AN ABSTRACT OF THE THESIS OF

Siew Bee Tang for the degree of Master of Science in Horticulture presented on August 14, 2017.

Title: Physiological effects of non-nutritive sugars on the survivorship and fecundity of *Drosophila suzukii* (Diptera: Drosophilidae)

Abstract approved:

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Man-Yeon Choi

Spotted wing drosophila (SWD), *Drosophila suzukii*, is an economically damaging pest on small fruits. The estimated economic impact is hundreds of millions of dollars annually in the U.S. alone, and increasing every year. Current control of SWD relies heavily on chemical insecticides which have many negative impacts on environmental and human health. Therefore, this should be replaced or at least complemented with biologically-based alternatives. The current project has focused on investigating non-nutritive sugars including erythritol as an environmentally-friendly control agent against SWD.

The study found a potential insecticidal effect from non-nutritive sugars and sugar alcohols, erythritol and erythrose, to decrease the survivorship of *D. suzukii*. In a dose-dependent feeding, erythritol and erythrose significantly reduced the fly longevity with 1 M - 0.05M doses for 7 days. When sugar solutions were provided separately to flies, there was no effect on survivorship regardless of erythritol concentrations. However, with a serial combination of sucrose and erythritol solutions, fly survivorship was significantly decreased. In a no-choice feeding assay, the fly ingested more erythritol than sucrose or water, and erythritol and sucrose-fed flies gained more weight than water-fed flies. However, in two-choice assays, the amount of erythritol ingested was less than sucrose or water.

Total sugar and glycogen levels among the body of erythritol and erythrose-fed flies were significantly less than flies fed nutritive sugars of mannitol, sorbitol, and xylitol. The result indicates that the two non-nutritive sugars can't be used as a substrate for enzymes involved in sugar metabolism. Although the metabolism of erythritol and erythrose is unknown in insects, the mortality of *D. suzukii* flies ingesting these sugars might be caused by two potential physiological changes: 1) starvation from the feeding of non-metabolizable erythritol and erythrose; and 2) abnormal osmotic pressure increased in the hemolymph with erythritol transported from the midgut.

Chapter 3, sucrose and erythritol were applied to blueberries and effects of these combinations on fly mortality and fecundity were monitored in the lab and greenhouse. In the lab test, two sucrose/erythritol combinations (0.5M sucrose/2M erythritol, 1M sucrose/2M erythritol) resulted in the highest mortality and the lowest fecundity in SWD adults. Two combinations, therefore, were selected for further evaluation with blueberry bushes and fruits in the greenhouse. The fly fed on 0.5M sucrose/2M erythritol significantly decreased their survivorship than 1M sucrose/2M erythritol-fed flies in the greenhouse. This result indicates that flies could move more in the bigger cage accelerating the exhaustion of energetic reserves in the body.

The presence of erythritol in the hemolymph and frass was determined to investigate the nutritional metabolism and absorption of erythritol in *D. suzukii*. Unlike sucrose, a large amount of erythritol was observed in the hemolymph of the fly ingested the 0.5M sucrose/0.5M erythritol. Not sucrose, erythritol was found in the frass in the same fly. The results imply that erythritol might be directly transported from the midgut without being metabolized and stored, but is accumulated in the hemolymph which in turn elevates the osmotic pressure in the fly hemolymph.

Overall, this research found the sucrose/erythritol combination would be more effective than erythritol alone for practical application, because the combination tastes sweeter to elicit more feeding. This erythritol formulation can be a potential insecticide used alone or as a delivery agent combined with conventional or

biological insecticides to enhance their efficacy. While the present research focuses on *D. suzukii*, it can be expanded to other Dipteran pests as well.

Physiological effects of non-nutritive sugars on the survivorship and fecundity of *Drosophila suzukii* (Diptera: Drosophilidae)

by
Siew Bee Tang

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented August 14, 2017
Commencement June 2018

Master of Science thesis of Siew Bee Tang presented on August 14, 2017.

APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Siew Bee Tang, Author

ACKNOWLEDGEMENTS

I would like to express my immense gratitude to my major professor and mentor, Dr. Man-Yeon Choi for granting this precious opportunity to pursue my master degree with his research team. I am very grateful for his insight, guidance, patience and willingness to assist at non-official hours in this research. Without his full support and mentoring, this project would not be possible.

I would also like to thank Drs. Jana Lee, Vaughn Walton and Ronald Wrolstad for being in my committee. I hold my deepest thanks to Dr. Jana Lee for contributing useful input in Chapter 2 and assisting in statistical analysis in both Chapter 2 and 3. Dr. Jana Lee supplied SWD samples throughout the entire research period, providing facilities and equipment for bioassays and greenhouse studies. A great thanks goes out to everyone in the Choi's lab and Jana's lab especially to Kelly Donahue, Dr. Seung-Joon Ahn, Victoria Skillman and Jessica Wong for providing technical and colony support.

Lastly, I would like to thank my family and friends for their endless support, encouragement and understanding that helps me being grounded. A very special thanks goes out to Ian Kellems for his genuine support and constant motivation throughout this process. Thanks to Jennifer Kling, Ching Ling Song and Balkis Fatomer bt Abu Bakar for being supportive and accommodative. Thanks to my parents for strengthening me with their unconditional love. You are the ultimate reason for not losing faith.

My master degree completion would not have been possible without a scholarship from Malaysian Agricultural Research and Development Institute (MARDI) and research funding support from Northwest Center for small Fruits Research Grant, Oregon Blueberry, Raspberry & Blackberry Commissions, Agricultural Research Foundation, base funding from USDA ARS CRIS 2072-22000-040-00D.

CONTRIBUTION OF AUTHORS

Dr. Man-Yeon Choi oversaw the planning and design of this project, instrumental in development and writing of Chapter 2 and 3 as well as editing this paper and assisted with outcomes interpretation. Dr. Jana Lee was a collaborator and conducted carbohydrate analysis in Chapter 2 as well as assisted in statistical analyses of both Chapter 2 and 3. Drs. Seung-Joon Ahn, Peter Shearer and Kaushalya Amarasekare instrumental in editing Chapter 2. Dr. Jin-Kyo Jung instrumental in editing Chapter 3.

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DEDICATION

To my family and friends.

Chapter 1

General introduction

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Introduction

Biology of *Drosophila suzukii*

Spotted wing drosophila (SWD), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) belongs to the subgenus *Sophophora*. SWD is commonly known as a vinegar fly and is attracted by fermented odor. SWD adults are characterized by a body length of 2-3mm, red eyes and pale brown thorax (Walsh et al., 2011). SWD is distinctive with two obvious morphological traits from other *Drosophila* species. The male flies have a vivid dark spot on each fore wing, and the female is equipped with a serrated ovipositor which enables it to oviposit eggs into fresh and ripe fruits (Walsh et al., 2011). SWD has a short life cycle duration with high fecundity. It takes approximately 12.8 ± 0.2 days to complete from egg to adult stage (egg = 1.4 days, larva: 1^{st} instars = 1.1 days, 2^{nd} instars = 1.5 days, 3^{rd} instars = 3.1 days, pupa = 5.8 ± 0.05 days) at 22 ºC under a photoperiod of L:D 15:9 h, and a relative humidity of 25% RH. The adult lifespan ranges between 50 – 154 days with an average 86 ± 4.25 days (male = 93.6 ± 6.88 days, female = 79.5 ± 4.86 days) (Emiljanowicz et al., 2014). The short developmental cycle contributes to make 7-15 generations annually with average 400 eggs laid per female during the entire adult stage (Cini et al., 2012). Development time, fecundity and longevity may vary by host and environmental factors such as temperature.

It is difficult to control SWD due to high dispersal ability of the flies and a well adaptability to survive in extreme temperatures (Cini et al., 2012). According to Jacobs et al. (2015), SWD is a chill-susceptible insect but shows acclimation responses to extreme temperature changes. Adult flies are unable to survive under extreme cold temperature; no mortality was observed when exposed to 0 to -4˚C but there was 100% mortality when exposed to -10˚C. Adults that were reared under fluctuating conditions (Week one: 9˚C/21˚C, Week 2: 5.5˚C/19˚C) that mimic autumn conditions show better acclimation ability with shorter chill coma recovery time (CCR), approximately 10 minutes on average. In contrary, flies reared under constant temperature, 21.5˚C, took 40 minutes on average to regain movement ability from

chill coma regardless of time exposure ranging from 4h to 48h at 0˚C. (Jacobs et al., 2015). In addition to a short life cycle, high fecundity rates and cold tolerance, SWD has a high dispersal ability due to its broad host range including blueberries, strawberries, raspberries, cherry, peach, grapes, blackberries and other soft fruits (Cini et al., 2012).

Pest status and economic importance

The spotted wing drosophila (SWD), *Drosophila suzukii*, originally from Asia is now an important small fruits and cherry pest (Asplen et al., 2015). The infestation areas of SWD are rapidly spreading across the U.S., Canada and Europe due to its extraordinarily high reproductive ability, extreme adaptability to environment, and difficulties of control at the larval stage. As well, there is currently a lack of knowledge regarding natural enemies. (Hauser, 2011; Cini et al., 2012; Rota-Stabelli et al., 2013). SWD was first observed on the mainland US in California in 2008. SWD's invasion to the California raspberry industry led to revenue losses of US $ 36.4 million and US $ 3.43 million to conventional and organic producers, respectively (Farnsworth et al., 2017). The economic impact by SWD is also an expected 13% revenue loss in the northeastern berry production area of Italy (De Ros et al., 2015). In Switzerland, it was first detected in 2011 and within just 3 years, SWD caused severe economic destruction to the fruit industry, particularly in berries, stone fruit and grapes (Mazzi et al., 2017). In Europe, the total potential damage costs could be up 1 million EUR per year with all management approaches combined including cost of materials, labor and infrastructures (De Ros et al., 2015). Although many pest management options have been carried out to combat SWD in the small fruits industry, it is still causing huge revenue losses and high management costs in US and elsewhere. Therefore, better pest management to suppress SWD population is required.

Pest management for *Drosophila suzukii*

Although various control options including biological, cultural, mechanical, and chemical controls are being applied for *D. suzukii*, chemical insecticides are the major

control tool. To date, the most effective chemical insecticides used to suppress SWD populations are organophosphates, pyrethroids and spinosyns (Andreazza et al., 2017, Haye et al., 2017, Beers et al., 2011). These chemicals are very toxic to SWD at both adult and larval stages (Beers et al., 2011, Andreazza et al., 2017). High mortality of adult flies were reported in both contact (topical application) and ingestion (toxic bait bioassay) bioassays after 24 HEAT (hour after exposure to treatment) while 85% of larval mortality was observed in dipping bioassay (Andreazza et al., 2017). Chemical control is effective, and promising in most circumstances. However insecticides may cause off target effects to beneficial insects such as honey bees. Current chemical insecticides being used for SWD control are very toxic to honey bees (Hooven *et al*., 2013). Although the residual period of chemicals is short, approximately 2-3 days, it is still toxic to honey bees even though it is not sprayed during bee foraging (Miles et al., 2011). For example, dry residue on flowers of Spinosad (96g a.i./ha) at 7 day after treatment is sufficient to cause lethal effect to honey bees (Miles et al., 2011). Introducing trapping with or without integration of insecticide use could reduce pesticide usage and minimize off target effect.

In addition to chemical approach, trapping methods that utilize semiochemicals, including attractants, are a more environmental friendly strategy to mitigate SWD attacks. In general, trapping serves as a monitoring tool for insect populations while mass trapping is used to suppress insect populations via 'lure and kill' strategy. The survey shows that trapping methods could be an alternative measure to facilitate SWD control in both open and netted fields (Mazzi et al., 2017). Yeast, sugar, wine, apple cider vinegar and fermented fruit solutions are frequently used in SWD traps (Lee et al., 2012; Asplen et al., 2015; Haye et al., 2017).

Efficacy of SWD traps is not only determined by the type of bait, but is also affected by environmental factors. Performance of trap with same bait substance may vary upon crops and/or fruits producing different odors over the baits (Lee et al. (2011). Placement space and time are other factors that determine the successfulness of the trapping system (Cini et al., 2012). According to Walsh et al. (2011), cool and shaded

areas are the best placement spot for effective trap performance; while performance from different designs varied under warmer or cooler climate conditions largely due to the volatilization rates (Lee et al., 2012). Furthermore, traps designed with a wider entry may increase the number of flies caught (Lee et al., 2012). Some studies suggest SWD adults are more attracted towards red and black colored traps (Lee et al., 2013). However, a separate study with contrary findings reported that color does not give a significant difference in flies captured (Lee et al., 2012). Although trapping is a reliable tool for both monitoring and mitigating the SWD population, fermentation baits and vinegar attract all Drosophila species and currently there is no trap available that solely targets SWD. For example, SWD comprised approximately 26-31% SWD from total flies captured (Lee et al., 2012).

Implementation of trapping for SWD control had been extended from conventional approach with semiochemical or pheromone in chemical insecticide, then used with entomopathogenic fungus as a biopesticide. In the Netherlands, a laboratory test was conducted to assess the feasibility of microbial-based bait to kill SWD adults via "lure and infect" strategy, and found that *Metarhizium robertsii* inoculums on blueberry (food and oviposition site) resulted in 94% reduction of adult's reproduction within 2 weeks (Van Tol, 2016). Although this is a fascinating finding, more virulent biological control agent (BCA) is require to increase the lethal effect and disruption of the reproduction cycle.

Biocontrol is another alternative way to control this pest. Numerous studies have been conducted to discover natural enemies of SWD and the possible use of existing BCA to help reduce this pest problem. Under laboratory studies in United Kingdom, various commercial predators such as *Orius majusculus, Orius laevigatus, Atheta coriaria, Hypoaspis miles* and *Anthocoris nemoralis* have been tested for efficacy on SWD larval, pupal and adult stages. Although predatory activities have been investigated, none of these predator candidates were able to decrease SWD population (Cuthbertson , 2014). SWD control had become more challenging when SWD larvae displayed strong immune response towards indigenous larval parasitoids,

Leptopilina heterotoma and *Leptopilina boulardi*, which could contribute to suppress SWD population. Although these larval parasitoids show high parasitism rates on SWD larvae, emergence of adult wasps was unsuccessful (Chavert et al., 2012). Thus, searching for better BCA candidates is crucial for successful biocontrol on SWD.

In order to enhance existing control measures for SWD, discovering potential strategies to combat this invasive pest in multifaceted aspects is the most important. The utilization of natural substances such as non-nutritive sugars can be another potential strategy for SWD control. Screening of various sugars including non-nutritive sugars is a new avenue to develop non-toxic or environmentally-friendly insecticides.

Nutritive and non-nutritive sugars

A variety of non-nutritive sweeteners including erythritol have been approved for use as a food additive and sugar alternative labeled as zero-calories in the United States (FDA, 2014). Erythritol is a four carbon-structured sugar alcohol with 75% of the sweetness of sucrose, and is produced from corn or wheat starch by enzymatic process of either yeast or fermentative microorganisms (Munro et al., 1998). The non-nutritive sugar is also known a sweet antioxidant to help against high blood sugar (hyperglycemia) affecting diabetes (Den Hartog et al., 2010; Munro et al., 1998). Erythrose is also a tetrose carbohydrate and belongs in the aldose family with aldehyde group (Nelson and Cox, 2000). Little is known about the metabolism of erythrose. It has been suggested the initial reduction of erythrose to erythritol (Hiatt and Horecker, 1956). Erythrose was recently offered as an anti-cancer agent that inhibits tumor growth (Liu et al., 2015).

A number of sugars, sucrose, glucose and fructose, etc. obtained from soft skin fruits as food sources provide an effective energy. Sugar acts as a phagostimulant increasing ingestion of insecticides and may play an important role by increasing insecticide effectiveness. To enhance the effectiveness of insecticides, sucrose can be added to conventional or organic insecticides targeting *D. suzukii* (Cowles et al., 2015).

Although the non-nutritive sugars such as erythritol failed to increase the feeding activity of the ant (Vander Meer et al., 1995), recently they were shown to carry insecticidal properties against Dipteran pests (Baudier et al., 2014; O'Donnell et al., 2016; Sampson et al., 2016; Zheng et al., 2016). In the lab, *D. melanogaster* (Meigen) had reduced longevity and motor coordination when fed erythritol or the artificial sweetener that contained erythritol (Baudier et al., 2014). Moreover, *D. melanogaster* actively consumed erythritol in the presence of the sucrose, and had decreased longevity. This is important because erythritol could have an insecticidal effect if the insect pest readily consumes it. Although erythritol showed some insecticidal activity on *Drosophila* (Baudier et al., 2014; Sampson et al., 2016), the corresponding physical changes following erythritol ingestion and mechanism leading to death remains unknown.

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Chapter 2

Effect of non-nutritive sugars to decrease the survivorship of spotted wing drosophila, *Drosophila suzukii*

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Styled for Journal of Insect Physiology
by ELSEVIER
Received 7 December 2016; Received in revised form 2 April 2017;
Accepted 4 April 2017; Available online 5 April 2017

Abstract

In this study, we investigated the effects of non-nutritive sugars and sugar alcohols on the survivorship of spotted wing drosophila, *Drosophila suzukii*, and found erythritol and erythrose as potentially insecticidal effect to the fly. In a dose-dependent study, erythritol and erythrose significantly reduced fly longevity, with 100% mortality with 1, 0.5, 0.1 & 0.05M doses after feeding for 7 days. When sugar solutions were provided separately to flies for 7 days, there was no effect on survivorship regardless of erythritol concentrations. However, with a serial combination of sugar and erythritol solutions, fly survivorship was significantly decreased for the same period. Also, the higher dose of erythritol regardless of the sucrose dose combined showed in greater mortality. In a no-choice assay, *D. suzukii* ingested more erythritol than sucrose or water, indicating the fly continuously fed on erythritol for 72 h. Also under no-choice conditions, erythritol and sucrose-fed flies gained more weight than water-fed flies. However, in two-choice assays, the amount of erythritol ingested was less than sucrose or water. Total sugar and glycogen levels among erythritol and erythrose-fed flies were significantly less than mannitol, sorbitol, xylitol, and sucrose-fed flies after 48 h. This indicates that these two non-nutritive sugars can't be used a substrate for enzymes involved in sugar metabolism. Although the metabolism of erythritol and erythrose is unknown in insects, the mortality of *D. suzukii* flies ingesting these sugars might be caused by two potential physiological changes. The fly is starved by feeding of non-metabolizable erythritol and erythrose, or experiences abnormally high osmotic pressure in the hemolymph with erythritol molecules diffused from the midgut. Non-nutritive sugars might be used as an insecticide alone or combined with conventional or biological insecticides to enhance efficacy. If other sugar sources are present, a palatable sugar might be mixed with erythritol to elicit feeding.

Keywords: non-nutritive sugar, sweetener, insecticidal activity, *Drosophila suzukii*

Introduction

A variety of non-nutritive sweeteners including erythritol have been approved for use as a food additive and sugar alternative labeled as zero-calories in the United States (FDA, 2014). Erythritol is a four carbon-structured sugar alcohol with 75% of the sweetness of sucrose, and is produced from corn or wheat starch by enzymatic process of either yeast or fermentative microorganisms (Munro et al., 1998). The non-nutritive sugar is also known a sweet antioxidant to help against high blood sugar (hyperglycemia) affecting diabetes (Den Hartog et al., 2010; Munro et al., 1998). Erythrose is also a tetrose carbohydrate and belongs in the aldose family with aldehyde group (Nelson and Cox, 2000). Little is known about the metabolism of erythrose. It has been suggested the initial reduction of erythrose to erythritol (Hiatt and Horecker, 1956). Erythrose was recently offered as an anti-cancer agent that inhibits tumor growth (Liu et al., 2015).

A number of sugars, sucrose, glucose and fructose, etc. obtained from soft skin fruits as food sources provide an effective energy for adult flight in *Drosophila*. The detection and selection of sugars by flies was decided with palatability and nutrient value of sugars, that flies leaned the appetitive memory to continuous feeding (Burke and Waddell, 2011; Fujita and Tanimura, 2011). Non-nutritious sugars although they contain some sweet taste were eventually excluded by the section of food from a long term memory learned by *Drosophila* fed these sugars.

Sugar acts as a phagostimulant increasing ingestion of insecticides and may play an important role by increasing insecticide effectiveness. To enhance the effectiveness of insecticides, sucrose can be added to conventional or organic insecticides targeting *Drosophila suzukii* (Matsumura) (Cowles et al., 2015). Although the non-nutritive sugars such as erythritol failed to increase the feeding activity of the ant (Vander Meer et al., 1995), recently they were shown to carry insecticidal properties against Dipteran pests (Baudier et al., 2014; O'Donnell et al., 2016; Sampson et al., 2016; Zheng et al., 2016). In the lab, *Drosophila melanogaster* (Meigen) had reduced longevity and motor coordination when fed erythritol or the artificial sweetener that

contained erythritol (Baudier et al., 2014). Moreover, *D. melanogaster* actively consumed erythritol in the presence of the sucrose, and had decreased longevity. This is important because erythritol could have an insecticidal effect if the insect pest readily consumes it. Although erythritol showed some insecticidal activity on *Drosophila* (Baudier et al., 2014; Sampson et al., 2016), the corresponding physical changes following erythritol ingestion and mechanism leading to death remains unknown.

The spotted wing drosophila, *D. suzukii*, originally from Asia is now an important small fruit and cherry pest in Asia, Europe, and North America (Asplen et al., 2015). If the pest is left unmanaged, annual losses have been estimated at US$421 million for the soft fruit and cherry industry of California, Oregon, and Washington combined (Bolda et al., 2010), and 13% revenue loss in the northeastern berry production area of Italy (De Ros et al., 2015). While numerous biological, cultural, mechanical, and chemical strategies are being developed for *D. suzukii* control, insecticides are used in both conventional and organic programs. While generally effective and convenient, the application of insecticides must be repeatedly every 10-14 days based on their residual activity (Beers et al., 2011), and even more if rainfall occurs (Diepenbrock et al., 2016; Van Timmeren and Isaacs, 2013). For insecticides to be part of a more sustainable program, efforts are underway to make insecticide applications more effective and reduce overall use, such as reduced spray programs (Klick et al., 2016), and also to develop environmentally-friendly insecticides.

The first objective of our study was to determine whether erythritol, erythrose and other sugars impact on the survivorship of *D. suzukii* with a dose assay. The second objective tested erythritol feeding in various combinations with water or sucrose to determine whether mortality was caused by starvation or other physiological changes. The third objective confirmed that erythritol was ingested in capillary no-choice and choice assays. Lastly, the fourth objective measured the sugar and glycogen content of *D. suzukii* adults fed various sugars to determine whether these foods were converted for carbohydrate storage.

Materials and Methods

Flies, sugars and sugar alcohols

Drosophila suzukii used in these experiments were from a colony maintained at 22±5ºC under a photoperiod of L:D 16:8 h, and a relative humidity of 60±5% RH at the Horticultural Crops Research Unit, USDA ARS in Corvallis, Oregon, USA. Wildtype flies collected from infested fruits in Corvallis in October 2015 and July 2016 were used to start the colony. Standard rearing methods and diet are described by Woltz et al. (2015). Newly emerged adult males and females were collected daily and maintained in cages with water and diet until they were specific ages for experimentation. Sugars used in this study, meso-erythritol (>99%), D-erythrose (75% syrup), D-mannitol (>99%), sorbitol (>99%), sucrose (>99%), and xylitol (>99%), were purchased from Fisher Scientific (Hampton, USA).

Dose-dependent effects of sugars on fly survivorship

Ten 5-day old flies (5 males and 5 females) were introduced into a plastic vial (28 mm id x 95 mm height) and fed a dose of 1, 0.5, 0.1 or 0.05M of either sucrose, erythritol, erythrose, xylitol, mannitol, or sorbitol. Each sugar solution was soaked on a cotton stud in a 1.5 ml centrifuge tube. Survivorship of flies was checked daily for 7 days. All treatments were replicated at least three times (vials). Water and sucrose solution were used for a negative and positive controls, respectively in this and subsequent studies.

Age-dependent effects of sugars on fly survivorship

Once survivorship was confirmed to be lower on erythritol and erythrose, the age-specific susceptibility on sugars was observed to refine future assays. Ten flies (5 males and 5 females) from 1- to 7-day old were introduced into a vial as described above and given 0.5M sucrose, erythritol, erythrose, xylitol, mannitol, or sorbitol. Survivorship of flies was checked daily for 7 days.

Separate or combined sugars on survivorship

Ten 5-day old flies (5 males and 5 females) were introduced into a plastic vial and given two separate tubes (1.5 ml) containing different solutions (Fig. 2.2A). Various pairs were tested: water + 0.5M sucrose, water + 0.5M erythritol, 0.5M sucrose + 0.5M erythritol, 0.5M sucrose + 1M erythritol, 1M sucrose + 1M erythritol, or 1M sucrose + 2M erythritol. Survivorship of flies was checked daily for 7 days. Each pair was replicated three times.

To test combined solutions, ten 5-day old flies were introduced to feed on different ratios of a mixed sucrose and erythritol solution placed in one tube (Fig. 2.2C). The combination of sucrose/erythritol ratios (0.5M/0.5M, 0.5M/1M, 0.5M/2M, 1M/2M, or 1M/2M) were soaked into a cotton stud in a 1.5 ml centrifuge tube. Survivorship of flies was checked daily for 7 days. Each combination was replicated three times.

Measurement of sugar consumption and body weight gain in no-choice assay

A single fly was introduced into a glass vial (15 mm id x 45 mm length, Thermo Scientific, Rockwood, TN, USA). As a lid, a half-cut centrifuge tube with a few holes for aeration had a central capillary glass tube (70 µl, 11 mm id x 70 mm height, Fisher Scientific) (Fig. 2.3A). The solution (0.5M) of erythritol or sucrose, or water was filled in the capillary tube, then a mineral oil (Thermo Scientific) layer covered the treatment solution to prevent evaporation. Three identical vials without a fly were served as controls to measure actual evaporation. The amount in the capillary tube was checked daily before and after feeding. The consumed amount was calculated by subtracting the evaporated amount in the control vial from the reduced amount in the fly vial.

To measure fly body weight gain, five male and female flies were separately introduced into a glass vial with a capillary as described above. Flies were allowed to feed sucrose, erythritol, or water, and weighed before and after feeding for 48 h using a microbalance (Thermo Scientific). The average body weight from five flies in each treatment was measured, and four replications (vials) were conducted for each treatment.

Measurement of consumption amount of sugars in choice assay

Five female flies aged 5-day old were introduced into a glass vial as described above with a modified lid with 2 aeration holes and 2 glass capillary tubes (Fig. 2.3C). Water + sucrose (0.5M), water + erythritol (0.5M), or sucrose (0.5M) + erythritol (0.5M) solutions were filled up in the capillary tubes. The set-up with mineral oil and control vials were similar to as above as well as the weight gain calculation.

Anthrone test for carbohydrate content

Adults were fed various diets: 1) water, 2) erythritol, 3) erythrose, 4) xylitol, 5) mannitol, 6) sorbitol, and 7) sucrose. To set up assays, 5-day old flies were transferred into plastic vials (28 mm id x 95 mm height). Each vial contained 4 males or 4 females with a diet treatment for 24 h, or 5 males or 5 females held for 48 h. More flies were held for 48 h in case of mortality. In each vial, a 0.5M solution was provided in a 0.5 ml centrifuge tube plugged with a cotton wick. For all treatments, live flies were frozen at -80°C after 24 h and 48 h of exposure; dead flies in the vial were not collected. Each diet treatment and hour combination was replicated in three vials.

A standard procedure refined for parasitic wasps (Olson et al., 2000) was used to determine the amount of glycogen and sugar in each fly. This procedure has been used for *D. suzukii*, Tochen et al. (2016) describes the calibration and final determination of nutrient values. The same procedures were used except that 200 µl of the final solution was pipetted into a 96-well ELISA plate and read on an absorbance reader (ELx808, BioTek).

Statistics

For the dose study, a separate survivorship analysis was conducted at each dose. First, a Kaplan-Meier log-rank comparison with censored observations for flies alive on day 7 tested all seven treatments together for survivorship. Next, means comparisons were done by testing each treatment pair by log-rank and using an

adjusted Bonferroni P-value (α/no. pairwise comparisons = 0.05/21 = 0.00238). For the separate or combined sugar survivorship assay, a similar analysis as described was used with an adjusted P-value of 0.0033 and 0.000238 for means comparisons, respectively. For the no-choice consumption and weight gain study, a standard ANOVA tested the effect of treatment; the assumptions of an ANOVA were met with untransformed data. For the choice consumption study, a separate repeated analyses was run for each paired choice, the assumptions of a parametric model were met. The effect of diet choice, hour, and diet x hour were fixed effects, and vial was the random subject effect. Means comparisons were done by Tukey HSD or t-test. These analyses were conducted in JMP® 12.1.0 (SAS, 2015).

For the carbohydrate assay, separate analyses were conducted for males and females and for glycogen and sugar. Each nutrient level was the dependent variable with a lognormal distribution. The effect of diet treatment, hour, diet x hour were fixed effects. The vial where 4-5 flies were held together was a random effect, each fly was a replicate. Nutrient levels of flies from the feeding treatments were compared with Tukey HSD test. If the effect of treatment was significant but diet x hour was non-significant, diets were compared pooling both hours. If the diet x hour interaction was significant, a separate Dunnett's test compared treatments at 24 h and at 48 h. These analyses were conducted in Proc Glimmix in SAS 9.3 (SAS, 2010).

Results

Dose-dependent effects of sugars on fly survivorship

Erythritol, erythrose and water-fed flies had lower survival given 1M, 0.5M, 0.1M and 0.05M dosages compare with xylitol, mannitol and sorbitol-fed flies for 7 days (Fig. 2.1, Table 2.1). No flies survived after 6-days of feeding on erythritol, erythrose and water. The survivorship from xylitol, mannitol and sorbitol-fed flies appeared lower, but was not significantly different compared with the sucrose treatment in 1M, 0.5M and 0.1M solution. At the 0.05M concentration, mannitol, sorbitol and xylitol-fed flies had significantly lower survivorship than sucrose-fed flies by 68%, 50%, and

30%, respectively on the 7th day (Fig. 2.1). Also, xylitol-fed flies had significantly lower survivorship than mannitol and sorbitol-fed flies.

Age-dependent effects of sugars on fly survivorship

After confirming the effects of sugar doses on fly survivorship, the 0.5M dose was selected for subsequent studies, and was introduced to 1 – 7 day old flies for 7 days (Supplementary data 1). Fly survivorship appeared to decrease with erythritol, erythrose and water from all age ranges. The most susceptible age on the sugar treatments appeared to be the 2-day old adult followed 1-day, 3-day, 4-day, 6-day, and then either 5-day or 7-day olds. Therefore, we used 5-day old flies as the most tolerant age on erythritol for following assays in this study unless stated. Survivorship from the same dose of sucrose, mannitol, sorbitol and xylitol did not appear to vary by age (data not shown).

Separate or combined sugars on fly survivorship

To determine the cause of mortality from erythritol ingestion, various concentrations of erythritol and sucrose were introduced in separate or combined tubes (Fig. 2.2, Table 2.1). When solutions were given separately, there was no effect on survivorship occurred regardless of erythritol concentrations, except for only erythritol + water (*) as a negative control (Fig. 2.2B). Mortality from erythritol was at 80% within 4 days (Fig. 2.2B). However, with a serial combination of sugar and erythritol solutions (Fig. 2.2C), fly survivorship was significantly ($P < 0.0001$) decreased for 7 days (Fig. 2.2D). The fastest and highest mortality occurred in the 1M sucrose/2M erythritol and 0.5M sucrose/2M erythritol combinations, followed by the 1M sucrose/1M erythritol and 0.5M erythritol (= a negative control), and then the 0.5M sucrose/1M erythritol combination. But no significant mortality occurred from combinations with 0.5M sucrose/0.5M erythritol as well as 0.5M sucrose as a positive control. The higher dose of erythritol regardless of the sucrose dose combined showed greater fly mortality (Fig. 2.2D).

Capillary no-choice and choice assays

To measure the amount of sugar ingested by the fly, a glass capillary tube was filled with 0.5M erythritol, sucrose or water for the fly to access in a glass container for 72 h (Fig. 2.3A, Table 2.1). The amount of erythritol ingested by the fly was significantly greater (0.12 µl per fly) than sucrose (0.01 µl per fly) and water (-0.02 µl per fly). Choice assays with paired capillary tubes filled with water + sucrose, water + erythritol, or sucrose + erythritol were exposed to five files for 72 h (Fig. 2.3C, Table 2.1). Overall, the sucrose consumed by the flies was greater than the erythritol choice (Fig. 2.4D). Between a choice of sucrose or water, there was no significant difference in 24 h ($P > 0.05$), 48 h ($P > 0.05$) and 72 h ($P > 0.05$) (Fig. 2.4D). Between a choice of water or erythritol, water was ingested more than erythritol in 24 h ($P=0.0038$) and 72 h ($P=0.0166$), but not in 48 h ($P > 0.05$). Between sucrose and erythritol, sucrose was more ingested than erythritol in 72 h ($P=0.0287$), but not different in 24 h ($P=0.3095$) and 48 h ($P=0.6771$).

Fly weight gain from the feeding sugars

After feeding for 48 h, the average male gained 1.41 mg from sucrose which was significantly heavier than 1.2 mg from water, and similar to that of 1.41 mg from erythritol (Fig. 2.4). The average female gained 1.96 mg from the sucrose and 2.01 mg from erythritol, these were significantly greater than 1.72 mg gained from water (Fig. 2.4). Overall, both male and female flies gained more weight with sucrose or erythritol than water, and female flies gained ~40% more weight than male flies (Fig. 2.4).

Sugar content after fed various sugars

Both males and female had significantly elevated total sugar levels in their entire body when fed mannitol, sorbitol, xylitol, or sucrose compared with water-fed flies after 48 h. Only those fed sucrose had significantly higher sugar levels at 24 h than water controls. We confirmed that our bioassay test does not react with sugar alcohols including mannitol, sorbitol or xylitol as well as erythritol and erythrose. Flies fed erythritol and erythrose had similar sugar levels as water-fed flies (Fig. 2.5, Table

2.1). The observed difference in the sugar levels between male and female flies is expected because females are heavier than males (Fig. 2.4).

Glycogen content after fed various sugars

Like sugar content, both males and females had significantly elevated glycogen levels when fed sorbitol, xylitol, and sucrose compared with water-fed flies (Fig. 2.6, Table 2.1). Males had elevated glycogen when fed mannitol, whereas females did not. However, glycogen levels remained low in males and females fed erythritol and erythrose for 48 h.

Effect of time on carbohydrate metabolism

Time affected both sugar and glycogen levels in males (Table 2.1). Time affected glycogen levels in females, but not sugar levels. Sugar and glycogen levels were higher at 24 h than 48 h. For males and females, there was also a diet x hour interaction with resulting sugar. Only those fed sucrose had significantly higher sugar levels at 24 h than water controls (Fig. 2.5). By 48 h, those fed mannitol, sorbitol, xylitol, and sucrose had higher sugar levels than water. This result indicated that some time is needed before the ingested diet is metabolized into other forms of sugar.

Discussion

In this study, we tested various sugars including erythritol under a variety of dosages, sugar combinations, fly ages, and feeding choices to investigate its potential insecticidal activity on *D. suzukii* adults. The previous study suggested erythritol acts an insecticidal effect on *D. melanogaster* (Baudier et al., 2014). We also observed fly mortality from various doses of ingested erythritol or erythrose in *D. suzukii*. In this study, therefore we investigated whether the fly mortality is caused by starvation because these sugars could not be metabolized into nutritional carbohydrates, or by hyperosmotic imbalance in the hemolymph in *D. suzukii*. In mammals, tetra-carbon molecules are normally passed through intestinal membranes at faster rate than hexose sugars (Munro et al., 1998; O'Donnell and Kearsley, 2012). Likely in *D. suzukii*, both erythritol and erythrose could be simply absorbed and diffused through

the midgut membrane. This may increase the osmotic pressure in the hemolymph before being excreted out. Further physiological studies are need to elucidate the possible mechanism.

The lowest concentration (0.05M) of xylitol and sorbitol ingested in this study led to decreased survivorship at 30% and 50% after feeding for 7 days. Death may have resulted from these sugar alcohols being slowly metabolized. Sorbitol is converted to fructose by the sorbitol dehydrogenase in mammalian tissues (El-Kabbani et al., 2004), and its metabolism is relatively slower than glucose and sucrose (O'Donnell and Kearsley, 2012). Therefore, lower doses of xylitol and sorbitol might not provide *D. suzukii* enough nutritional carbohydrates due to a slow metabolic process. A proboscis extension reflex (PER) assay to evoke the food response of *D. melanogaster* showed that flies learned sweet taste for a short period, then more likely chose sweet sugars (Burke and Waddell, 2011; Fujita and Tanimura, 2011). The survivorship on flies fed sorbitol or xylose like tasteless or less sweet sugar, was lower than sucrose-fed flies. The same 0.05M dose of mannitol did not significantly reduce longevity in *D. suzukii* compared with sucrose but appeared lower. Mannitol is metabolized to the mannose-6-phosphate from the reduction of mannose to be utilized for the glycolysis (Nelson and Cox, 2000).

Worker honey bees that ingested mannose had died, which suggests that mannose is toxic to honey bees (Sols et al., 1960). Mannose toxicity in the honey bee was due to a large accumulation of mannose-6-phosphate and decreasing ATP by a shortage of mannosephosphate isomerase, however, this was not found to occur in *D. melanogaster* (De la Fuente et al., 1986). Like *D. melanogaster*, *D. suzukii* in this study was also not susceptible to 0.1 - 1M mannitol, indicating it is readily metabolized to mannose. Recently, *D. melanogaster* that ingested 1M mannitol over 10 days had reduced the longevity of female flies more than males, but it was not clear about the sex specific toxicity (O'Donnell et al., 2016).

When given a choice, *D. suzukii* ingests more sucrose than erythritol in 72 h (Fig. 2.3D). When sucrose and erythritol were both available as separate solutions to *D. suzukii*, the fly survivorship was not affected regardless of the erythritol dose (Fig. 2.2B). This indicates that *D. suzukii* could be more preferred to sucrose than erythritol, and will feed sufficiently on sucrose to maintain itself in the presence of erythritol. This may not be surprising because the sweetness of sucrose is known to be 30% higher than erythritol (Munro et al., 1998). However, when *D. melanogaster* were given access to both 1M sucrose and 2M erythritol as separate solutions, fly longevity was reduced to 50% after two weeks compared with sucrose alone (Baudier et al., 2014). Thus, *D. melanogaster* will feed on erythritol sufficiently in the presence of sucrose, and erythritol could be a detrimental.

While erythritol may not be detrimental to *D. suzukii* if sucrose is also available separately, erythritol was confirmed to have insecticidal properties against *D. suzukii*. In our survivorship assay that combined sucrose and erythritol together, the mortality of *D. suzukii* rapidly increased with several ratios of the two sugars combined (Fig. 2.2D). Actually, the most significant result obtained is when sucrose/erythritol was provided at a 1:2 or 1:4 ratio. When *D. suzukii* were given 0.5M sucrose/water or 0.5/0.5M sucrose/erythritol, they had high survivorship. In this case, the sucrose concentration is 0.5M and the same as the sucrose concentration in the 0.5/2.0M sucrose/erythritol solution which induced a high mortality.

Although there is nothing known about the absorption and biochemical metabolism of ingested erythritol, it might be conceivable that a feedback loop of food intake regulates the crop emptying and hemolymph osmolality in the fly. In Diptera, both digestion and absorption are predominantly accomplished in the midgut and the control of food intake is closely related to the rate of crop emptying and hemolymph osmotic pressure in insects (Bernays and Simpson, 1982). Diffusion of sugars from the midgut to the hemolymph elevates the osmotic pressure, which in turn decreases the rate of crop emptying in the blowflies (Thomson and Holling, 1977).

Therefore, it is possible that the non-metabolizable and non-storable erythritol in *D. suzukii* might maintain high osmotic pressure in the hemolymph that decreases the rate of crop emptying resulting in feeding inhibition followed by starvation. Another postulation is that an abnormally high concentration of sugars from erythritol and sucrose diffused to the hemolymph from the midgut should demand a large amount of water. The water dilutes to reduce the osmotic pressure, and may also be used to hydrolyze the breakdown of sucrose to glucose and fructose, or to immediately excrete excess erythritol from the body. This extreme change in osmotic pressure and over-regulated physiological change could be a critical for the fly body. Sucrose ingested with erythritol should be metabolized through a specific enzymatic process, and then utilized or stored in the glycogen form. The increasing rate of the osmolality in the hemolymph has been discussed and different depending on metabolizable or non-metabolizable carbohydrate fed in the honey bee (Roces and Blatt, 1999).

Although flies would be more preferred to sucrose than erythritol in choice conditions (Fig. 2.3D), the actual amount of erythritol consumed was greater than sucrose for 72 h under no-choice conditions (Fig. 2.3B). This could be interpreted that if flies were fully satiated after feeding sucrose, they stopped feeding, whereas the fly continuously fed on erythritol for this period. Interestingly, the actual amount of ingested water by fly was a negative value which means the evaporation of water exceeded water consumption by the fly. When *D. suzukii* feed on non-nutritive erythritol, they continue to feed because they are still hungry. A similar result has been observed in *D. melanogaster* that fed more on erythritol than sucrose (Baudier et al., 2014). Under no-choice conditions, *D. suzukii* fed the least amount on water compared with sucrose and erythritol. *D. suzukii* may have fed more on erythritol than water under no-choice because the fly responded to the sweet taste from erythritol which is 60–80% as sweet as sucrose, or table sugar (Munro et al., 1998). In contrast to the no-choice test, in the choice test with erythritol and water, flies consumed more water than erythritol. This result implies that additional water feeding might protect the fly against the high physiological osmotic pressure caused by erythritol in the hemolymph.

Since *D. suzukii* ingested more erythritol than sucrose in no-choice tests (Fig. 2.3B), flies would be expected to have more weight gain from erythritol than sucrose feeding. However, weight gain was similar among sucrose and erythritol-fed flies (Fig. 2.4). This may have resulted if flies excreted more erythritol after feeding. In mammals, erythritol as a sweet antioxidant could be converted to erythrose in the intestinal membranes and excreted by urination because erythrose has been found in the urine of erythritol-consuming rat (Den Hartog et al., 2010). In insects, the physiological process of absorption, metabolism and excretion from ingested erythritol would be interesting for future research.

By measuring the sugar and glycogen content of flies after feeding, we could infer whether ingested erythritol or erythrose was metabolized into certain carbohydrates. The levels of sugars and glycogen were significantly elevated in mannitol, sorbitol, and xylitol-fed flies after 24 h and/or 48 h, but did not changed among erythritol and erythrose-fed flies. The result implies that those sugar alcohols were utilized for substrates to be converted into sugar metabolisms (Nelson and Cox, 2000). Yet, erythritol and erythrose might be absorbed into midgut and were not converted or synthesized into long chain carbohydrates such as glycogen, a common storage form. Interestingly, only flies fed sucrose had significantly higher sugar levels at 24 h while those fed mannitol, sorbitol, and xylitol had higher sugar levels at 48 h. This suggests that these sugar alcohols may be slowly converted to sugars and should have more metabolic pathways than sucrose metabolism. The lack of carbohydrate metabolism with an erythritol or erythrose diet, and slower storage with sorbitol or xylitol in this assay may be related to the complete mortality with erythritol or erythrose and lowered survivorship with sorbitol or xylitol in the dose assay.

Acknowledgements

We thank Adam Cave, Kelly Donahue, Kathleen Knight, Anne Snell and Ann You for technical and colony support. This work was supported in part by a Northwest Center for Small Fruits Research Grant, and base funding from USDA ARS CRIS 2072-22000-040-00D.

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Figures

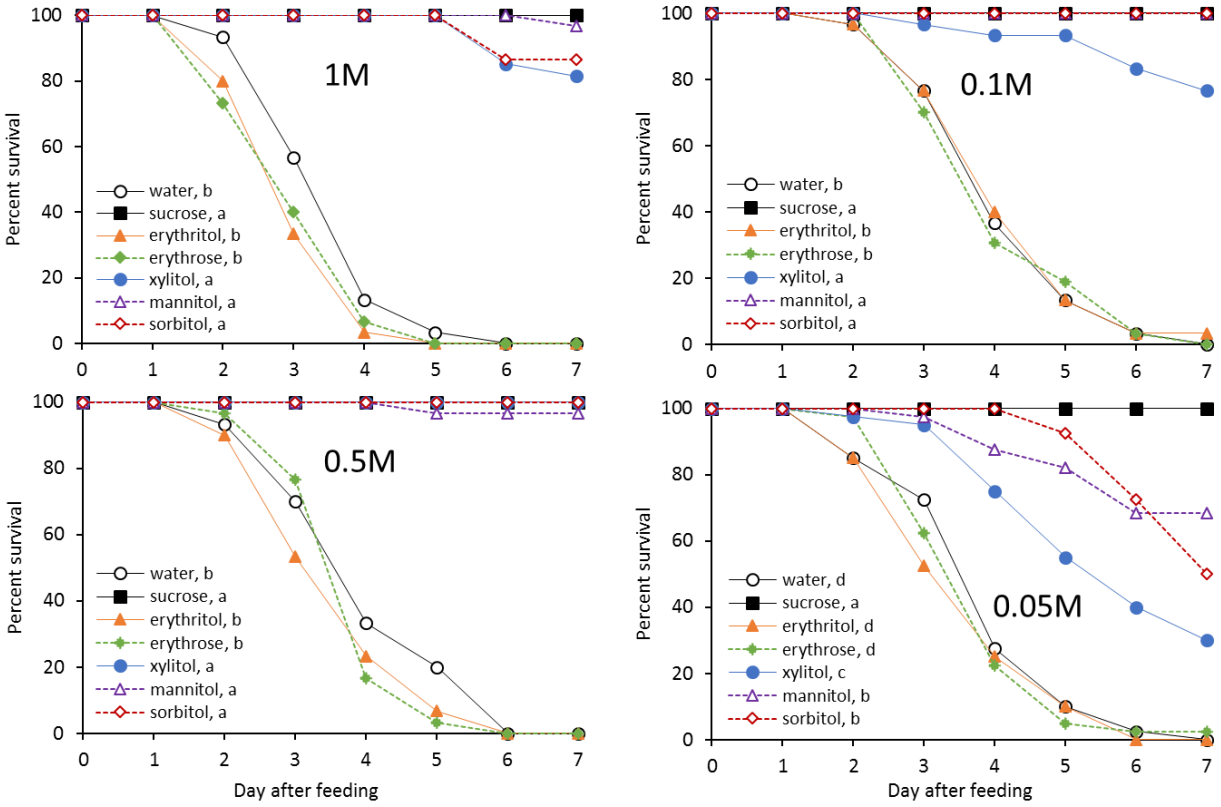


Figure 2.1. Survivorship of *D. suzukii* in different doses of sucrose, erythritol, erythrose, xylitol, mannitol and sorbitol provided as a sole food source. Different letters in figures denote significant differences by log-rank analyses (statistical analysis in Table 2.1).

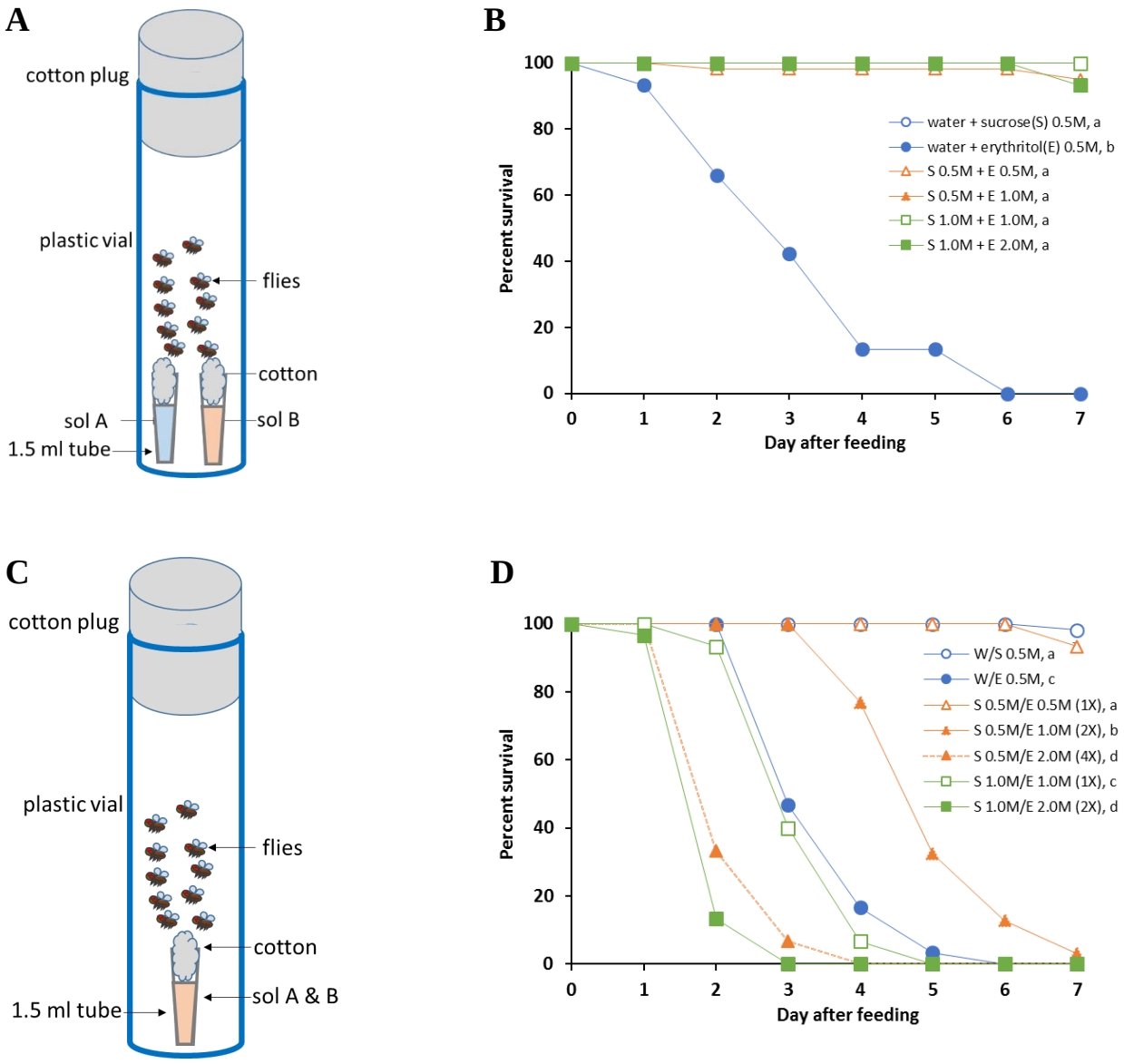


Figure 2.2. Survivorship of *D. suzukii* in the separate sugar (A and B) and combined sugar (C and D) tests. Different letters denote significant differences by log-rank analyses (statistical analysis in Table 2.1).

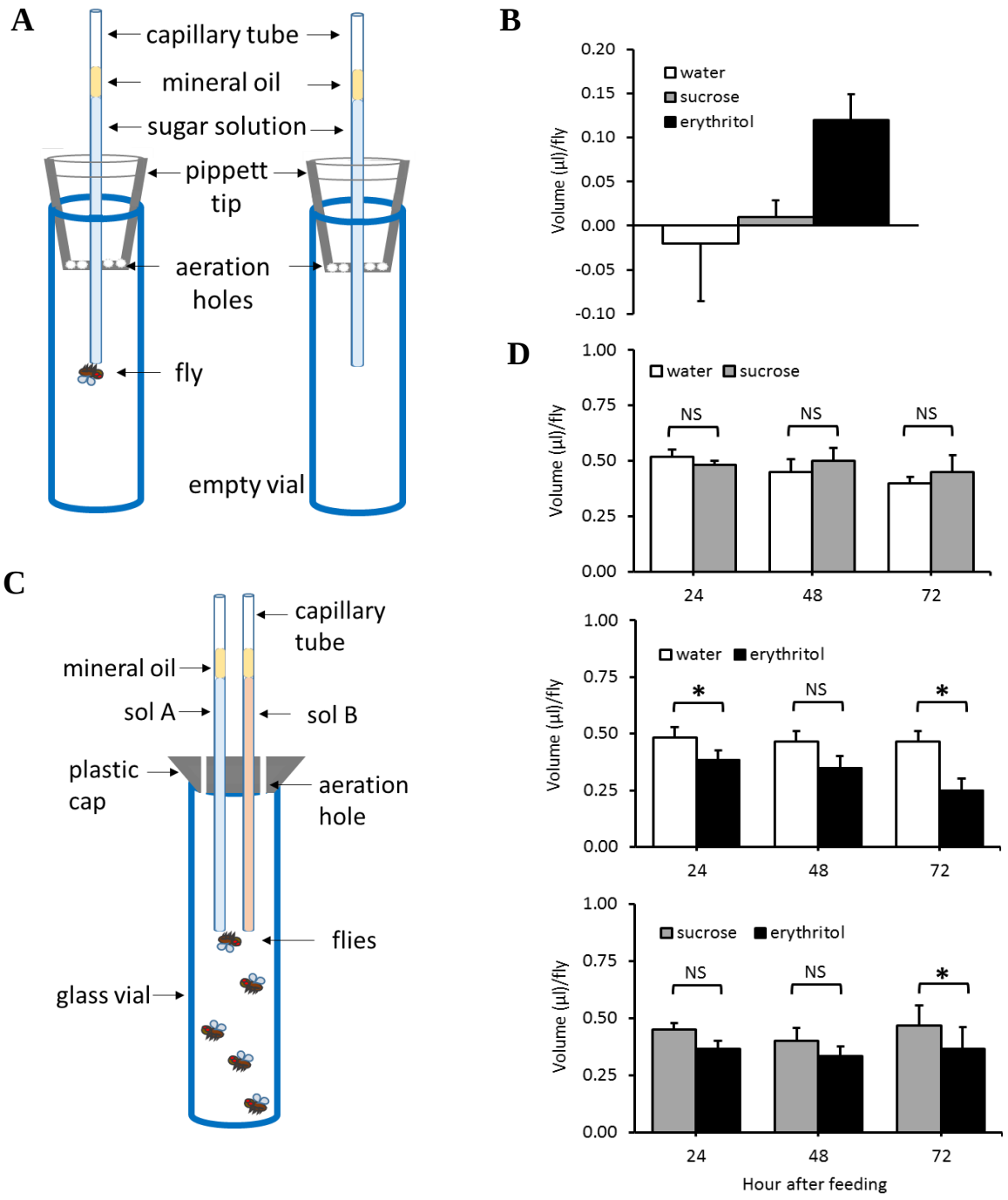


Figure 2.3. Sugar feeding tests in different test regimes. (A) Diagram of no-choice test. (B) Consumed amount of water, sucrose and erythritol per *D. suzukii* adult at 72 h. (C) Diagram of choice test. (D) Consumed amount per SWD fly at 24, 48, and 72 h when two solutions out of water, sucrose and erythritol were provided. Asterisks denote a higher volume consumed for the given choice when data were analyzed for each hour. Asterisk (*) in D indicates a significant difference ($P < 0.05$) by Tukey HSD between two sugars. NS: no significance (statistical analysis in Table 2.1).

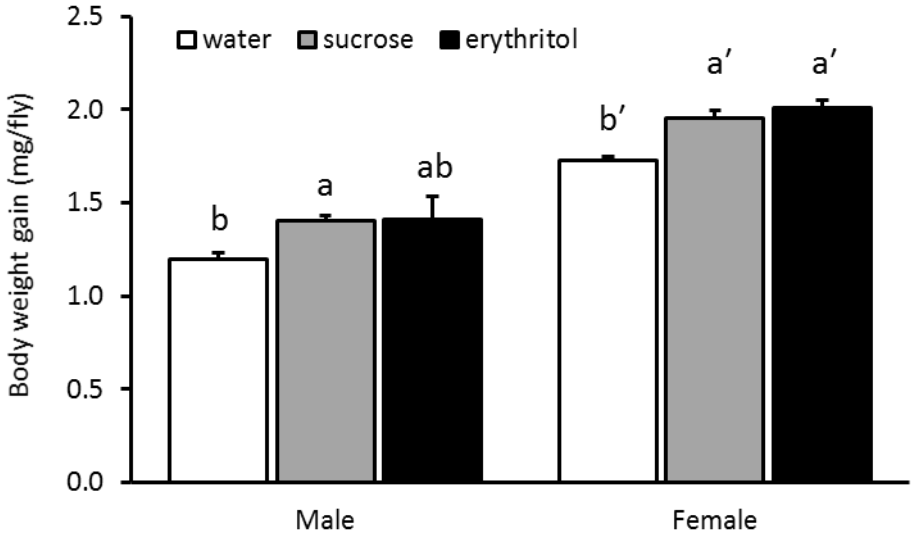


Figure 2.4. Change of body weight gain of SWD feeding water, sucrose and erythritol. Fly fed 1M sucrose and 1M erythritol for 48h. Male: $F_{2,6} = 6.0$, $P = 0.037$. Female: $F_{2,7} = 21.2$, $P = 0.001$. Different letters on the bars denote significant differences by Tukey HSD.

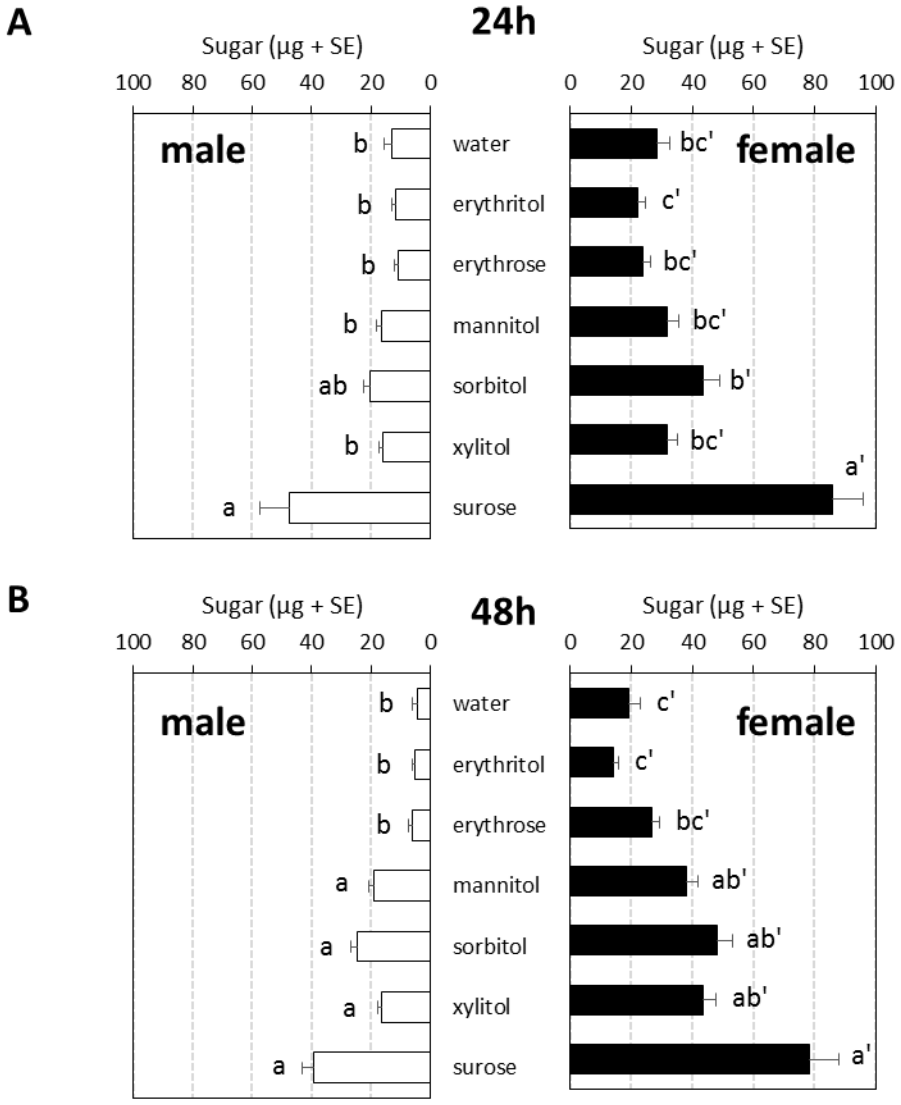


Figure 2.5. Average level of sugar in *D. suzukii* males and females when exposed to various sugar diets for 24 h (A) and 48 h (B). Different letters indicate significant differences analyzed by Tukey HSD test (statistical analysis in Table 2.1).

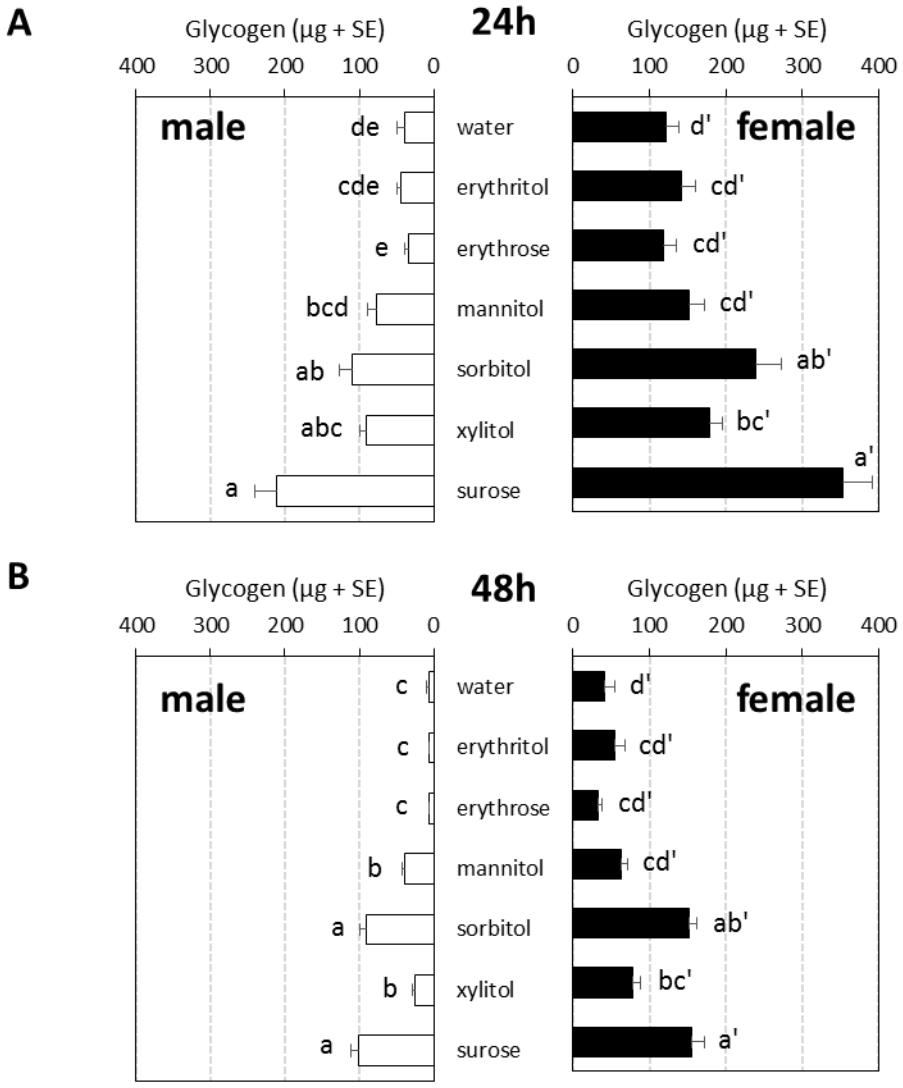
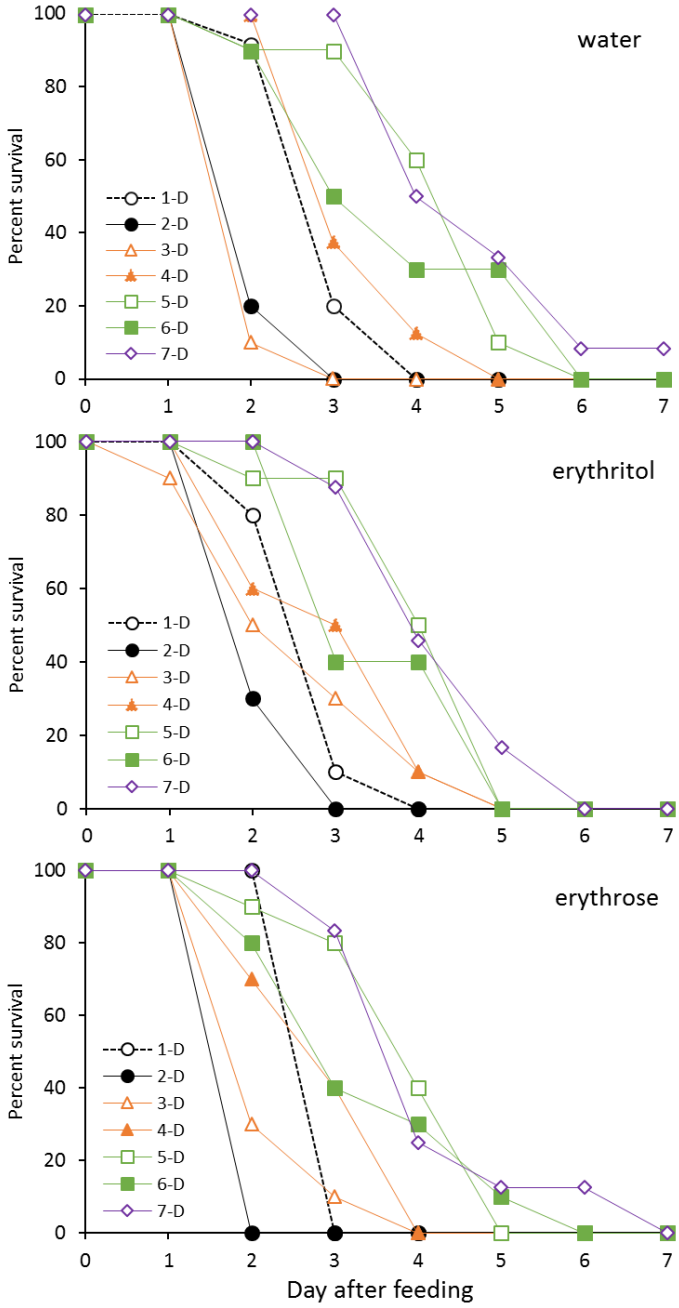


Figure 2.6. Average level of glycogen in *D. suzukii* males and females when exposed to various sugar diets for 24 h (A) and 48 h (B). Different letters indicate significant differences analyzed by Tukey HSD test (statistical analysis in Table 2.1).



Supplementary figure 2.1. Survivorship of differently-aged adults of *D. suzukii* in water, erythritol, and erythrose. Flies reared in standard diet for 1 day to 7-days from emergence. Ten flies were provided with either water, erythritol, or erythrose as a sole food source.

Table 2.1. Kaplan-Meier log-rank test on survivorship between treatments, or the effects of diet treatment, time and their interaction on the volume ingested in the no-choice/choice study, and on sugar and glycogen content in the carbohydrate assay.*

Study	Dependent variable	Effect	df	F	P	df	F	P
Dose 1 M	Survival	Diet	6	250.7	<0.001			
Dose 0.5 M	Survival	Diet	6	251.2	<0.001			
Dose 0.1 M	Survival	Diet	6	231.4	<0.001			
Dose 0.05 M	Survival	Diet	6	230.0	<0.001			
Sep. solution	Survival	Diet	5	382.5	<0.001			
Comb. solution	Survival	Diet	6	291.4	<0.001			
No-choice/choice study			**No-choice**			**Choice: Water or sucrose**		
	Volume ingested	Diet	2, 12	2.2	0.15	1, 4	0.36	0.58
		Hour				2, 8	1.4	0.30
		Diet x Hour				2, 8	1.6	0.26
			Choice: Water or eryth.			**Choice: Sucrose or eryth.**		
	Volume ingested	Diet	1, 4	12.8	0.023	1, 4	13.2	0.022
		Hour	2, 8	13.1	0.003	2, 8	0.08	0.93
		Diet x hour	2, 8	2.6	0.13	2, 8	1.7	0.26
Carbohydrate assay			**Male**			**Female**		
	Sugar	Diet	6, 138	24.2	< 0.0001	6, 143	17.8	< 0.0001
		Hour	1, 138	14.16	0.0002	1, 143	1.43	0.233
		Diet x hour	6, 138	5.26	< 0.0001	6, 143	2.41	0.030
	Glycogen	Diet	6, 138	69.4	< 0.0001	6, 143	14.4	< 0.0001
		Hour	1, 138	174.5	< 0.0001	1, 143	81.6	< 0.0001
		Diet x hour	6, 138	9.63	< 0.0001	6, 143	1.88	0.088

* Measured total amounts of sugar and glycogen from 5-day old flies fed sugars in Figs. 2.1, 2.2, 2.3, 2.5 and 2.6.

Chapter 3

Effect of erythritol formulation on the mortality, fecundity and physiological excretion in *Drosophila suzukii*

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Styled for Journal of Insect Physiology
by ELSEVIER
Received 12 June 2017; Received in revised form 24 July 2017;
Accepted 28 July 2017; Available online 29 July 2017

Abstract

Previously, we studied various combinations of non-nutritive sugars including erythritol and erythrose having a potentially insecticidal effect on *Drosophila suzukii*. The study suggested two potential physiological changes causing fly mortality: 1) starvation from the feeding of non-metabolizable erythritol and erythrose; 2) abnormal osmotic pressure increased in the hemolymph with erythritol transported from the midgut. In the present study, sucrose and erythritol were applied to blueberries and effects of these combinations on fly mortality and fecundity were monitored in the lab and greenhouse. In the lab, two sucrose/erythritol formulations (0.5M sucrose/2M erythritol, 1M sucrose/2M erythritol) resulted in the highest mortality and the lowest fecundity among *D. suzukii* adults. Two formulations, therefore, were selected for further evaluation with blueberry bushes and fruits in the greenhouse; fly survival with 0.5M sucrose/2M erythritol was significantly lower than 1M sucrose/2M erythritol for 7 days. Unlike the smaller container, mortality occurred faster in the greenhouse probably because flies moved more in the bigger cage accelerating the exhaustion of energetic reserves in the body. We examined presence of erythritol in the hemolymph and frass to determine the nutritional metabolism and absorption of erythritol in *D. suzukii*. Unlike sucrose, a large amount of erythritol was observed in the hemolymph of the fly ingested 0.5M sucrose/0.5M erythritol. Erythritol was also found in the frass in the same fly. The results imply that erythritol might be directly transported from the midgut without being metabolized and stored, but is accumulated in the hemolymph which in turn elevates the osmotic pressure in the fly hemolymph. For practical application, the sucrose/erythritol combination would be more effective than erythritol alone because the combination tastes sweeter to elicit more feeding. This erythritol formulation can be a potential insecticide used alone or as a delivery agent combined with conventional or biological insecticides to enhance their efficacy.

Keywords: non-nutritive sugar, erythritol, insecticidal activity, mortality, fecundity, *Drosophila suzukii*

Introduction

Sugar mixed in insecticide formulations can increase their effectiveness by acting as a phagostimulant, causing pests to ingest more insecticide. Sugars are detected and selected by *Drosophila* flies based on the sugar's nutritional value, that flies recognize from continuous feeding on the nutritional sugar through learning and appetitive memory (Burke and Waddell, 2011; Fujita and Tanimura, 2011). A similar result was found from a choice test with spotted wing drosophila, *Drosophila suzukii*, that flies preferred sucrose to the non-nutritional sugar (Choi et al., 2017). To enhance the effectiveness of insecticides, sucrose has been added to conventional or organic insecticides targeting *D. suzukii* (Cowles et al., 2015).

In the lab, nonnutritive sugars applied to *Drosophila melanogaster* had reduced the fly longevity and motor coordination when fed erythritol or the artificial sweetener that contained erythritol (Baudier et al., 2014; O'Donnell et al., 2016). Moreover, *D. melanogaster* actively consumed erythritol in the presence of the sucrose, and had decreased longevity (Baudier et al., 2014). This is important because erythritol could have an insecticidal effect if the insect pest readily consumes it. Recently, erythritol was also shown to carry insecticidal properties against *D. suzukii* (Choi et al., 2017; Sampson et al., 2017; Sampson et al., 2016), and the oriental fruit fly, *Bactrocera dorsalis* (Zheng et al., 2016), and the house fly, *Musca domestica* (Fisher et al., 2017).

Although erythritol had insecticidal properties on Diptera pests including *Drosophila*, the corresponding physiological changes following erythritol ingestion and mechanism leading to death remains unknown. Erythritol is a four carbon-structured sugar alcohol with 75% of the sweetness of sucrose, and is produced from corn or wheat starch by enzymatic process of either yeast or fermentative microorganisms (Munro et al., 1998). Our previous study showed various concentrations of erythritol and sucrose combinations to affect the mortality of *D. suzukii* (Choi et al., 2017). From the study, two mechanisms for the fly death were hypothesized: that fly mortality is caused by starvation because erythritol cannot be metabolized into

nutritional carbohydrates, and by hyperosmotic imbalance in the hemolymph in *D. suzukii*. Such knowledge could be used to develop a non-toxic chemical alternative to control SWD in the field.

This study aims to improve management of *D. suzukii,* a serious economic pest of small fruits and cherries in Asia, Europe and North America (Asplen et al., 2015). *D. suzukii* could spread to additional temperate and subtropical areas including South America, Australia and Africa where the environment is suitable (Dos Santos et al., 2017). Chemical insecticides, while effective and used in both conventional and organic programs, have negative environmental and health consequences. Insecticides must be reapplied after 10-14 days as the residual activity wanes (Burke and Waddell, 2011), with rainfall (Van Timmeren and Isaacs, 2013), or as a new generation of adults emerge since most spray programs target the adult population (Wiman et al., 2016). Recent research has sought to reduce insecticide inputs by reducing the area sprayed while maintaining crop protection (Klick et al., 2016), and reducing the amount of insecticides used in an "attract and kill" approach (Rice et al., 2017).

In the present study, various combinations of erythritol and sucrose were applied on blueberry fruits and compared for their effects on the mortality and fecundity of *D. suzukii*. Also, the two most effective formulations were further evaluated for their insecticidal effect on blueberry bushes inside a screen cage in the greenhouse. To determine whether erythritol is being metabolized in *D. suzukii*'s body, erythritol was measured in the hemolymph and frass of flies that had ingested erythritol. Our project uses a sugar formulation as both a phagostimulant and non-toxic insecticide that may be used alone or combined to enhance other insecticidal agents.

Materials and Methods

Flies, sugars and sugar alcohols

The colony of *Drosophila suzukii* was maintained at 22±5 ºC under a photoperiod of L:D 16:8 h, and a relative humidity of 60±5% RH at the Horticultural Crops Research Unit, USDA ARS in Corvallis, Oregon, USA. Flies were the F_2 generation from

wildtype parents; wild parents were regularly collected from infested fruits in the field and used to lay eggs in the colony. Standard rearing methods and diet are described by Woltz et al. (2015). Newly emerged adult males and females were collected daily and maintained in cages with water and diet until they were specific ages for experimentation. Sugars used in this study, meso-erythritol (>99%), D-erythrose (75% syrup), D-mannitol (>99%), and sucrose (>99%) were purchased from Fisher Scientific (Hampton, USA).

Effect of erythritol formulations on fly mortality and fecundity in cup arena

Ten 5-day old flies (5 males and 5 females) starved for 24 h in advance were introduced into a plastic cup (0.5 L) to feed on different ratios of sucrose/erythritol solution in a tube. A total of 10 fresh blueberries (provided 5 blueberries twice) per container were provided to flies lay egg on blueberries for 7 days. The combination of sucrose/erythritol ratios (0.5M/0.5M, 0.5M/1M, 0.5M/2M, 1M/0.5M, 1M/1M, or 1M/2M) were soaked into a cotton stud in a 1.5 ml centrifuge tube. Survivorship of flies was checked daily for 7 days. Eggs laid on the blueberries in the cup were investigated under a dissecting microscope. Each combination was replicated three times.

Effect of erythritol formulations on fly mortality in cage

One-day old flies colony were set up in a greenhouse located in USDA-ARS, Corvallis under controlled environmental conditions with temperature and relative humidity (RH) maintained at 22 ± 5 ºC, 35 ± 5 % RH under a photoperiod of L:D 16:8 h with a LED light in the greenhouse. Two-year old southern highbush blueberry plants (Fall Creek Farm and Nursery, Inc. Lowell, OR, USA) were introduced in a bug tent (L60 x W60 x H60 cm^3, MegaView Science, Taiwan). The pot was wrapped with a plastic bag and tied shut at the plant stem to prevent flies from accessing the soil on or moving under the pot. This ensured that flies could only use the food or water resource given to them. Twenty fresh organic blueberries in a plastic weigh boat were hung from a branch. Ten ml of sucrose/erythritol ratios (0.5M/2M, 1M/2M), 0.5M sucrose, 0.5M erythritol or water was evenly sprayed on the entire bush with

leaves and fruits using a hand atomizer. Then, thirty 5-day old flies (15 females and 15 males) were released into the tent. From then on, 10 ml water was sprayed on the bush and fruits once a day for 6 days. Survivorship of flies was checked daily for 7 days. Each combination was replicated three times.

Preparation of hemolymph and frass from flies

More than twenty females flies (5-day old) starved for 24 h in advance were introduced into a plastic cup (0.5 L) to feed on 0.5M sucrose, 0.5M sucrose/0.5M erythritol, or 0.5M sucrose/0.5M erythrose solutions for 24 hrs. To collect hemolymph, fed-flies were anesthetized by CO_2 on a Flypad (8.1 x 11.6 cm^2, Genesee Scientific, San Diego, CA, USA). A tiny hole was punctured through the scutum above mesopleuron laterally (above joint of wing) while avoiding the crop and gut area. All legs were removed before puncturing and bled to avoid potential sugar contamination from leg tarsi if a fly had landed on a cotton stud soaked in sugar. Amputated flies were then transferred into a 0.5 ml tube with a tiny hole at the bottom (served like a column) that was inserted in a 1.5 ml tube chilled on ice. Hemolymph was collected by a centrifugation at 5000 rpm, 4 ºC for 5 min, and stored at -20 ºC until the sugar analysis below.

To collect frass from the fly, 5-day old flies were starved for 24 h earlier and allowed to feed on sugar solutions above in a plastic container (3 cm id x 10 cm height). A transparent plastic film (9 cm x 8 cm) was rolled and placed into the tube. The film was exposed for 24 h to flies depositing their frass on the wall. After the film was carefully removed from the container using a forceps, 20 dots of frass and 20 empty spots as a control were marked on the other side of the film under light. An RNase-free water (2 µl) was dropped on the fecal dot, and gently washed out by pipetting few times to collect the frass, then transferred it into 20µl RNAse-free water. This step was repeated to collect 20 fecal dots. The frass samples were then stored under -20 ºC and until the precipitation and derivatization process prior to GC-MS analysis. The collection of hemolymph and frass were repeated three times.

Derivatization of sugars in hemolymph and frass and GC-MS analysis

Derivatization for sugars collected from the hemolymph and frass was conducted according to method described by Wahjudi *et al*., (2010). Mannitol (100 µg) as an internal standard (IS) was added in the each sample tube for sugar derivatization. A hundred µl of methoxylamine hydrochloride (0.18M in pyridine) was added, capped and heated at 70 ˚C for 1 h in fume hood as the first step of derivatization and followed by adding 100 µl acetic anhydride, heated under 45 ˚C for 1 hour. The end product were leave to air dried in fume hood and redissolved in 50 µl ethyl acetate prior to gas chromatography-mass spectrometer (GC-MS) analysis.

Sugar derivatives were analyzed by GC (HP6890; Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a mass-selective detector (HP 5975; Agilent) and a HP-5MS column (30 m × 0.25mmID, 0.25 µm film thickness; Agilent). Helium was the carrier gas. The oven temperature was programed at 80 °C for 1 min, increased to 300 ºC at 10 °C/min, and held for 10 min. Injector temperature was 250 ºC. Retention times for erythritol and other sugars were identified by the GC-MS after the sugar derivatization. Quantification of sugars was obtained by comparing its total ion area with the total ion area of the IS. Thus, the concentrations are not absolute but, instead, relative to the IS.

Statistics

For analyzing survivorship in a cup or cage, a Kaplan-Meier log-rank comparison with censored observations for flies alive on day 7 tested all treatments together. Next, means comparisons tested each treatment pair by log-rank and using the Sidak multiple comparison adjustment. For analyzing fecundity, the total number of eggs laid over seven days was summed. Treatments were compared using a generalized linear model with a Poisson distribution, and Tukey HSD means separation. Lastly, the number of eggs laid was regressed with the average number of days that the 10 flies lived on a per cup (replicate) basis to determine the relationship between longevity and fecundity. Survivorship analyses were done in PROC LIFETEST,

fecundity analysis in PROC GLIMMIX, and linear regression in PROC REG in SAS 9.3 (SAS 2012).

Results

Effect of erythritol formulations on fly mortality and fecundity in cup arena

Various combinations of sucrose/erythritol dose were introduced to flies with blueberries in cup for 7 days (Fig. 3.1A). The fly survivorship was significantly decreased for 7 days (Fig. 3.1B). The fastest and highest mortality occurred in the 1M sucrose/2M erythritol and 0.5M sucrose/2M erythritol combinations, followed by 0.5M erythritol only (= a negative control), the 1M sucrose/1M erythritol, the 0.5M sucrose/1M erythritol, 1M sucrose/0.5M erythritol, and then 0.5M sucrose/0.5M erythritol. The total higher concentration of two sugars likely showed greater fly mortality (Fig. 3.1B).

The fecundity of female adults was significantly decreased for 7 days (Fig. 3.1C). The lowest fecundity (< 2 eggs from 5 females) was observed in 0.5M sucrose/2M erythritol, and 1M sucrose/1M erythritol combinations for 7 days, followed by 0.5M erythritol and 0.5M sucrose/1M erythritol, and intermediate fecundity in 1M sucrose/2M erythritol, 1M sucrose/0.5M erythritol, and 0.5M sucrose/0.5M erythritol. Egg numbers in all treatments were significantly lower than 0.5M sucrose as a positive control (~22 eggs). The number of eggs laid significantly increased as flies within the cup lived longer (regression $F_{1,46}$= 23.6, P < 0.0001, r^2 = 0.34).

Effect of erythritol formulations on fly mortality in cage

Two combinations of sucrose/erythritol that caused the highest mortality in Fig. 3.1B were selected for a larger scale test on in a greenhouse cage (Fig. 3.2A). Each solution made with 0.5M sucrose/2M erythritol, 1M sucrose/2M erythritol, was sprayed on blueberry bushes and blueberries provided separately in a hanging basket (inset photo in Fig. 3.2A). The highest mortality was obtained from 0.5M sucrose/2M erythritol, then followed 0.5M erythritol and 1M sucrose/2M erythritol (Fig. 3.2B). Two controls, 0.5M sucrose and water only, however were not significantly different

in survival rates. The resulting fly mortality from each treatments was more pronounced than the cup test above.

Sugar contents in hemolymph and frass of flies

To determine the presence and quantity of sugars in the fly's hemolymph and frass following sugar ingestion, each sugar was analyzed by GC-MS through a sugar derivative process. Four to twelve carbon sugars derivatized were clearly detected and measured with their retention times by GC-MS (Fig. 3.3A). Although the molecular weights of sucrose and trehalose are the same, they were separated by their retention times. In hemolymph (approx. 20 µl) a large amount of erythritol (avg. 59.5 µg) was measured (Figs. 3.3B, 3.4), but amounts of sucrose (avg. 3.5 µg) and trehalose (avg. 1.9 µg) were relatively low (Figs. 3.3B, 3.4). Erythritol (avg. 2.1 µg) was also clearly detected from the frass (approx. 20 drops) of the same fed-flies although the level was much lower than in the hemolymph (Figs. 3.3C, 3.4). The other two sugars, however, were not observed in the frass.

Discussion

Once erythritol was found to have an insecticidal effect on *D. melanogaster* (Baudier et al., 2014), the effects were investigated on other flies such as *D. suzukii* (Choi et al., 2017; Sampson et al., 2017; Sampson et al., 2016), *Bactrocera dorsalis* (Zheng et al., 2016) and *Musca domestica* (Fisher et al., 2017). Previously, a variety of dosages of erythritol and other sugars in mixed or separate solutions had significantly decreased the survival of *D. suzukii* adults, and suggested that erythritol alone or with sucrose had potential insecticidal activity (Choi et al., 2017). The present study builds on the prior study by monitoring the impacts on fecundity, testing at a larger scale in greenhouse cages, and examining the nutritional pathway of ingested erythritol in the fly body. Meanwhile, a feasible application and possible mode of action of erythritol as an insecticidal agent are discussed.

Survival rates of *D. suzukii* in a cup arena with blueberries were similar to previous tests in vials without fruits when flies were fed various sucrose/erythritol solutions

(Choi et al., 2017). While keeping sucrose concentrations constant at 0.5 or 1.0 M, higher concentrations of erythritol at 2 M elicited quicker mortality than lower doses at 0.5 or 1M. Ratios of 0.5M sucrose/2M erythritol and 1M sucrose/2M erythritol led to the highest mortality. Both results have been consistent in the current and previous studies. These studies revealed that 0.5M sucrose provides a sufficient carbohydrate dose for sustaining *D. suzukii* adults.

Two sucrose/erythritol ratios of 0.5M sucrose/2M erythritol and 1M sucrose/2M erythritol were selected for further evaluation in cages with blueberry bushes and fruits in the greenhouse. Unlike the smaller cup container, the cage space was more open, and mortality occurred more quickly in the greenhouse than the lab. There was 100% survival in the positive sucrose control in the lab but almost no survival by 7 days in the greenhouse. The rapid death could result from the fly actively moving around the cage, which accelerated the exhaustion of energetic reserves in the body. Interestingly, 0.5M sucrose/2M erythritol was more detrimental than 1M sucrose/2M erythritol on *D. suzukii* in the cage experiment but not in the lab experiment. Future evaluations should consider the practical convenience of the formulation. Notably, the 1M sucrose/2M erythritol contains a high concentration of sugars (w/v, approx. 60%) that becomes a very sticky solution. When sprayed on a plant, the fly might have difficulty drinking the viscous droplet. Since the 0.5M sucrose/2M erythritol combination elicited high mortality, has a lower sugar concentration, is less viscous and more likely to be fed upon by *D. suzukii*, it will be chosen for future evaluations. In addition to field trials, greenhouse trials should test the effectiveness of this formulation when fed upon before or after feeding on other naturally-occurring sugar sources such as floral nectar or wounded fruit. This would clarify potential impacts in the field, and whether initial consumption of erythritol followed by other sugars leads to continual unbalanced osmolarity.

Non-nutritive sugars such as erythritol have been introduced to increase the feeding activity of the ant although the ants were not attracted very much (Vander Meer et al., 1995). Although Drosophila will initially feed on many non-nutritive sugars

(Gordesky-Gold et al., 2008), long-term feeding studies demonstrate that they will eventually reject non-nutritive sweeteners due to a process involving long-term memory (Burke and Waddell, 2011; Fujita and Tanimura, 2011). For a feasible application, therefore, the sucrose/erythritol combination will be more effective than erythritol alone because it is sweeter and more nutritious and would be chosen by the fly.

In the previous study, we suggested two mechanisms that might explain how erythritol caused fly mortality: by starvation with a nonnutritive food source, or by physiological imbalance with an abnormal osmotic pressure in the hemolymph (Choi et al., 2017). Starvation can be explained in the case when erythritol is provided alone without sucrose. However, *D. suzukii* still died when fed 0.5M sucrose and erythritol solutions, which cannot be explained by starvation. Therefore, erythritol in the sucrose solution likely contributes to negative impacts such as increasing an osmotic pressure in the hemolymph of the fly. Total molarities of sugars from 0.5M sucrose/1M erythritol and 1M sucrose/0.5M erythritol solutions are the same, but the total percentage of sugars (w/v) are different between the former (approx. 30%) and the latter (approx. 40%) combinations. Though not statistically different, the higher erythritol dose (0.5M sucrose/1M erythritol) consistently appears to elicit greater mortality and lower fecundity than the lower erythritol dose (1M sucrose/0.5M erythritol). As sucrose molarity increased from 0.5M sucrose/1M erythritol (~30%) to 1M sucrose/1M erythritol (~46%), fly mortality was similar.

While fly mortality varied more among the eight combinations of sucrose/erythritol, the adult fecundities under the same sugar combinations resulted in four statistical groups. Rates of egg laying decreased by 3 to 100 times in all treatments contained erythritol compared to the sucrose-only positive control. Female fecundity might be reduced due to shorter lifespan as supported by the significant regression between the two variables, and/or a physiological change in the fly. Also, behavioral avoidance or deterrence could have contributed to the observed reduction in numbers of eggs laid.

To clarify physiological effects, ovarial dissection of female flies that ingested erythritol should be done the in future.

To our knowledge, nothing is known about the nutritional pathway and absorption of erythritol in insects. This requires tracking the presence of erythritol in the digestive system of the insect. In our study, a large amount of erythritol (avg. 60 µg) was detected in the hemolymph of *D. suzukii*, which was ~17 times that of sucrose (avg. 3.5 µg) among flies fed 0.5M sucrose/0.5M erythritol (Figs. 3.3B, 3.4). Erythritol was also found in the frass (Figs. 3.3C, 3.4). In contrast, sucrose was found at very low levels in the hemolymph. This observation is consistent with sucrose being metabolized, and then utilized as carbohydrate energy, or converted into a storage form like glycogen. Our observations may suggest that erythritol is directly transported from the midgut without being metabolized and stored, but is accumulated in the hemolymph which in turn elevates the osmotic pressure in the fly hemolymph. The increasing rate of osmolarity in the hemolymph has been discussed among honeybees, and differs depending on whether metabolizable or non-metabolizable carbohydrates are ingested (Roces and Blatt, 1999).

To avoid the serious physiological imbalance caused by the high osmotic pressure, excess erythritol should be removed immediately from the body. Excretion is the only way to dispose of non-metabolized wastes and ions in insects. We, indeed, observed more frass droplets from the erythritol-fed flies than sucrose-fed flies on the surface of the plastic film (pers. obs.). The specific behavioral activity would be for the removal of the excess erythritol from the body. An abnormally high concentration of sugars in the hemolymph demands a large amount of water consumption to restore physiological homeostasis. With water consumption, sucrose is hydrolyzed into glucose and fructose, and excess erythritol is excreted through the hindgut and Malpighian tubules. This ultimately reduces the osmotic pressure in the body. Erythritol was clearly found in the fly frass, but relatively small compared to amounts in the hemolymph. This implies that the excretion process might be slow and delayed in the fly body, resulting in high osmotic pressure for a while. In mammals, tetra-

carbon molecules are normally passed through intestinal membranes at a faster rate than hexose sugars (Munro et al., 1998; O'Donnell and Kearsley, 2012). Likely in *D. suzukii*, erythritol could be simply absorbed and diffused through the midgut membrane to be transported into the hemolymph. This may quickly increase the osmotic pressure in the hemolymph before being excreted out. Momentarily, the extreme changes and overreacted physiology by osmotic pressure in the fly body could be a lethal.

In mammals, erythritol as a sweet antioxidant could be converted to erythrose in the intestinal membranes and excreted by urination because erythrose has been found in the urine of erythritol-consuming rat (Den Hartog et al., 2010). The metabolic pathway of erythritol in the fly would be interesting to elucidate even though it is not utilized for nutritional energy. In this study, we could not find erythrose in the frass from erythritol-fed flies and vice versa. However, a certain level of the erythrose was apparently detected in the hemolymph and frass from the erythrose-fed flies (data not shown). Our contrasting result might be due to different metabolisms between vertebrate and invertebrate animals.

Undoubtedly, erythritol combined with sucrose reduced the survival and fecundity of *D. suzukii,* which is caused by the physiological imbalance with the osmolarity in the body. To elicit more feeding, erythritol should be mixed with sucrose which is sweeter than erythritol. While the present research focuses on *D. suzukii*, it can be expanded to other Dipteran pests. This erythritol formulation can be a potential insecticide used alone or as a delivery agent combined with conventional or biological insecticides to enhance their efficacy. To develop this novel control application for growers, the mode of action of erythritol, and its toxicity on non-target insects need to be explored in future.

Acknowledgements

We thank Kelly Donahue and Young Koh for technical, Jessica Wong for colony support and Jon Umble for blueberry bushes. This work was supported in part by a

Northwest Center for Small Fruits Research Grant, Oregon Blueberry, Raspberry& Blackberry Commissions, Agricultural Research Foundation, Malaysian Agricultural Research and Development Institute, and base funding from USDA ARS CRIS 2072-22000-040-00D.

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Figures

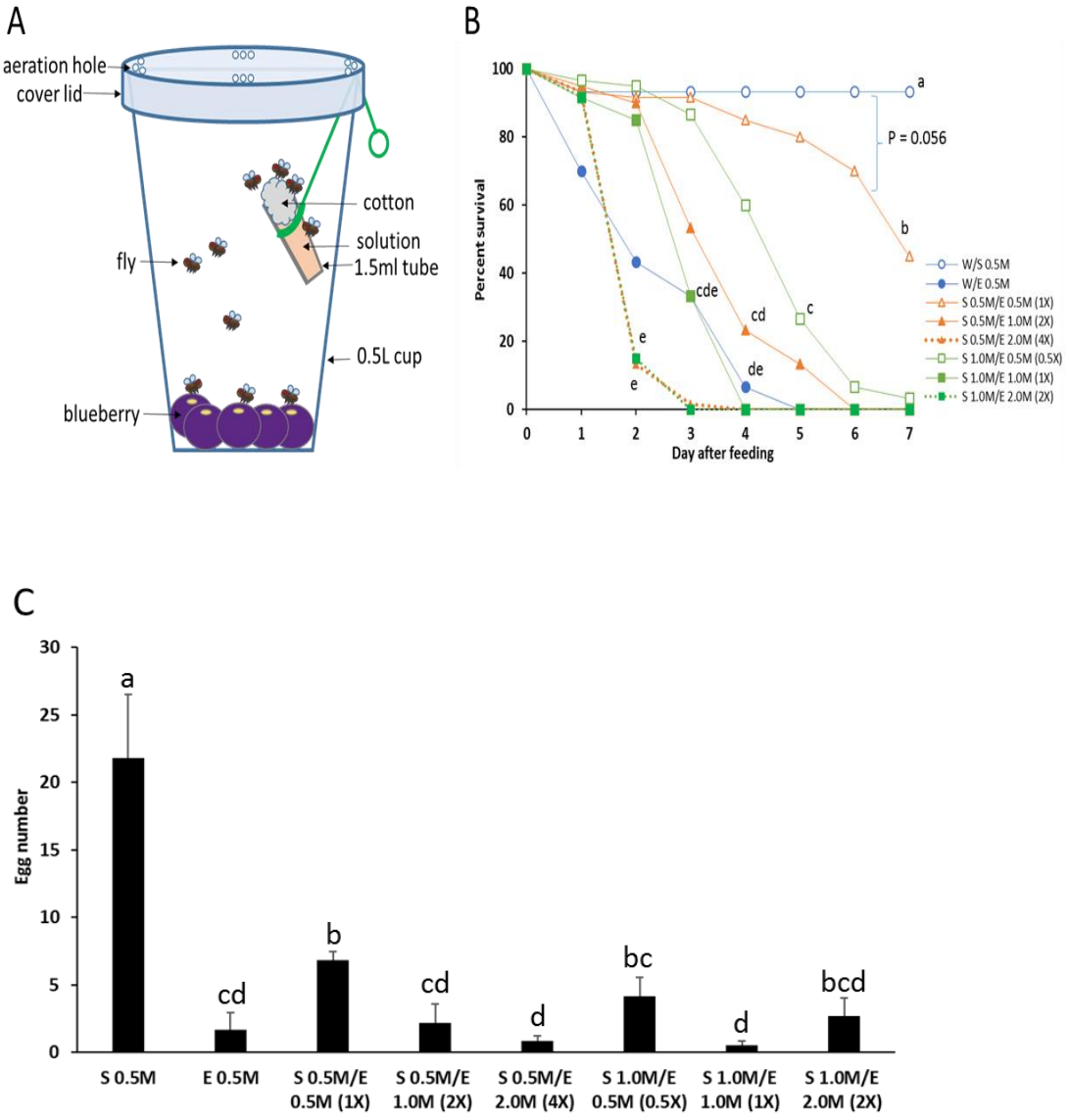

Figure 3.1. Diagram of the cup test with blueberries (A), survivorship of *D. suzukii* adults with combinations of sucrose/erythritol solutions, $\chi^2 = 440.9$, df = 7, P < 0.0001 (B), and fecundity $F_{7,40} = 36.0$, P < 0.0001 (C) of the female flies. Different letters denote significant differences by log-rank pairwise comparisons (B) and Tukey HSD (C).

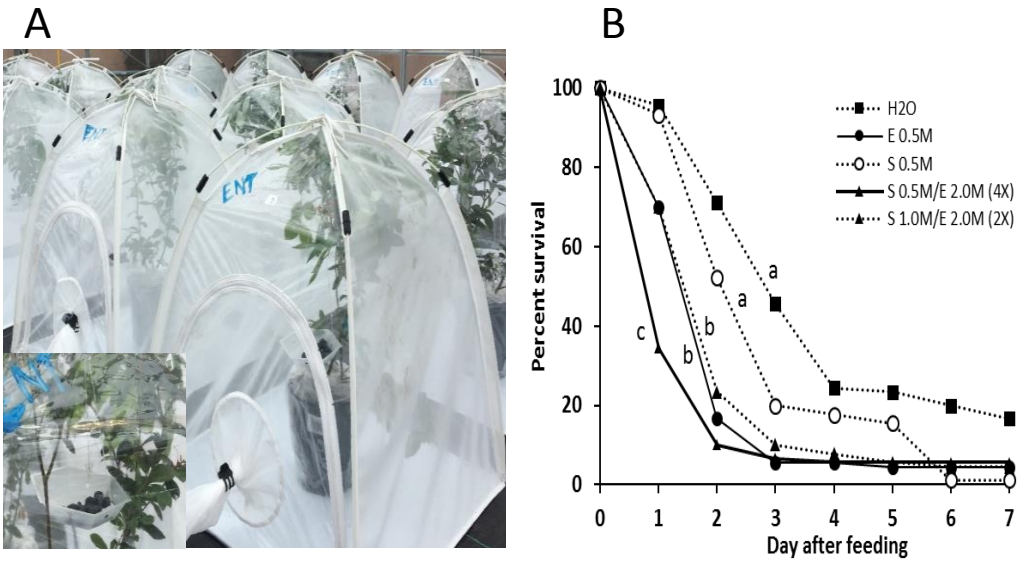

Figure 3.2. Photo of the greenhouse cage contained the blueberry bush and blueberries (inset photo) (A), and survivorship of *D. suzukii* adults with combinations of sucrose/erythritol solutions, $\chi^2 = 130.9$, df = 4, $P < 0.0001$ (B). Different letters denote significant differences by log-rank pairwise comparisons.

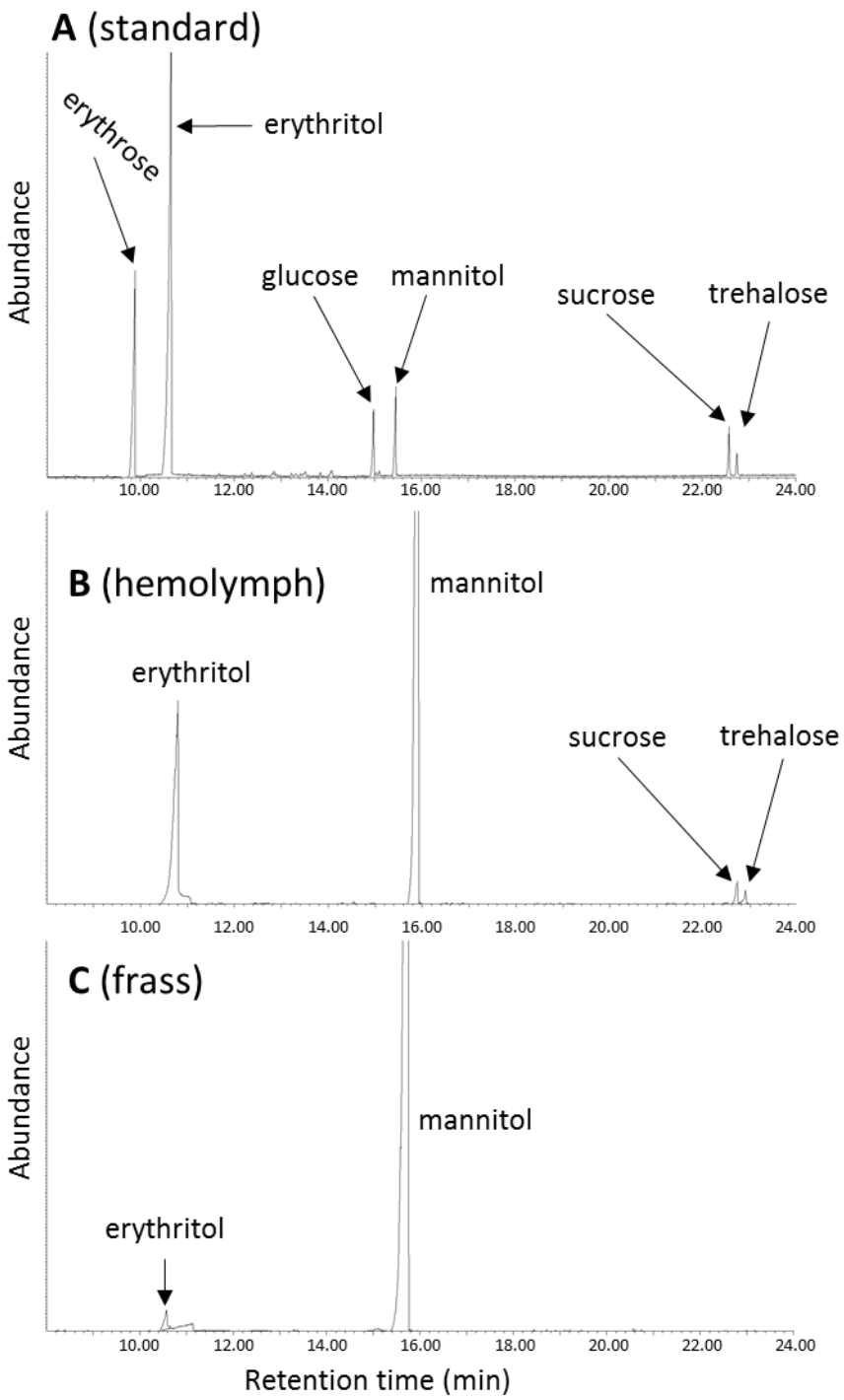

Figure 3.3. GC-MS profile of sugar derivatives in sugar standards (A), hemolymph (B) and frass (C) collected from 0.5M sucrose/0.5M erythritol-fed flies. Mannitol was used for an internal standard. Sugars were derivatized for the GC-MS analysis as described in Methods.

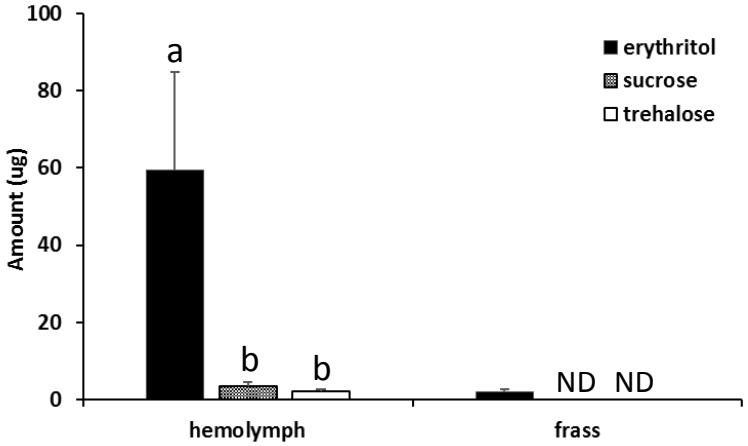

Figure 3.4. Amounts of sugars determined in the hemolymph and frass of the erythritol-fed flies. The measurement of sugars were determined with the mannitol internal standard added in the experimental samples. Sugars were derivatized for the GC-MS analysis as described in Methods. Different letters denote significant differences ($P < 0.05$). Replicated three times. Bars indicate mean + SEM. ND: not detected.

Chapter 4

Conclusion

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General Discussion and Conclusion

Spotted wing drosophila (SWD), *Drosophila suzukii*, is a serious agricultural crop pest infesting almost all berry crops, cherries, grapes (Lee et al., 2011a), and various wild and ornamental fruiting plants (Lee et al., 2015). With a short life cycle, high fecundity, larval development inside the fruit, a wide dispersal ability and adaptability to climatic conditions, control of this pest has been challenging (Cini et al., 2012).

Currently, SWD control methods are mainly the use of conventional chemical insecticides although they have negative impacts on the environment and human health, and do not represent a sustainable pest management. Recent research has more empathized to find chemical insecticide alternatives such as biologically-based control plus cultural and physical methods. Although alternative controls (reviewed by Lee et al., 2011) are being applied and under development, there is still a gap to fully protect small fruits from SWD damage. Therefore, we need a combination of approaches to enhance the IPM program to be introduced for growers.

Sugar acts as a phagostimulant increasing ingestion of insecticides and their effectiveness. To enhance the effectiveness of insecticides, sucrose can be added to conventional or organic insecticides targeting SWD. Although the non-nutritive sugar was not effective at increasing the feeding activity of ants (Vander Meer et al., 1995), recently artificial sweeteners, including erythritol, were shown to carry insecticidal effect against Dipteran pests (Baudier et al., 2014; Choi et al., 2017; Fisher et al., 2017; O'Donnell et al., 2016; Sampson et al., 2017; Sampson et al., 2016; Tang et al., 2017; Zheng et al., 2016).

Erythritol as a food grade non-nutritive sweetener has been reported to cause a lethal effect as well as disrupting motor coordination to *Drosophila melanogaster* (Baudier, 2014, O'Donnell, 2016). Although erythritol had been described as a toxic agent to *D. melanogaster*, little has been known about the effects to other drosophila species such as *D. suzukii*. The mechanism underlying insect mortality has yet to be discovered. In

this project, a series of experiments has been designed to investigate the effect of erythritol towards SWD survivorship and the physiological mode of action.

In Chapter 2, the effects of non-nutritive sugars and sugar alcohols were investigated on the survivorship of *D. suzukii*. Dose-dependent and age dependent studies show that the lethal effect of erythritol and erythrose on adult flies are not age dependent but do significantly reduce longevity when fed higher concentrations. A similar mortality trend was illustrated to all ages (1 – 7-day old) of adult flies fed on 0.5M erythritol and erythrose; while higher concentrations of erythritol and erythrose speed up the lethal effect. Low survivorship of SWD fed on both erythritol and erythrose shared a similar trend in flies fed on water only, indicating the possibility that these compounds are non-nutritive sugars.

Total sugar and glycogen level remain low in flies fed on water, erythritol and erythrose show that these sugars are not metabolized. Erythritol and erythrose might be absorbed in the midgut and were not converted into long chain carbohydrates such as glycogen, a common storage form. Therefore they are non-nutritive and are causing mortality in SWD via starvation. Although there is nothing known about the absorption and biochemical metabolism of ingested erythritol, it might be a feedback loop of food intake regulating the crop emptying and hemolymph osmolality in the fly. SWD survivorship was not affected when sucrose and erythritol were provided separately, but the survivorship was significantly reduced when flies fed on a mixture of erythritol and sucrose. This result is evidence of an insecticidal effect of erythritol that might be caused by an abnormal physiological unbalance such as a high osmotic pressure due to high erythritol content in hemolymph.

In Chapter 3, the study continues to build on the previous by monitoring the impacts on mortality and fecundity with blueberries at a larger scale using a cage in a greenhouse setting , and examining the nutritional pathway of ingested erythritol in the fly body. In addition, a feasible application and possible mode of action of erythritol are discussed. Survival rates of *D. suzukii* with blueberries were similar to

previous tests without fruits. While keeping constant sucrose solutions, higher concentrations of erythritol elicited quicker mortality than lower doses. Ratios of 0.5M sucrose/2M erythritol and 1M sucrose/2M erythritol turned out to lead to the highest mortality with blueberries. Both results have been consistent through this project. These results reveal that 0.5M sucrose or above provide a sufficient carbohydrate amount for sustaining *D. suzukii* adults.

The two sucrose/erythritol ratios mentioned above were selected for further evaluation in cages with blueberry bushes and fruits in the greenhouse. Unlike the smaller cup container, mortality occurred more quickly in the greenhouse than the lab trials. The rapid death could be a result of the fly actively moving around the larger space of the cage, which accelerated the exhaustion of energetic reserves in the body. Interestingly, 0.5M sucrose/2M erythritol was more detrimental than 1M sucrose/2M erythritol on *D. suzukii* in the cage experiment but not in the lab experiment. The high concentration of sugars (1M sucrose/2M erythritol), makes for a very viscous solution, which may be difficult for the fly to feed upon. Future evaluations should consider the practical convenience of the formulation. Long-term feeding studies demonstrated that flies remember non-nutritive foods, then eventually reject non-nutritive sweeteners (Burke and Waddell, 2011; Fujita and Tanimura, 2011). For a feasible application, therefore, the sucrose/erythritol combination will be more effective than erythritol alone because it is sweeter and more nutritious.

While fly mortality varied more among the various combinations of sucrose/erythritol, the fecundity under the same combinations resulted in four statistical groups. Rates of egg laying decreased by 3 to 100 times in all treatments containing erythritol compared to the positive control. The reduction of female fecundity might be due to shorter lifespan and/or a physiological change in the fly. Also, behavioral avoidance or deterrence could have contributed to reduced numbers of eggs laid.

In this study, two possibilities causing fly mortality were suggested: 1) starvation from the feeding of non-metabolized erythritol; 2) increased osmotic pressure in the hemolymph with erythritol. Starvation can be explained in the case when erythritol is provided alone without sucrose. However, *D. suzukii* still died when fed 0.5M sucrose and erythritol solutions, which cannot be explained by starvation. Therefore, erythritol in the sucrose solution likely contributes to other physiological impact(s) such as increasing an osmotic pressure in the hemolymph of the fly.

The nutritional pathway and absorption of erythritol in insects is still unknown. This requires tracking the presence of erythritol in the digestive and circulative systems of the insect. From GC-MS analysis a large amount of erythritol was detected in the hemolymph, which was ~17 times that of sucrose among flies fed the sucrose/erythritol solution. The results may indicate that erythritol is directly transported from the midgut without being metabolized and stored, but is accumulated in the hemolymph which in turn elevates the osmotic pressure. To avoid the serious physiological imbalance caused by the high osmotic pressure, excess erythritol should be removed immediately from the body. Erythritol was clearly found in the fly frass, but relatively small compared to amounts in the hemolymph. This implies that the excretion process might be slow and delayed in the fly body, resulting temporarily in high osmotic pressure.

An abnormally high concentration of sugars in the hemolymph demands a large amount of water consumption to restore physiological homeostasis. Likely in *D. suzukii*, erythritol could be simply absorbed and diffused through the midgut membrane to be transported into the hemolymph. This may quickly increase the osmotic pressure in the hemolymph before being excreted out. Momentarily, the extreme changes and overreacted physiology by osmotic pressure in the fly body could be lethal.

In conclusion, erythritol combined with sucrose reduced the survival and fecundity of *D. suzukii,* which is caused by the physiological imbalance with the osmolarity in the

body. To elicit more feeding, erythritol should be mixed with sucrose which is sweeter than erythritol. This erythritol formulation can be a potential insecticide used alone or combined with other insecticides to enhance IPM. While the present research focuses on *D. suzukii*, it can be expanded to other Dipteran pests. To develop this novel control application for growers, the mode of action of erythritol, and its toxicity on non-target insects need to be explored in the future.

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