µm film (Figure 3.2). Helium was used as a carrier gas at a rate of 1 mL/min through a gas chromatograph/mass spectrometry instrument (GC/MS) (Agilent Technologies, 5975C Series GC/MSD). 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 minutes once 300°C was reached.

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 amount per fly.

2.5 Experiment 1: Ozonolysis of Hydrocarbons

Comparison of unsaturated CHCs, cuticular aldehydes and saturated CHCs 1 h after ozonolysis

The CHC profile of ozone treated, oxygen treated and untreated, 3-5 d old male and female flies were compared. Treatments consisted of an untreated control, oxygen treatment (99.5% purity) and an ozone treatment (45,000 ppm) (Table 3.1.). Treatments followed procedures outlined in section 2.3 Ozone Treatment Application. Total amount, in nanograms (ng), of unsaturated CHCs (5(Z)-tricosene, 7(Z)-tricosene, 9(Z)-tricosene/tricosane, 5(Z)-pentacosene, 7(Z)-pentacosene, 9(Z)- pentacosene), cuticular aldehydes (heptanal, nonanal, tetradecanal, pentadecanal, hexadecanal, octadecanal) and saturated CHCs (heneicosane, heptacosane, 2-methyl octacosane, nonacosane) peaks were quantified from CHC extracts from living flies 1 h after treatment application.

Collection and analysis of cuticular hydrocarbon extraction samples followed the procedures explained in section 2.4. Cuticular hydrocarbon extraction samples (5) were collected for quantitation and statistical analysis for each sex and treatment. Amounts of unsaturated CHCs, cuticular aldehydes and saturated CHCs did not fit normal distributions so non-parametric analyses were used to analyze data. A Kruskal-Wallis rank sum test analyzed unsaturated CHCs, cuticular aldehydes and saturated CHCs observations based on experimental treatment (ozone, oxygen, untreated). 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).

2.6 Experiment 1: Hydrocarbon Regeneration

Comparison of unsaturated CHCs, cuticular aldehydes and saturated CHCs 1, 12, 36, 108 h after ozonolysis

I measured *D. suzukii* CHCs over time (at 1, 12, 36, 108 h) with methodology as detailed in experiment 1 on 3-5 d and 9-11 d old flies (Table 3.1.). A 2 x 4 factorial ANOVA model analyzed unsaturated and saturated CHC observations based on experimental treatment (ozone and untreated) and CHC extraction time after treatment (1, 12, 36, 108 h) as fixed factors. ANOVAs were performed in R version 3.5.1 with the 'aov' function (R Core Team 2015). The 'TukeyHSD' function was applied for post-hoc analysis (R Core Team 2015). Aldehyde data were first fit to a linearized model, 'lm' function, in base R before ANOVA analysis and then a post-hoc Tukey test (R Core Team 2105).

2.7 Experiment 2: Desiccation Resistance Assessment

Two desiccation resistance trials were performed on male and female *D. Suzukii* (3-5 d), the first evaluated flies 1 h after ozonolysis and the second 108 h after ozonolysis. Methods were modified from Folk et al. (2001) desiccation study (Folk, Han, and Bradley 2001). Experimental arenas consisted of 50 mL polystyrene vials (Lab Express, Cat. # 8002-cs) containing 4.5 grams of drierite (W. A. Hammond Drierite Co. Ltd., Stock No: 11001) at their base with a permeable plastic barrier placed above the drierite. Nine to 11 single sex adult flies were placed into each arena and vials were capped with plastic wrap (Gordon Food Services, Item: 115193). Relative humidity and temperature was measured inside an experimental vial without flies during each trial using an electronic hygrometer sensor (Sensirion, EK-H4). Fly survival was assessed at 30 minute 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. Trials were completed when all

flies had died, so there were no surviving individual specimens at the end of the trial. This means no censored data were included in Kaplan-Meier analyses.

Data for both trials were analyzed using Kaplan-Meier survival curves followed 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 et al. 2020). Trial one compared the survival of male or female *D. suzukii* survival 1 hour 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.

3. RESULTS

Chromatograms of *D. suzukii* CHCs for untreated controls and ozonated flies reveal striking differences at 1 h following ozonolysis that disappeared after 108 h after ozonolysis. Figure 3.3 presents a comparison of *D. suzukii* CHCs for untreated flies and flies 1 h after ozonolysis. The peaks of 7-tricsosene and other unsaturated CHCs appear heavily reduced, while new cuticular aldehyde peaks (heptanal, nonanal, tetradecanal, pentadecanal, hexadecanal, octadecanal) were detected after ozonolysis on treated flies. Figure 3.4. presents a comparison of *D. suzukii* CHCs for untreated flies and flies 108 h after ozonolysis. The peaks for 7-tricosene and unsaturated CHC appear similar to those observed for the untreated flies, where the amounts of cuticular aldehydes (heptanal, nonanal, tetradecanal, pentadecanal, hexadecanal, octadecanal) were reduced or weren't present.

3.1 Experiment 1: Ozonolysis of Hydrocarbons

Comparison of unsaturated CHCs, cuticular aldehydes and saturated CHCs 1 h after ozonolysis

The amount of unsaturated CHCs, cuticular aldehydes and saturated CHCs at 1 h after treatment for 3-5 d and 9-11 d flies is presented in Table 3.2. Separate Kruskal Wallis models were performed for each fly age and sex combination for unsaturated CHCs, cuticular aldehydes and saturated CHCs, respectively (12 models total).

Unsaturated CHCs of 3-5 d old females were significantly reduced by ozonolysis with a 2.03 and 1.89 fold reduction compared to oxygen treated and untreated flies, respectively (Chisq=9.62, df=2, p=0.0081), while they were similar between oxygen and untreated flies. A similar pattern was observed for 9-11 d old females with a 2.79 and 2.63 fold reduction compared to oxygen treated and untreated flies, respectively (Chisq=10.22, df=2, p=0.0060), and no difference between oxygen and untreated flies. Likewise, 3-5 d old males demonstrated a 2.85 and 3.10 fold reduction of ozone treated unsaturated CHCs compared to oxygen treated and untreated flies, respectively (Chisq=11.18, df=2, p=0.0037), and similar CHC content between oxygen and untreated flies. 9-11 d old males presented a slightly different pattern resulting from ozonolysis, where the amount of unsaturated CHCs were reduced by 1.63 and 2.28 fold as compared to levels extracted in oxygen treated and untreated flies, and a 1.4 fold reduction in levels observed between the oxygen and untreated control flies (Chisq=11.52, df=2, p=0.0031).

The amount of cuticular 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 (Chisq=13.29, df=2, p=0.0013, Chisq=13.29, df=2, p=0.0013, Chisq=13.29, df=2, p=0.0013, Chisq=13.29, df=2, p=0.0013, respectively). No cuticular 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 amounts of saturated CHCs of 3-5 d old females were significantly increased after ozone treatment compared to oxygen treated and untreated flies (Chisq=9.42, df=2, p=0.0090), while they were similar between oxygen and untreated flies. Saturated CHCs of 9-11 d old females experienced no difference between ozone treated, oxygen treated and untreated flies (Chisq=1.46, df=2, p=0.4819). The saturated CHC amount of 3-5 d old males treated with ozone and oxygen were significantly reduced in comparison to untreated flies (Chisq=8.96, df=2, p=0.0113), while similar between ozone treated and oxygen treated flies. The saturated CHC amount of 9-11 d old males experienced no difference between ozone treated, oxygen treated and untreated flies (Chisq=4.58, df=2, p=0.1013).

3.2 Experiment 1: Hydrocarbon Regeneration

Comparison of unsaturated CHCs, cuticular aldehydes and saturated CHCs 1, 12, 36, 108 h after ozonolysis

The amount of unsaturated CHCs, cuticular aldehydes and saturated CHCs at 1, 12, 36, 108 h after treatment for 3-5 d and 9-11 d flies is presented in Table 3.3 and Figures 3..5-7. Separate ANOVA models were performed for each fly age and sex combination for unsaturated CHCs, cuticular aldehydes and saturated CHCs, respectively (12 models total). Amounts at 108 h for female 9-11 d flies were excluded from analysis due to contamination of CHC extractions.

Unsaturated CHCs of 3-5 d old females were significantly affected by the main effect of hour ($F=43.818$, $df=3, 32$, $p<0.0001$) as well as the treatment and hour interaction ($F=17.965$, $df=3, 32$, $p<0.0001$), but was not affected by the main effect of treatment ($F=0.094$, $df=1, 32$, $p=0.761$). The general trend for ozone treated flies was an increase in unsaturated CHCs over time, while untreated control flies did not demonstrate a consistent pattern (Figure 3.5a.). Unsaturated CHCs were significantly lower for ozone treated flies compared to untreated flies at 1 h after treatment with mean ± SEM values of 721.18 ± 52.37 and 1365.9 ± 44.91 ng, respectively. In contrast, unsaturated CHCs were

significantly higher for ozone treated flies compared to untreated flies at 36 h after treatment with mean ± SEM values of 2145.39 ± 79.08 and 1489.14 ± 112.27 ng, respectively.

Unsaturated CHCs of 9-11 d old females were significantly affected by the main effects of treatment ($F=73.10$, $df=1, 24$, $p<0.0001$), hour ($F=24.63$, $df=2, 24$, $p<0.0001$) and an interaction between treatment and hour ($F=24.92$, $df=2, 24$, $p<0.0001$). The general trend for ozone treated flies was an increase in unsaturated CHCs over time, while untreated control flies did not show a consistent pattern (Figure 3.5b.). Unsaturated CHCs were significantly lower for ozone treated flies compared to untreated flies at 1 h after treatment with mean ± SEM values of 1016.92 ± 85.03 and 2663.90 ± 107.83 ng, respectively, and at 12 h after treatment with mean ± SEM values of 1836.7 ± 81.97 and 2634.77 ± 140.26 ng, respectively.

Unsaturated CHCs of 3-5 d old males were significantly affected by the main effects of treatment ($F=173.26$, $df=1, 32$, $p<0.0001$), hour ($F=149.97$, $df=3, 32$, $p<0.0001$) and an interaction between treatment and hour ($F=27.38$, $df=3, 32$, $p<0.0001$). The general trend for ozone treated and untreated flies was an increase in unsaturated CHCs over time, however, untreated control flies remained unchanged between 12, 36 and 108 h (Figure 3.5c.). Unsaturated CHCs were significantly lower for ozone treated flies compared to untreated flies at 1 h (1016.92 ± 85.03 and 2663.90 ± 107.83 ng, mean ± SEM values respectively), 12 h (1634.62 ± 55.36 and 2658.51 ± 45.12 ng, mean ± SEM values respectively), and 36 h (2130.23 ± 96.70 and 2870.87 ± 71.65, mean ± SEM values respectively).

Unsaturated CHCs of 9-11 d old males were significantly affected by the main effects of treatment ($F=68.52$, $df=1, 32$, $p<0.0001$), hour ($F=83.37$, $df=3, 32$, $p<0.0001$) and an interaction between treatment and hour ($F=14.54$, $df=3, 32$, $p<0.0001$). The general trend for ozone treated flies was an increase in unsaturated CHCs over time, while untreated control flies consistently remained unchanged until an increase at 108 h (Figure 3.5d.). Unsaturated CHCs were significantly lower for ozone treated

flies compared to untreated flies at 1 h (893.95 ± 110.10 and 2036.24 ± 64.17 ng, mean ± SEM values respectively), 12 h (1313.85 ± 103.99 and 2071.64 ± 124.13 ng, mean ± SEM values respectively), and 36 h (1630.74 ± 87.71 and 2092.27 ± 27.64, mean ± SEM values respectively).

Cuticular aldehydes of 3-5 d old females were significantly affected by the main effects of treatment ($F=618.3$, $df=1, 32$, $p<0.0001$), hour ($F=201.4$, $df=3, 32$, $p<0.0001$) and an interaction between treatment and hour ($F=201.4$, $df=3, 32$, $p<0.0001$). The general trend for ozone treated flies was a decrease in cuticular aldehydes over time, while untreated flies consistently did not detect any cuticular aldehydes (Figure 3.6a.). Cuticular aldehydes were significantly higher for ozone treated flies compared to untreated flies at 1 h (573.67 ± 17.61 and 0 ± 0 ng, mean ± SEM values respectively) and 12 h (237.32 ± 31.46 and 0 ± 0 ng, mean ± SEM values respectively).

Cuticular aldehydes of 9-11 d old females were significantly affected by the main effects of treatment ($F=1320.9$, $df=1, 24$, $p<0.0001$), hour ($F=296.5$, $df=2, 24$, $p<0.0001$) and an interaction between treatment and hour ($F=296.5$, $df=2, 24$, $p<0.0001$). The general trend for ozone treated flies was a decrease in cuticular aldehydes over time, while untreated flies consistently did not detect any cuticular aldehydes (Figure 3.6b.). Cuticular aldehydes were significantly higher for ozone treated flies compared to untreated flies at 1 h (715.03 ± 28.34 and 0 ± 0 ng, mean ± SEM values respectively), 12 h (344.6 ± 12.48 and 0 ± 0 ng, mean ± SEM values respectively) and 36 h (89.79 ± 6.43 and 0 ± 0 ng, mean ± SEM values respectively).

Cuticular aldehydes of 3-5 d old males were significantly affected by the main effects of treatment ($F=1493.9$, $df=1, 32$, $p<0.0001$), hour ($F=396.9$, $df=3, 32$, $p<0.0001$) and an interaction between treatment and hour ($F=396.9$, $df=3, 32$, $p<0.0001$). The general trend for ozone treated flies was a decrease in cuticular aldehydes over time, while untreated flies consistently did not detect any cuticular aldehydes (Figure 3.6c.). Cuticular aldehydes were significantly higher for ozone treated flies

compared to untreated flies at 1 h (382.04 ± 11.14 and 0 ± 0 ng, mean ± SEM values respectively), 12 h (214.46 ± 9.14 and 0 ± 0 ng, mean ± SEM values respectively) and 36 h (58.77 ± 9.14 and 0 ± 0 ng, mean ± SEM values respectively).

Cuticular aldehydes of 9-11 d old males were significantly affected by the main effects of treatment ($F=340.8$, $df=1, 32$, $p<0.0001$), hour ($F=109.5$, $df=3, 32$, $p<0.0001$) and an interaction between treatment and hour ($F=109.5$, $df=3, 32$, $p<0.0001$). The general trend for ozone treated flies was a decrease in cuticular aldehydes over time, while untreated flies consistently did not detect any cuticular aldehydes (Figure 3.6d.). Cuticular aldehydes were significantly higher for ozone treated flies compared to untreated flies at 1 h (443.02 ± 31.79 and 0 ± 0 ng, mean ± SEM values respectively) and 12 h (189.79 ± 19.59 and 0 ± 0 ng, mean ± SEM values respectively).

Saturated CHCs of 3-5 d old females were significantly affected by the main effects of treatment ($F=93.355$, $df=1, 32$, $p<0.0001$), hour ($F=8.867$, $df=3, 32$, $p=0.0002$) and an interaction between treatment and hour ($F=3.995$, $df=3, 32$, $p=0.0159$). There is a slight increase in saturated CHCs for ozone treated and untreated flies over time (Figure 3.7a.). Saturated CHCs were significantly higher for ozone treated flies compared to untreated flies at 1 h (495.27 ± 12.35 and 319.13 ± 8.55 ng, mean ± SEM values respectively), 12 h (599.79 ± 29.93 and 423.88 ± 42.62 ng, mean ± SEM values respectively) and 36 h (653.88 ± 26.89 and 370.18 ± 33.98 ng, mean ± SEM values respectively).

Saturated CHCs of 9-11 d old females were significantly affected by the main effect of treatment ($F=16.564$, $df=1, 24$, $p=0.0004$), but was not affected by the main the effect of hour ($F=2.115$, $df=2, 324$, $p=0.1425$) or the treatment and hour interaction ($F=1.503$, $df=2, 24$, $p<0.2425$). The general trend for ozone treated flies was an increase in saturated CHCs over time, while untreated flies remained unchanged (Figure 3.7b.). Saturated CHCs were significantly higher for ozone treated flies compared to untreated flies at 36 h (860.37 ± 52.98 and 659.14 ± 36.62 ng, mean ± SEM values respectively).

Saturated CHCs of 3-5 d old males were significantly affected by the main effect of hour ($F=110.814$, $df=3, 32$, $p<0.0001$), but was not affected by the main the effect of treatment ($F=3.982$, $df=1, 32$, $p=0.0545$) or the treatment and hour interaction ($F=2.303$, $df=3, 32$, $p=0.0957$). The general trend for ozone treated and untreated flies was an increase in saturated CHCs over time (Figure 3.7c.).

Saturated CHCs of 9-11 d old males were significantly affected by the main effect of hour ($F=43.334$, $df=3, 32$, $p<0.0001$), but was not affected by the main effect of treatment ($F=4.037$, $df=1, 32$, $p=0.053$) or the treatment and hour interaction ($F=0.383$, $df=3, 32$, $p=0.766$). Saturated CHCs of ozone treated and untreated flies remained unchanged over time, except for an increase in both groups at 108 hours (Figure 3.7d.). The saturated CHCs in ozone treated and untreated flies increased 61% and 65%, respectively, from 36 h to 108 h.

3.3 Experiment 2: Desiccation Resistance Assessment

Kaplan-Meier survival curves for desiccation trials conducted 1h and 108 h after ozonolysis are presented in Table 3.4 and Figure 3.7. 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 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. Output from Cox Proportional Hazard models for the two trials is presented in Table 3.5. Optimized Cox Proportional Hazard models were developed for male and female flies treated 1h following ozonolysis, allowing the oxygen and untreated controls to be combined into a single survival group (Crawley 2007; RICH et al. 2010). Oxygen treated and untreated fly survivals were not significantly different for female and male flies at 1 h after treatment application ($z=0.5260$, $d.f.=2$, $p=0.5990$, $z=-0.4250$, $d.f.=2$, $p=0.6710$, respectively). No significant difference was observed between model 1 (separated control observations)

and model 3 (combined control observations) within female or male flies at 1 h after treatment application (Chisq=0.2761, d.f.=1, p=0.5993, Chisq=0.1809, d.f.=1, p=0.6706, respectively) (Table 3.5.).

Mantel-Haenszel log-rank tests of female and male survival 1 h after ozonolysis (trial 1) showed significant differences between ozone treated, oxygen treated and untreated flies (Chisq=158, d.f.=2, p<0.0001, Chisq=75, d.f.=2, p<0.0001, 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, d.f.=1, p<0.0001; z=8.411, d.f.=1, p<0.0001, respectively). Ozonated females had a Hazard Ratio of 4.267 compared to control flies with median times of death of 2.5 h and 4.5 h, respectively (Table 3.5). Similarly, ozonated males had a Hazard Ratio of 2.467 compared to control flies with median times of death of 2 h and 3 h, respectively (Table 3.5).

Mantel-Haenszel log-rank tests of female and male survival 108 h after ozonolysis showed significant differences between ozone treated and untreated flies (Chisq=4.2, d.f.=1, p=0.04, Chisq=22.4, d.f.=1, p<0.0001, respectively). Cox Proportional Hazard models of female and male survival 108 h after ozonolysis showed that ozone treated flies had significantly reduced survival times compared to untreated flies 1 h after ozonolysis (z=-1.9720, d.f.=1, p=0.0486, z=-4.9420, d.f.=1, p<0.0001, respectively). Ozonated females had a Hazard Ratio of 0.7525 compared to control flies with median times of death of 4.5 h and 4 h, respectively (Table 3.5). Similarly, ozonated males had a Hazard Ratio of 0.4695 compared to control flies with median times of death of 2.5 h and 2 h, respectively (Table 3.5).

4. DISCUSSION

My data demonstrates that ozonolysis of live *D. suzukii* significantly reduces the amounts of unsaturated CHCs, increases that of CHC aldehydes but does not affect the levels of saturated CHCs (Figure 3.5.) (Table 3.3.). Furthermore, the levels of CHCs on flies treated with ozone return to untreated CHC levels within 12 -108 h after treatment application (Figure 3.5.) (Table 3.3.). Flies

regenerated unsaturated CHCs at different rates depending on their sex and age (Figure 3.5.) (Table 3.3.). Desiccation resistance was correlated to changes in CHC abundance, with an immediate decrease followed by recovery over the same time period. (Figure 3.6) (Table 3.4). Surprisingly, desiccation resistance significantly increased in comparison to untreated controls at 108 h after treatment application, although a concurrent significant increase in unsaturated CHCs was only observed for females at 3-5 d at 36 h after treatment application (Figure 3.5.) (Table 3.3.).

My study is the first to quantify ozonolysis of CHCs on living insect specimens and may provide an important new method for exploring the generation, structure and function of these important constituents of the insect epicuticle. Current methodology for the modification of living insect CHCs include genetic modification and direct CHC application via perfuming (Ferveur 1997). 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. The methods developed in our paper could hypothetically be used to modify the unsaturated CHCs of any insect model and allow quantification of CHC generation time, potential for regeneration as well as how they modify behavior and survival.

While CHC generation in *Drosophila* species has been elucidated in previous work (Robert J. Bartelt et al. 1986; Jallon and David 1987; Toolson and Kuper-Simbron 1989; Dekker et al. 2015; Snellings et al. 2018), this is a novel study that provides data on the regeneration of unsaturated CHCs following their removal on living subjects. The regeneration of CHCs to untreated levels suggests that maintaining proper amounts of 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; A. G. Gibbs 1998; Howard and Blomquist 2005; Blomquist and Bagnères 2010; Ortiz-Urquiza and Keyhani 2013; Chung and Carroll 2015). The variety and importance of CHC functions may provide insight as to why CHCs regenerate within the period of a few days.

The results also provide evidence that *D. suzukii* of different sexes and ages have the ability to regenerate CHCs to untreated levels, albeit point estimates of CHC's vary across these groups (Figure 3.5.) (Table 3.3.). Females at 3-5 d and 9-11 d regenerated unsaturated CHCs to untreated fly amounts by 12 and 36 h after treatment application, respectively (Figure 3.5.) (Table 3.3.). Both males at 3-5 d and 9-11 d regenerated unsaturated CHCs to untreated fly amounts by 108 h after treatment application (Figure 3.5.) (Table 3.3.). This suggests that females either have a greater capacity to generate CHCs.

Insect CHCs have been found to decrease water permeability through the cuticle and, thus, increasing desiccation resistance (Quinlan and Hadley 1993; A. G. Gibbs 1998; Blomquist and Bagnères 2010; Chung and Carroll 2015). Desiccation resistance was shown to be greatly reduced immediately following ozone treatments, but returned to pre-treatment levels after regeneration of unsaturated CHCs (Figure 3.6.) (Table 3.4.). This finding supports the hypothesis that CHCs function to reduce cuticle water permeability (Ramsay 1935; Gibbs 1998) and that *D. suzukii* unsaturated CHCs play a significant role in desiccation resistance. This finding is of interest because it has been previously suggested that saturated CHCs are generally more correlated with desiccation resistance due to their higher melting points (A. Gibbs and Pomonis 1995). Additional supporting evidence suggests that a greater abundance of long-chained saturated CHCs impart greater desiccation resistance by reducing the water loss rate (WLR) in *D. pseodoobscura* and *Tibicen dealbatus* (Homoptera: Cicadidae) (Toolson 1984; Toolson and Kuper-Simbron 1989).

My 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 (A. G. 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 CHCs. 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 by showing that a layer of the epicuticle is extensively damaged after an ozone treatment.

While the data strongly suggest that desiccation resistance is directly linked to unsaturated hydrocarbon content, it is possible that differences were due to ozone induced off-target effects. The ozonolysis performed in this experiment, while largely non-lethal, did result in some mortality. This data was not collected but, dose response curves developed in Savage (2020) predict that a CT product of 3,750 ppm-min of gaseous ozone would result in 11% and 25% mortality immediately following ozonolysis for males and females, respectively (Benjamin A. Savage 2020). One potential, non-desiccation, source of mortality could be tracheal damage resulting from ozone exposure. However, Sousa et al. (2008) examined the respiration rates of *T. castaneum, R. dominica* and *O. surinamensis* and concluded that ozone toxicity and respiration rates did not correlate (Sousa et al. 2008). This study did not directly measure the respiration rate of specimens after ozone exposure.

Potential future applications of live ozonolysis of CHCs includes characterizing arthropod physiology and behavior in regards to desiccation, chemical communication and entomopathogen resistance (Quinlan and Hadley 1993; A. G. Gibbs 1998; Howard and Blomquist 2005; Blomquist and Bagnères 2010; Ortiz-Urquiza and Keyhani 2013; Chung and Carroll 2015). Unsaturated CHCs have been shown to be important in *Drosophila spp.* for identification of conspecifics and courtship/mating behaviors (Antony et al. 1985; Jallon and David 1987; Ferveur 1997; Howard and Blomquist 2005; Ferveur 2005). For example, 7,11-heptacosadiene has been shown to be an aphrodisiac for male *D. melanogaster* (Antony et al. 1985). Cleavage of 7,11-heptacosadiene at the 7 and 11 double bond 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 CHCs.

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 effects desiccation resistance in the absence of CHCs. Additionally, courtship and copulation is shown to be mediated by unsaturated CHCs, such as the anti-aphrodisiac 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 semio-chemicals in courtship and mate selection. Ozonolysis of unsaturated CHCs on female *D. suzukii* were correlated to reduced courtship and/or copulation by untreated males (unpublished data). This is counter-intuitive to the reduction of the 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 have been shown to be produced after ozone exposure, which cause mutagenicity and a reduced reproductive potential in *D. virilis* (Erdman and Hernandez 1982).

The amounts of *Drosophila suzukii* unsaturated CHCs are significantly reduced, 2-3 fold, after ozone treatment due to the process of ozonolysis. This creates aldehydes which remain on the cuticle for between 36 and 108 h. Saturated CHC amount on flies are largely unaffected by ozone treatment, except for female flies at 3-5 d where a significant increase in saturated CHCs were 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 CHCs. However, the desiccation resistance of ozone treated flies was elevated above untreated flies after unsaturated CHC regeneration. These findings provide

novel methodology for insect CHC reduction/modification, evidence for CHC regeneration after reduction/modification and evidence supporting the importance of unsaturated CHCs in desiccation resistance.

APPENDIX

Figure 3.1. Ozone generation and treatment arena set-up.

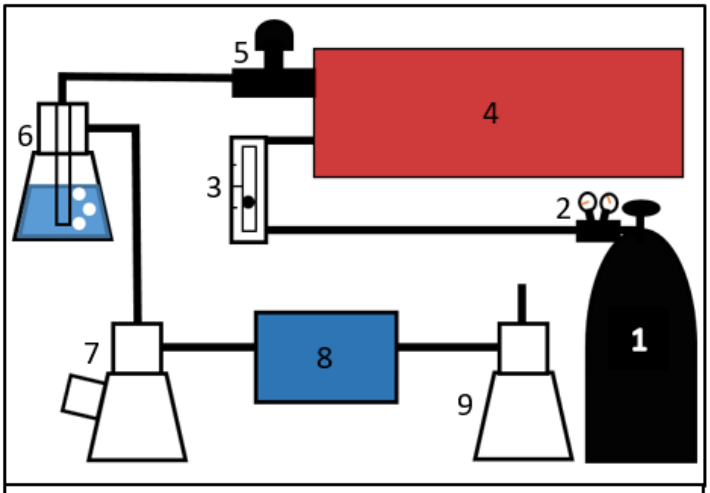

Figure 1. - Gaseous Ozone Experiment Setup
1. Oxygen Tank (99.5%)
2. Pressure Gauge
3. Airflow Meter
4. Nano Ozone Generator ™ (Absolute Ozone)
5. Needle Valve
6. Bubbler - Flask (250 mL)
7. Gas Washing Flask (250 mL)
8. Ozone Monitor - 106-H ™ (2B Technologies)
9. Flask (250 mL) – Relative Humidity Samples
*All parts in contact with ozone are under fume hood for safety

Figure 3.2. Work flow process. (1) Caging of flies in stainless steel cages. (2) Ozone and oxygen treatment. (3) Cuticular hydrocarbon extraction. (4) GC/MS analysis of extractions.

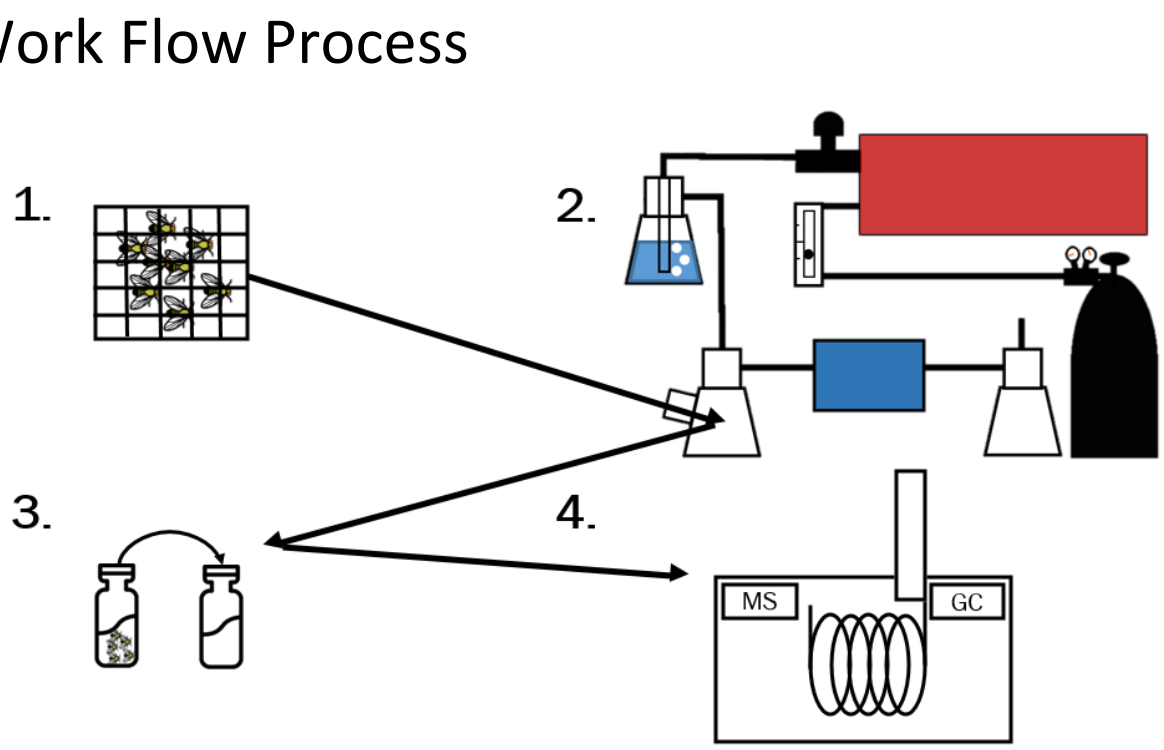

Figure 3.3. Comparison of a gas chromatogram of a control fly (top) versus an ozone fly (bottom) cuticular hydrocarbon profile at 1 h after ozonolysis. Hexacosane (25 ng/µL) was used as an internal standard (IS).

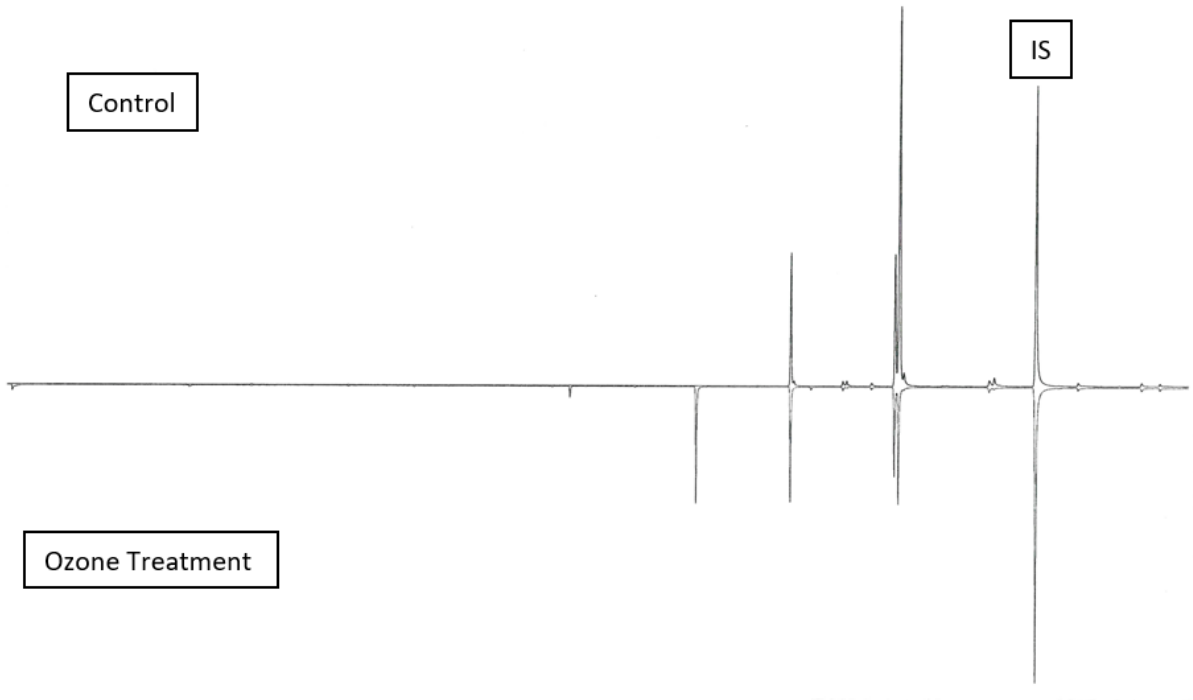

Figure 3.4. Comparison of a gas chromatogram of a control fly (top) versus an ozone fly (bottom) cuticular hydrocarbon profile at 108 h after ozonolysis. Hexacosane (25 ng/µL) was used as an internal standard (IS).

Figure 3.5. Female and male mean (±SEM error bars) amount (ng) of unsaturated CHCs (5(Z)-tricosene, 7(Z)-tricosene, 9(Z)-tricosene/tricosane, 5(Z)-pentacosene, 7(Z)-pentacosene, 9(Z)- pentacosene) from experiment 1. Graphs separated by fly age (3-5 d, 9-11 d) and CHC group. Samples were collected at 1, 12, 36 & 108 h after treatment. A significant difference between ozone treated and untreated flies within a CHC extraction hour was marked with a '*' (* : 0.05, ** : 0.01, *** : 0.001).

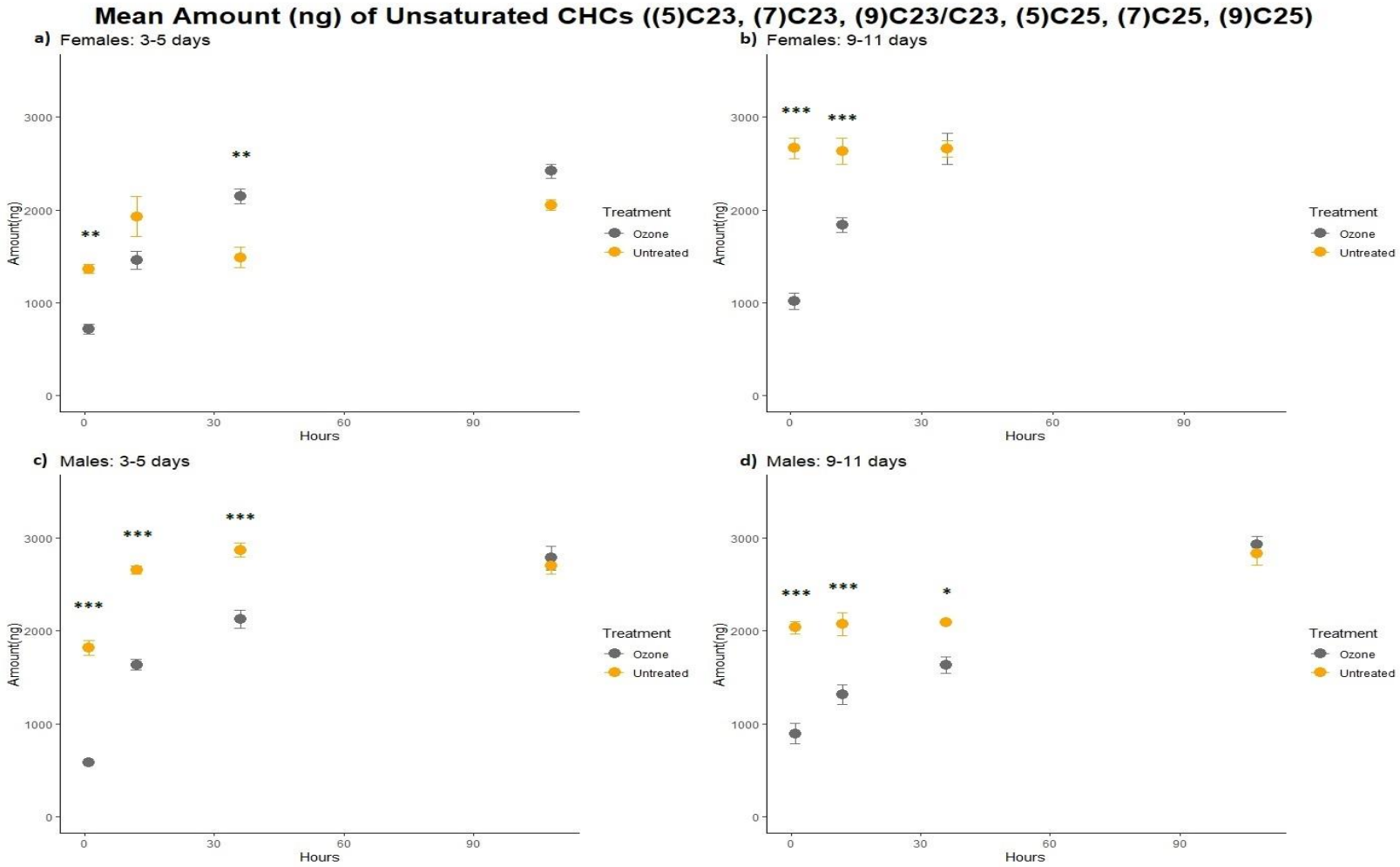

Figure 3.6. Female and male mean (±SEM error bars) amount (ng) of cuticular aldehydes (heptanal, nonanal, tetradecanal, pentadecanal, hexadecanal, octadecanal) from experiment 1. Graphs separated by fly age (3-5 d, 9-11 d) and CHC group. Samples were collected at 1, 12, 36 & 108 h after treatment. A significant difference between ozone treated and untreated flies within a CHC extraction hour was marked with a '*' (* : 0.05, ** : 0.01, *** : 0.001).

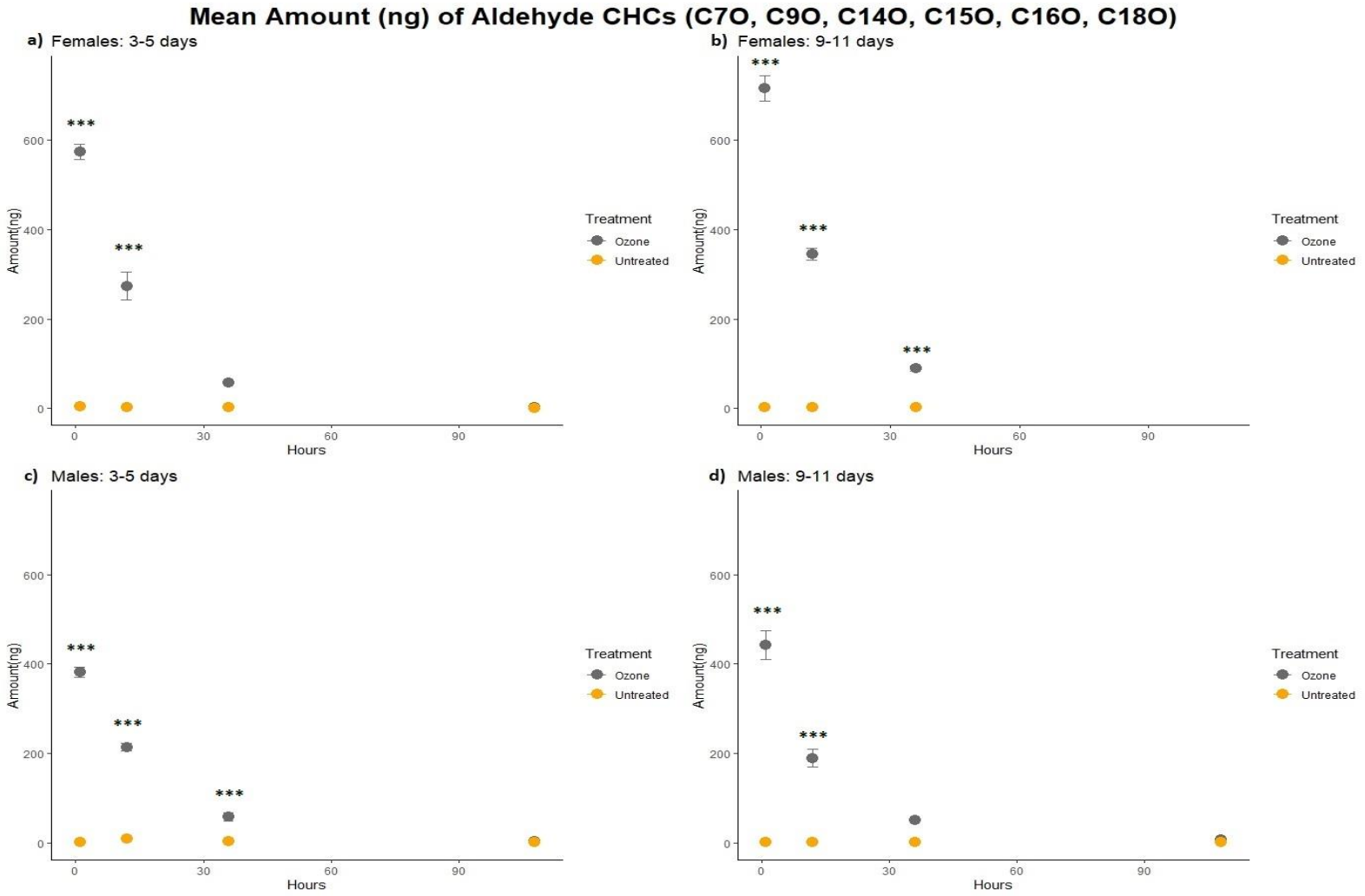

Figure 3.7. Female and male mean (±SEM error bars) amount (ng) of saturated CHCs (heneicosane, heptacosane, 2-methyl octacosane, nonacosane) from experiment 1. Graphs separated by fly age (3-5 d, 9-11 d) and CHC group. Samples were collected at 1, 12, 36 & 108 h after treatment. A significant difference between ozone treated and untreated flies within a CHC extraction hour was marked with a '*' (* : 0.05, ** : 0.01, *** : 0.001).

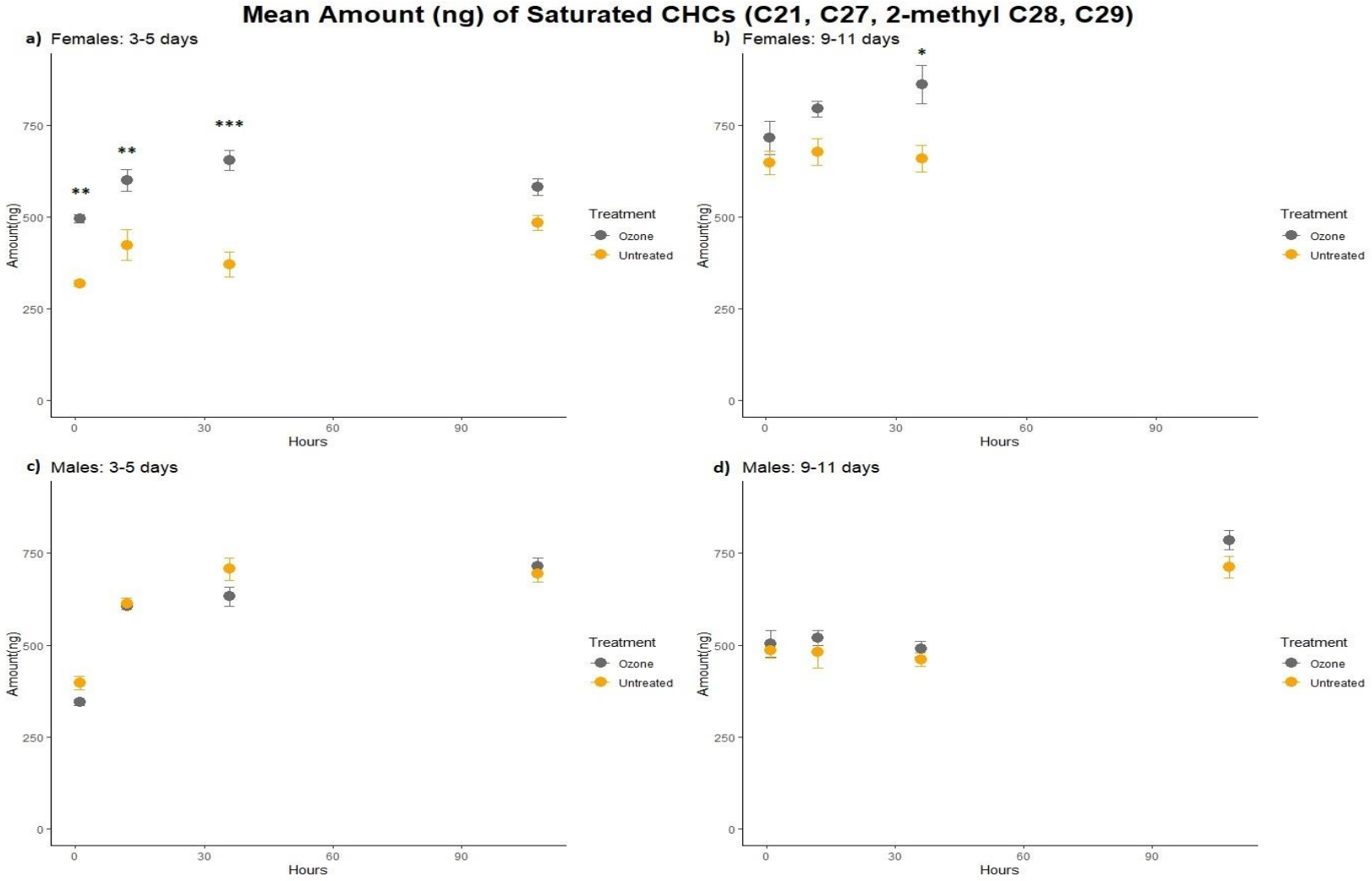

Figure 3.8. Kaplan-Meier Survival Curves of 3-5d old flies at 1 h (RH=18.13% (±1.54%), Temp=28.89°C (±0.20)) and 108 h (RH=15.93% (±2.12), Temp=28.32°C (±0.34°C)) after treatment application. Half-h sampling periods until 10 hours or all flies died.

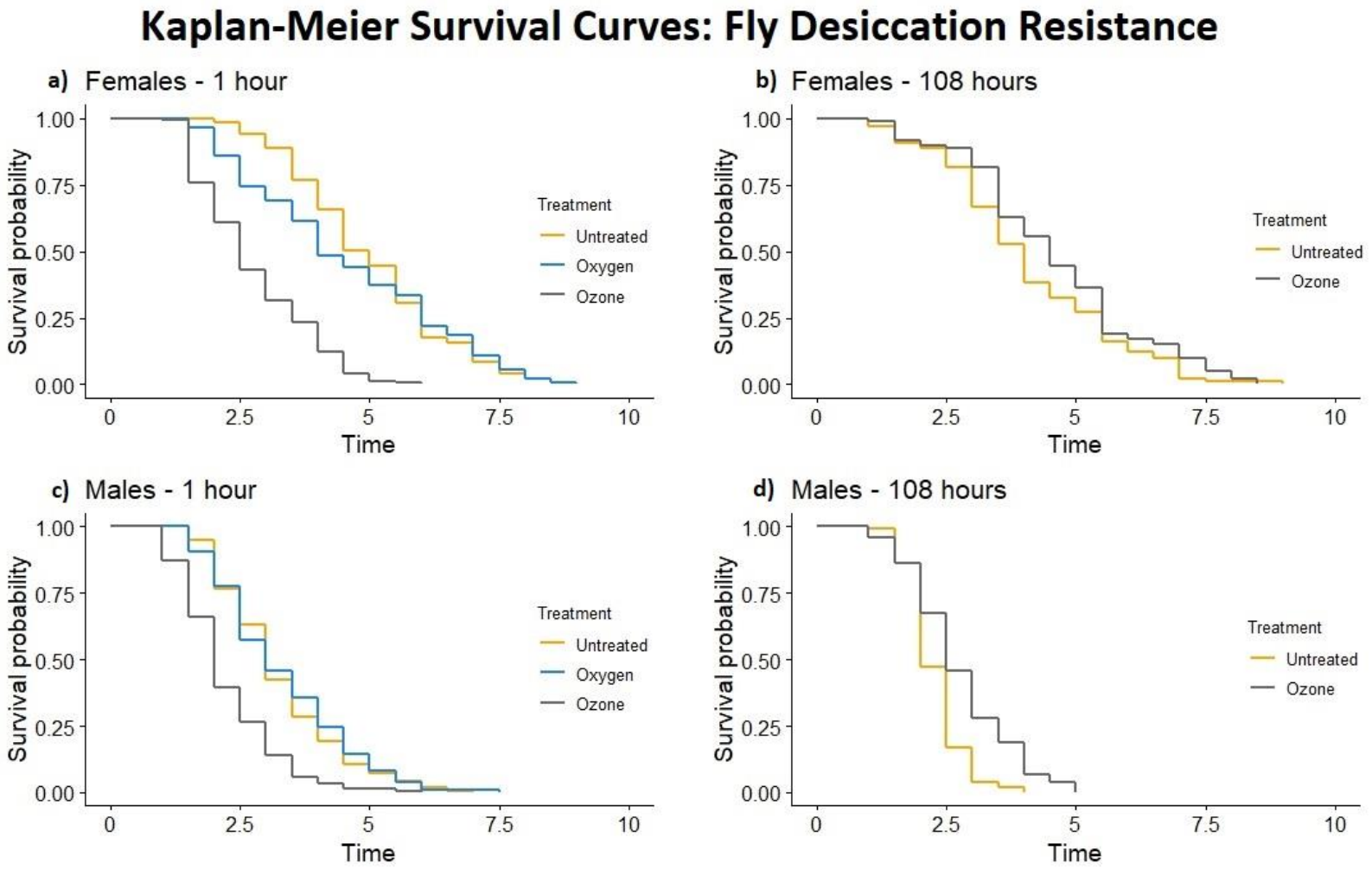

Table 3.1. Experiment 1, 2 & 3 data collection times, treatments, fly age and replication. An '*' in the 'Replication (Male, Female)' column indicates the same number of replications at every data collection time in the 'Data Collection Hour after Treatment Application' column and a ';' indicates a separation of replication numbers consistent with the data collection times in the 'Data Collection Time after Treatment Application (h(s))' 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

Table 3.2. Mean (ng) and standard error of mean (SEM) of unsaturated CHCs, cuticular aldehydes and saturated CHCs extracted from female and male flies at 1 h after treatment application (3-5 & 9-11 days old). Disparate letters signify differences within a population based Wilcoxon rank sum test with Holm p-adjustment.

Cuticular Compound Amount (ng) of *D. suzukii* at 1 h after treatment

Unsaturated CHCs

Females

	3-5 d			9-11 d		
Treatment	**Mean**	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	721.18	52.37	b	1016.92	85.03	b
Oxygen	1462.46	101.20	a	2831.19	77.54	a
Untreated	1365.90	44.91	a	2663.90	107.83	a

Males

	3-5 d			9-11 d		
	Mean	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	586.04	5.48	b	893.95	110.10	c
Oxygen	1673.55	32.46	a	1452.55	132.14	b
Untreated	1817.31	75.91	a	2036.24	64.17	a

Cuticular Aldehydes

Females

	3-5 d			9-11 d		
Treatment	**Mean**	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	573.67	17.61	a	715.03	28.34	a
Oxygen	0.00	0.00	b	0.00	0.00	b
Untreated	0.00	0.00	b	0.00	0.00	b

Males

	3-5 d			9-11 d		
	Mean	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	382.04	11.14	a	443.02	31.79	a
Oxygen	0.00	0.00	b	0.00	0.00	b
Untreated	0.00	0.00	b	0.00	0.00	b

Saturated CHCs

Females

	3-5 d			9-11 d		
Treatment	**Mean**	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	495.27	12.35	a	715.89	45.20	a
Oxygen	332.94	23.84	b	667.36	35.24	a
Untreated	319.13	8.55	b	647.63	32.46	a

Males

	3-5 d			9-11 d		
	Mean	**SEM**	**CLD**	**Mean**	**SEM**	**CLD**
Ozone	343.93	7.29	a	503.20	36.86	a
Oxygen	349.83	10.22	a	378.31	38.37	a
Untreated	396.56	17.60	b	485.25	20.41	a

Table 3.3. Unsaturated CHCs, cuticular aldehydes and saturated CHCs mean amount extracted from flies (3-5 & 9-11 days old). Cuticular hydrocarbon extractions occurred at 1 h, 12 h, 36 h and 108 h after treatment application. CLD marks differences between "Treatment - Hours" means based on an ANOVA p-values. Disparate letters only signify differences within a sex and age.

Cuticular Compound Amount (ng) of *D. suzukii* at 1, 12, 36 and 108 h after treatment

		3-5 d						9-11 d					
		Female			Male			Female			Male		
	Treatment - Hour	Mean	SEM	CLD	Mean	SEM	CLD	Mean	SEM	CLD	Mean	SEM	CLD
Unsaturated	Ozone - 1	721.18	52.37	e	586.04	5.48	d	1016.92	85.03	c	893.95	110.10	d
	Untreated - 1	1365.90	44.91	d	1817.31	75.91	bc	2663.90	107.83	a	2036.24	64.17	be
	Ozone - 12	1460.44	99.84	cd	1634.62	55.36	c	1836.70	81.97	b	1313.85	103.99	cd
	Untreated - 12	1929.09	215.34	bc	2658.51	45.12	a	2634.77	140.26	a	2071.64	124.13	be
	Ozone - 36	2145.39	79.08	ab	2130.23	96.70	b	2659.84	169.52	a	1630.74	87.71	ce
	Untreated - 36	1489.14	112.27	cd	2870.87	71.65	a	2659.06	88.02	a	2092.27	27.64	b
	Ozone - 108	2417.21	73.93	a	2786.27	127.81	a				2927.78	90.21	a
	Untreated - 108	2051.53	58.81	ab	2700.23	84.59	a				2832.26	126.06	a
Aldehyde	Ozone - 1	573.67	17.61	a	382.04	11.14	a	715.03	28.34	a	443.02	31.79	a
	Untreated - 1	0.00	0.00	c	0.00	0.00	d	0.00	0.00	d	0.00	0.00	c
	Ozone - 12	273.32	31.46	b	214.46	9.14	b	344.60	12.48	b	189.79	19.59	b
	Untreated - 12	0.00	0.00	c	0.00	0.00	d	0.00	0.00	d	0.00	0.00	c
	Ozone - 36	57.04	5.62	c	58.77	9.14	c	89.79	6.43	c	51.60	2.73	c
	Untreated - 36	0.00	0.00	c	0.00	0.00	d	0.00	0.00	d	0.00	0.00	c
	Ozone - 108	3.43	0.67	c	4.27	0.33	d				7.48	1.58	c
	Untreated - 108	0.00	0.00	c	0.00	0.00	d				0.00	0.00	c
Saturated	Ozone - 1	495.27	12.35	bc	343.93	7.29	c	715.89	45.20	ab	503.20	36.86	b
	Untreated - 1	319.13	8.55	e	396.56	17.60	c	647.63	32.46	b	485.25	20.41	b
	Ozone - 12	599.79	29.93	ab	606.74	9.09	b	793.86	21.28	ab	518.96	20.60	b
	Untreated - 12	423.88	42.62	cde	612.92	15.18	b	676.67	36.45	b	481.13	42.11	b
	Ozone - 36	653.88	26.89	a	632.04	25.31	ab	860.37	52.98	a	489.02	20.95	b
	Untreated - 36	370.18	33.98	de	707.58	30.85	a	659.14	36.62	b	460.38	17.36	b
	Ozone - 108	581.79	23.36	ab	714.16	22.01	a				785.69	25.32	a
	Untreated - 108	482.95	20.48	bcd	694.02	22.87	ab				711.50	29.64	a

Table 3.4. Kaplan-Meier survival analyses of desiccation resistance from flies (3-5 days old). Flies (10) were placed into a plastic vial with a desiccant, drierite, and sealed. Flies were consider 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 included in 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

Table 3.5. Cox proportional hazard analysis models performed on fly survival within a sex and h after treatment application. All model parameters compared to untreated fly survival within the same sex and h. 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(s)	Model	Parameters Baseline:Comparison	β	SE	D.f.	*z*	*P*	HR	95% CL (Lower-Upper)
Female	1	1	Untreated:Oxygen	0.0629	0.1198	2	0.5260	0.5990	1.0650	0.8421-1.3470
		1	Untreated:Ozone	1.4801	0.1320	2	11.2100	<0.0001	4.3932	3.3915-5.6910
		2	Oxygen:Ozone	1.4171	0.1352	2	10.4830	<0.0001	4.1252	3.1650-5.3770
		3	Untreated/Oxygen:Ozone	1.4509	0.1194	1	12.1500	<0.0001	4.2670	3.3770-5.3920
	108	4	Untreated:Ozone	-0.2843	0.1442	1	-1.9720	0.0486	0.7525	0.5673-0.9982
Male	1	1	Untreated:Oxygen	-0.0512	0.1205	2	-0.4250	0.6710	0.9500	0.7502-1.2030
		1	Untreated:Ozone	0.8776	0.1225	2	7.1670	<0.0001	2.4052	1.8920-3.0580
		2	Oxygen:Ozone	0.9289	0.1238	2	7.5050	<0.0001	2.5317	1.9863-3.2270
		3	Untreated/Oxygen:Ozone	0.9030	0.1074	1	8.4110	<0.0001	2.4670	1.9990-3.0450
	108	4	Untreated:Ozone	-0.7561	0.1530	1	-4.9420	<0.0001	0.4695	0.3479-0.6337

CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

The objectives of my thesis were to evaluate gaseous and aqueous ozone as a potential insecticide using *D. suzukii* as a model organism (Chapter 2) and to evaluate sub-lethal effects of ozone on *D. suzukii* cuticular hydrocarbon (CHC) profile composition and desiccation resistance (Chapter 3). My major findings were that while gaseous ozone has insecticidal potential, aqueous ozone does not have adulticidal potential. In addition, ozonolysis cleaves unsaturated CHCs on living specimens, and survival based desiccation resistance correlates to CHC reductions and recovery. Furthermore, my finding that ozonolysis can be used to modify living insect CHC composition could be used to develop a greater understanding of CHC function and CHC mediated behaviors.

In chapter 2, ozone was applied to *D. suzukii* in gaseous and aqueous forms to evaluate the potential of ozone as an insecticide. Insect mortality from ozone has been recorded in studies evaluating ozone's potential to control stored product pest populations, however, lethality has been sparsely evaluated on dipteran species (Erdman and Hernandez 1982; Kells et al. 2001; Sousa et al. 2008; M. X. McDonough et al. 2010; Marissa X. McDonough, Mason, and Woloshuk 2011; Isikber and Athanassiou 2015). It was hypothesized that ozone would cause variable fly mortality dependent on dose, thus, a dose response curve was created for gaseous ozone. Aqueous ozone did not demonstrate any insecticidal potential after treating flies (Figure 2.4.), while gaseous ozone showed potential in confined environments where its concentration can be controlled (Table 2.3, Figure 2.3.).

As an insecticide, gaseous ozone has a good capacity for "knock-down" activity at the tested concentrations with flies succumbing in a matter of minutes to 14,600 ppm ozone and in mere seconds to 30,100 ppm ozone, however, ozone has low residual mortality effects (Figure 2.3.). It has been reported that fast knock-down activity is a common characteristic of currently recommended insecticides for *D. suzukii* control (Isaacs, Tritten, et al. 2013). Ozone lethal concentration-time products

should be evaluated for for lethal and sub-lethal effects *on D. suzukii* host crops that are cultured in confined areas, such as greenhouses or high tunnels. If lethal ozone concentrations do not negatively affect selected crops, future research regarding ozone lethality could lead to gaseous ozone exposure on flies in larger confined areas. Gaseous ozone treatments in greenhouses could potentially lead to arthropod, fungal and bacterial pest control, but it is too early to determine the viability of ozone as a pest management option.

My experiments provide definitive evidence that while gaseous ozone has insecticidal potential, aqueous ozone does not (Figure 2.4.). Exposure of flies to ozone dissolved in water (18.52 ppm) did not provide a detectable increase in mortality as compared to that observed in distilled water or untreated control. Thus we feel it is safe to conclude that aqueous ozone has very little potential to develop lethal activity in insects using current application methodologies in agricultural pest management. This is important because agricultural entrepreneurs have been marketing radial airblast sprayers to control fungal, bacterial, and arthropod pests, but data suggests that the sprayers provide no pest control (Grieshop et al., 2019). The data outlined in this thesis discredits the feasibility and viability of aqueous ozone as a potential insecticide.

Changes in the *D. suzukii* CHC profile and subsequent desiccation resistance were evaluated as potential sub-lethal impacts of ozonolysis in chapter 3. Previous studies, have used ozonolysis to identify insect unsaturated hydrocarbons extracted with a non-polar solvent (Beroza and Bierl 1967, Antony et al. 1985; Bartelt et al. 1982; Bartelt et al. 1986). My research was the first to evaluate the impact of ozonolysis on living insect CHCs. My results indicated that ozonolysis of living flies significantly reduces the amount of unsaturated CHCs. Desiccation resistance correlated with CHC amounts after ozonolysis (Table 3.2-4., Figures 3.5-8.). This sub-lethal interaction is of importance as a novel methodological tool to modify living insect unsaturated CHCs, which may allow the determination of new CHC functions.

Lethal effects of ozone proved to be fairly straightforward and conclusive, while sub-lethal interactions of ozone on flies demonstrated various undocumented consequences. While complications may exist, this study is the first to quantify ozonolysis of CHCs on living insect specimens and may provide an important new method for exploring the generation, structure and function of these important constituents of the insect epicuticle. The methods developed in our paper could hypothetically be used to modify the unsaturated CHCs of any insect model and allow quantification of CHC generation time, potential for regeneration as well as how they modify behavior and survival.

The potential application of CHC reduction methodology via ozonolysis developed in this thesis could allow for 1) insight into the importance of CHC regeneration, 2) the difference in CHC regeneration capacity between sexes, 3) the water loss rate (WLR) after ozonolysis through the cuticle or other sources, 4) the potential interactions of unsaturated CHC reduction to unsaturated CHC mediated behaviors, and 5) the ability to achieve ozonolysis on any arthropod with unsaturated CHCs. The future directions of CHC ozonolysis on living specimens opens many routes of exploration for determining the function of insect CHCs. *Drosophila melanogaster* may be of particular interest in regards to CHC ozonolysis due to the extensive literature already published on their CHC profile, CHC generation, CHC function and varying experimental methodologies specifically designed for the fly.

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

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 effects desiccation resistance in the absence of CHCs. Additionally, courtship and copulation is shown to be mediated by unsaturated CHCs, such as the anti-aphrodisiac 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 semio-chemicals in courtship and mate selection.

Complications of ozone exposure may exist in various documented and undocumented forms due the ability of ozone to oxidize a multitude of organic compounds and tissues. For example, dominant lethals have been shown to be produced after ozone exposure, which causes mutagenicity and a reduced reproductive potential in *D. virilis* (Erdman and Hernandez 1982). Mitosis was shown to be delayed in grasshopper, *C. viridifasciata*, embryos after ozone exposure and was hypothesized that free radicals produced by ozone oxidation could be the cause of mitosis delay (Fetner 1963). Respiration rates were evaluated by a study from Sousa et al. (2008), which did not find any correlation between ozone exposure and respiration rates of stored product pests (Sousa et al. 2008). However, respiration rates may change between different species after ozone exposure and should be evaluated in future experiments. Finally, future studies would benefit from determining non-lethal concentrations that also result in unsaturated CHC reduction as well. Reducing initial mortality after ozone exposure may help to reduce non-target effects if present.

The variety of documented applications for ozone are numerous, including drinking/waste water sanitation, medical treatments, food processing sanitation, insecticides and analytical chemistry (Kim et al. 1999; Masten and Davies 1994; Rico et al. 2007; Kells et al. 2001; Beroza and Bierl 1967; Ikehata and El-Din 2005; Siedler et al. 2008). My thesis outlines gaseous ozone toxicity on adult *D. suzukii*, which

shows potential as an insecticide within confined areas. The potential of ozone application in the field is low due to the inability to effectively control ozone concentrations in open-air environments. Sub-lethal interactions between *D. suzukii* and gaseous ozone were identified, specifically an alteration of CHCs and a simultaneous reduction in desiccation resistance. The major implication of this discovery is a non-lethal method for the modification of unsaturated CHCs. This novel methodology may lead to increased understanding of CHC generation, functions, and CHC mediated behaviors.

APPENDICES

APPENDIX A: RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2020 - 02

Author and Title of thesis: Benjamin Alexander Savage. "EXPLORING THE LETHAL AND SUB-LETHAL INSECTICIDAL PROPERTIES OF OZONE USING SPOTTED WING DROSOPHILA, *DROSOPHILA SUZUKII* (MATSUMURA) (DIPTERA: DROSOPHILIDAE) AS A MODEL ORGANISM."

Museum(s) where deposited:

Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Family	Genus-Species	Life Stage	Quantity	Preservation
Drosophilidae	*Drosophila-suzukii*	adult	5 Female	75% EtOH
Drosophilidae	*Drosophila-suzukii*	adult	5 Male	75% EtOH

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