LARINE DE PAIVA MENDONÇA

HOST FRUITS, DENSITY CARRY-OVER EFFECT AND POTENTIAL OF OZONE AS AN ALTERNATIVE FOR CONTROLLING *Drosophila suzukii* IN THE NEOTROPICAL REGION

Dissertation presented to the Universidade Federal de Viçosa, as part of the requirements of the Entomology Graduate Program, to obtain the title of *Magister Scientiae*.

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Abstract

MENDONÇA, Larine, M.Sc., Universidade Federal de Viçosa, February, 2019. **Host fruits, density carry-over effect and potential of ozone as an alternative for controlling *Drosophila suzukii* in the Neotropical region.** Advisor: Lêda Rita Faroni D'antonino. Co-Advisors: Eugênio Eduardo de Oliveira and Haddi Khalid.

Invasive pests are a constant threat to agricultural production. The adequate management of invasive pests, such as *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), requires a better understanding of potential hosts and the establishment of control practices. *Drosophila suzukii*, differently from other drosophilids, is considered a pest due to its capacity to oviposit in ripe soft skinned fruits. In this way, this study was divided in three chapters aiming to evaluate new potential hosts, effect of density in biological traits of *D. suzukii* and finally, the susceptibility of the flies to ozone treatment. Once that *D. suzukii* is a polyphagous insect, its capacity of dispersion is facilitated in the presence of new possible hosts. The attempt to mass rearing of this insect in laboratory can expose the pest to stresses affecting its biology as well as the results of toxicological tests. Lastly, it is always important to research new and safer options to add to control strategies of invasive pest. Thus, in the current study, the capacity of *D. suzukii* to oviposit and complete its life cycle in fruits of jabuticaba, *Plinia cauliflora*, Barbados cherry, *Malpighia emarginata*, bonnet pepper, *Capsicum chinese* and coffee, *Coffea arabica* was evealuted. Once confirmed this capacity, field surveys were realized to look for possible infestations. The new host and also two different food sources were assessed for their effects on biological and physiological traits of *D.suzukii*. Furthermore, the transgenerational effect of density was measured by the exposure of insect to high and low density on artificail diet under laboratory conditions. The effect on this stress was evaluated by the toxicological bioassays and how this affected the survival and the average fecundity of the pest. Finally, the toxic effect of ozone gas was tested in egg phase of *D. suzukii*, where it was tested four different concentrations of ozone (i.e. 0.2, 1.2, 2.3 and 3.0 mg/L) for 30 minutes of exposure. Our results showed the capacity of the fly to lay eggs in all the substrates that were offered, but it was only capable of putting their eggs inside fruits of *M. emarginata*. Furthermore, field surveys confirmed the occurrence of *D. suzukii* in fruits of Barbados cherry. The effect of density was possible to notice in the survival reduction of insects whose parents where reared in high density. The susceptibility was not affected, but the sublethal exposure associated with parental density, affected the survival and the fecundity of the flies following a hormetic pattern. Finally, even the exposure to the higher concentration of ozone (3.0 mg/L) was not capable to prevent the emergence of *D. suzukii* and the treatments also did not affect the weight of insects. As conclusions, the studies showed the suitability of Barbados cherry as host for *D. suzukii*, the stress of density can affect adaptive traits of these flies, and alternatives, such as ozone in the conditions used in these experiments, was apparently not effective to control the egg phase of *D. suzukii*.

Resumo

MENDONÇA, Larine, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Frutos hospedeiros, efeito carry-over de densidade e ozônio como potencial alternativa para o controle de *Drosophila suzukii* na região Neotropical.** Orientadora: Lêda Rita Faroni D'antonino. Coorientadores: Eugênio Eduardo de Oliveira e Haddi Khalid.

Pragas invasivas são uma ameaça constante a produção agrícola. O manejo adequado de pragas invasivas, como *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), requer um melhor entendimento dos potenciais hospedeiros e o estabelecimento de práticas de controle. A *Drosophila suzukii*, diferente de outros drosofilídeos, é considerada uma praga devido à sua capacidade de oviposição em frutos maduros de casca fina. Desta forma, este estudo foi dividido em três capítulos, com o objetivo de avaliar novos hospedeiros potenciais, o efeito da densidade em caracteres biológicos de *D. suzukii* e, finalmente, a susceptibilidade das moscas ao tratamento com ozônio. Uma vez que a *D. suzukii* é um inseto polífago, sua capacidade de dispersão é facilitada quando na presença de novos potenciais hospedeiros. A tentativa de criação massal desse inseto em laboratório pode expor a praga a estresses que afetam sua biologia, bem como os resultados de testes toxicológicos. Por fim, é sempre importante pesquisar opções novas e mais seguras para adicionar ao controle de pragas invasivas. Assim, no presente estudo, avaliou-se a capacidade da *D. suzukii* de ovipositar e completar seu ciclo de vida em frutos de jabuticaba, *Plinia cauliflora*, acerola, *Malpighia emarginata*, pimenta biquinho, *Capsicum chinesa* e café, *Coffea arabica*. Uma vez confirmada essa capacidade, foram realizados levantamentos a nível de campo para procurar possíveis infestações. Os efeitos do novo hospedeiro e também duas fontes alimentares diferentes foram avaliados em características biológicas e fisiológicas de *D.suzukii*. O efeito transgeracional da densidade foi medido pela exposição do inseto à alta e baixa densidade. O efeito deste estresse foi avaliado pelos bioensaios toxicológicos e como isso afetou a sobrevivência e a fecundidade média da praga. Finalmente, o efeito tóxico do gás ozônio foi testado na fase de ovo de *D. suzukii*, onde foram testadas quatro diferentes concentrações de ozônio (isto é, 0,2; 1,2; 2,3; e 3,0 mg / L) por 30 minutos de exposição. Nossos resultados mostraram a capacidade da mosca de colocar ovos em todos os substratos que foram oferecidos, mas só foi capaz de romper a casca, colocando seus ovos no interior, em frutos de *M. emarginata*. Além disso, levantamentos de campo confirmaram a ocorrência de *D. suzukii* em frutos de acerola. O efeito da densidade foi possível notar pela redução da sobrevivência de insetos cujos pais foram criados em alta densidade. A suscetibilidade não foi afetada pelo efeito, mas a exposição subletal associada ao efeito de densidade parental afetou a sobrevivência e a fecundidade do inseto seguindo um padrão hormético. Finalmente, mesmo a exposição da maior concentração de ozônio não foi capaz de prevenir a emergência de *D. suzukii* e os tratamentos também não afetaram o peso dos insetos. Como conclusões, os estudos mostraram a adequação da acerola como hospedeira de

D. suzukii, o estresse da densidade pode afetar as características adaptativas da mosca e alternativas, como o ozônio, nas condições utilizadas nesses experimentos, aparentemente não foram eficazes para controlar a fase de ovo de *D. suzukii*.

Contents

General introduction

Drosophilidae is a large family of insects belonging to the order diptera. In this family we can highlight the specie *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) that, in contrast to the majority of the species in this family (with exception of *Drosophila pulchrella*), shows a capacity of puncturing the skin of intact fruits to lay its eggs (Atallah et al. 2014; Mitsui et al. 2006; Sasaki and Sato 1995; 1996). Another important characteristics of *D.suzukii* that can be pointed out is its propensity to preferncially ovioposit in healthy fruits, (Mitsui et al. 2006), and the modification in female's ovipositor being serrated, which upon insertion can cause physical damage to the host fruit (Walsh et al. 2011). In addition to the damage caused by the deposition of its eggs inside the fruit, where the larvae will develop, the *D. suzukii* leaves an opening in the fruit's shell, which may favor the entry of pathogens and secondary pests (De Camargo and Phaff 1957; Louis et al. 1996).

The arrival of new invasive pest means a threat to agricultural crops. The current invasive pest that has growing importance in the Neotropical region is *D. suzukii,* mainly due to its rapid expansion in geografical range (Cini et al. 2012). It was first observed in Japan since 1916 but only around 1930 it was found in wild strawberries and cultivated cherries (Kanzawa et al. 1939). However, its worldwide recognition occurred in 2008, when it was reported in the state of California in the United States and the pest started to spread throughout the world (Bolda et al. 2010). *D. suzukii* is a polyphagous insect able to develop on a very wide range of soft-skinned fruits, with berries being the preferred host (Cini et al. 2012; Lee et al. 2011). This ability to use different fruits to complete its life cycle has been investigated (Andreazza et al. 2015; Geisler et al. 2015a; Müller and Nava 2014; Nunes et al. 2014; dos Santos et al. 2017). Thus, the study of invasive species can facilitate the understanding of the development and standard of dispersion of this pest.

One of the benefits to perform experiments and studies using drosophila flies as a model organism was in the fact that this insect did not show a conflict of interest allowing experiments with this insect without the fear of damage the human health or the natural environment (Ashburner et al. 1989). However, the potential to threat the humans interest occurs with the identification of the new invasive pest *D. suzukii* (Hauser 2011). In the bright side, as a specie from the same family as *Drosophila melanogaster* that already have been extensively investigated gives a chance to extend these studies to *D. suzukii*. However, keeping these insects under lab rearing conditions can expose the flies to stresses, like density effect. Then, great care must be taken to extrapolate the lab results to field reality (Vereijssen and Goldson 2016).

Due to its high damaging potential associated with the low knowledge about *D. suzukii* bioecology, the most used strategy by producer to combat this pest invasion is chemical control (Van Timmeren and Isaacs 2013; Haye et al. 2016). A huge problem attached to the unconscious use of chemicals to pest control is the selection of resistant population, being a common phenomenon to invasive pests (Haddi et al. 2012; 2017; Campos et al. 2014). At the same time, consumers' interest in the search for healthier foods and with fewer chemical residues is increasing (Pandiselvam et al. 2019). To overcome this problem, alternative ways to control pest are needed to be investigated (Andreazza et al. 2018). A promising alternative is the use of ozone gas that has already been suggested as a product with insecticide action (Işikber and Öztekin 2009; McDonough et al. 2011; Sousa et al. 2008; 2016; Velasquez et al. 2017).

Brazil as a country in the Neotropical region has a variety of fruits still unexplored as potential hosts for this pest, and their identification will aid in the management of *D. suzukii*. So, in the current study, we aimed to identify the capacity of *D. suzukii* to complete its life cycle in new potential hosts (e.g. Barbados cherry, bonnet pepper, coffee and jabuticaba) what was accomplished in the studies described in chapter 1. Then, in chapter 2, we evaluated the effect of density stress aiming to identify the impact of this stress in the toxicology and development of *D. suzukii*. Finally, in chapter 3, the ozone toxicity to egg phase of *D. suzukii* was tested to be suggested as an alternative way to manage this pest in strawberry orchards, preventing in this way its dispersion by trade.

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Chapter 1

Host potential and adaptive responses of *Drosophila suzukii* on Barbados cherries

Host potential and adaptive responses of *Drosophila suzukii* on Barbados cherries

Larine P. Mendonça, Eugenio E. Oliveira, Felipe Andreazza, Sarah M. Rezende, Lêda R. D. Faroni, Raul N. C. Guedes, Khalid Haddi.

Abstract

Biological invasions are global threat to agricultural crops worldwide. In the Neotropical region, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) has rapidly expanded its geographical range since its first occurrence. Beside climatic factors, its remarkable success in the establishment and subsequent distribution in this region are closely dependents on the diversity and availability of host plants. Here, laboratory experiments and field surveys were conducted to identify potential new hosts as well as their potential impacts on biological and physiological traits of *D.suzukii*. Firstly, the fruits of jabuticaba, *Plinia cauliflora*, Barbados cherry, *Malpighia emarginata*, bonnet pepper, *Capsicum chinese* and coffee, *Coffea arabica*, were tested under laboratory conditions, as food and oviposition sources for *D. suzukii*. Then effects of different food sources (*e.g.*; Barbados cherry, strawberry and artificial diet) were assessed on biological and physiological traits of *D.suzukii*. Our results revealed that *D. suzukii* laid eggs on the surface of all the tested fruit species but oviposition inside fruits and completion of life cycle occurred only on Barbados cherries. Furthermore, field surveys confirmed preferential use of undamaged ripe fruits of Barbados cherry as host by *D. suzukii*. Moreover, although feeding substrates did not alter the respiratory activities, flies developing on Barbados cherries and artificial diet presented earlier emergence, shorter development time, lower number of adults per female and female biased sex-ratio compared to flies developing on strawberries. Collectively, our findings demonstrated the suitability of Barbados cherry as host for *D. suzukii*, which turn its management on the neotropics an even more complex task.

Keywords: Spotted wings drosophila, Barbados cherry, Neotropical hosts.

1.1 Introduction

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae) also called the spotted wing Drosophila (SWD) is a major pest for stone fruits and berries crops, causing considerable damage for the fruit production economy in the infested crops (Lee et al. 2011) and with a remarkable ability to invade a diverse range of environments. Native to Southeast Asia in regions from Japan to Pakistan (Ometto et al. 2013), it was recorded simultaneously in North America and Europe in 2008 (Rota-Stabelli et al. 2013; Hauser 2011; Cini et al. 2012; Calabria et al. 2012), and has since presented fast geographical expansion across the world (Asplen et al. 2015a; Andreazza et al. 2017; dos Santos et al. 2017).

In southern Neotropical region and since its first occurrence in south Brazil in 2013 (Deprá et al. 2014), the ability of *D. suzukii* to attack and use as host many native fruit species has been established by several investigations (Andreazza et al. 2015; Geisler et al. 2015a; Müller and Nava 2014; Nunes et al. 2014; Souza et al. 2017). Countries with vast territorial extensions, such as Brazil, present a wide array of climate types and biomes resulting in large amount and diversity of resources suitable for the development of invasive insect pests such as *D. suzukii* which raise serious concerns about how and when to mitigate the losses caused by this pest (Schlesener et al. 2014b; 2015). Using climatic variables, Benito et al. (2016) and dos Santos et al. (2017) predicted the potential spread of *D. suzukii* in the southern Neotropical and suggested that the potential distribution areas included central region of southern Brazil, the southern half of Paraguay, all of Uruguay, the east and south of Argentina as well as the entire coastline of Chile. It was also concluded that future expansions and establishment of *D. suzukii* in Brazil, as well as in the other potential parts of the southern Neotropical region, will be mainly under limiting action of the combination of adequate temperature and relative air humidity (Benito et al. 2016). However, knowing that this species is highly polyphagous (Kenis et al. 2016), the development and establishment of *D. suzukii*, can be facilitated by the existence of several suitable native and exotic fruits.

Thus, studies attempting to investigate the suitability of native plant species as food and oviposition sources for *D. suzukii* are urgently needed and understanding the mechanisms allowing this species to use new hosts is critical for the area-wide management programs of the populations of such invasive species. Here, four native fruits species were tested under laboratory conditions for their suitability as hosts to *D. suzukii*. Once a potential host was found, field surveys were carried out to verify the natural occurrence of this pest on this host. Alterations in physiological and biological traits were verified when *D.suzukii* reared in this host compared to an artificial diet and a preferred host (*i.e.*; strawberries).

1.2 Material and Methods

1.2.1 *Insects*

Three populations of *D. suzukii* were used in the bioassays. A stock colony initially established from approximately 800 individuals obtained from a population maintained at Embrapa Clima Temperado (Pelotas, RS, Brazil). Two other populations that were started from infested fruits field collected in Minas Gerais State (Brazil), in the municipality of Ervália (Mar 2016), from an organic strawberry field (Andreazza et al. 2016) and in the municipality of Paula Cândido (Jan 2018) from Barbados cherry trees in a mixed cultivation of fruit trees in some rural house gardens.

The rearing in the laboratory was carried under controlled conditions (*i.e.*, temperature: 24 ± 2 °C; relative humidity: 55 ± 10 %; photophase: 12 h) using an artificial diet, composed of a mixture of water, brewer's yeast, corn flour, sugar, agar, propionic acid and methyl 4-hydroxybenzoate (NipaginTM) and prepared following methods described elsewhere (Andreazza et al. 2016; Emiljanowicz et al. 2014).

1.2.2 *Laboratory host screening bioassays*

Aiming to test the suitability of four Neotropical fruit species as hosts for the SWD we carried out an in-vivo host infestation bioassay. The species used were jabuticaba, *Plinia cauliflora*, Barbados cherry, *Malpighia emarginata*, bonnet pepper, *Capsicum chinese* and coffee berries, *Coffea arabica*.

The bioassay consisted of offering to adult flies (couples of females and males); originating from the stock colony; fruits as source of food and substrate for oviposition. Five replicates, each consisting of ten insects (five couples) less than 3 days old, were introduced in plastic cages of 0.5 L with vented openings and allowed to colonize the offered fruit for 24 hours. The fruits were changed daily by new ones for one week. All the offered fruits were successively kept in separate cages and monitored daily during two weeks to assess the emergence of the progeny.

Based on the results of in-vivo host suitability bioassay (see result section), a similar bioassay was conducted where the daily emergence, total number development time, sex-ratio of emerged insects were accessed when the flies couples were offered either fruits of Barbados cherry, *M.emarginata*, fruits of strawberry (*Fragaria ananassa*) or artificial diet. The experimental set-up followed the same methodology as described above.

1.2.3 *Field collection*

To verify the occurrence of the presence and attack of SWD on the Barbados cherry fruits in natural conditions, a field survey took place in the municipality of Paula Cândido, Minas Gerais (Latitude: 20º 52′ 27'' S; Longitude: 42º 58′ 49'' W) in January 2018. The survey targeted mainly fruits from trees of Barbados cherry in rural houses gardens. The trees are separated by a distance of approximately 20 meters with trees of other fruit species in the middle.

A total of 225 fruits were collected from the trees and divided into 3 groups of 75 fruits eacch, according to the observed damages at the moment of collection (i.e., visually undamaged, presence of small mechanical damage / natural cracking in the fruit epidermis, or heavily damaged fruits). In each group repetitions of 3 fruits were separated in 100mL cages covered by voile cloth. All the collected fruits were returned to the laboratory and monitored under controlled conditions (25 ± 2 ºC, 50 ± 5% RH and 12 h photoperiod) for new flies' emergence during two weeks.

A stereoscopic microscope (at 40×) (SZX-SDO2, Olympus Corporation, Tokyo, Japan) was used to identify and classify the insects that emerged in four groups: female *D. suzukii*, male *D. suzukii*, *Zaprionus indianus* and other species of Drosophila according to Vilela and Mori (2014), and Van der Linde (2010).

1.2.4 *Respirometry bioassays*

In order to capture potential changes in the metabolism of insects collected from different regions (Ervália, Rio Grande do Sul and Paula Cândido) when feeding on different food sources (artificial diet and Barbados cherries *M. emarginata*), measurements of respiration rates of males and females flies of *D. suzukii* were carried out.

Respirometry bioassays used a CO_2Analyzer TR2 respirometer (Sable Systems International, Las Vegas, NV), as previously described (Oliveira et al. 2005, 2007; Guedes et al. 2006; Haddi et al. 2015). Briefly, one day-old *D. suzukii* adults from each region x food source combination were individually placed in a 25 mL chamber connected to a closed system. The chambers were connected to the system 1 hour before injecting CO_2-free air into the chambers for 2 min at a rate of 600 mL/min. The CO_2 produced by each insect during one hour and carried by the airflow was immediately read by an infrared reader. A total of 48 flies (23 females and 24 males) from Ervália, 39 flies (19 females and 20 males) from Rio Grande do Sul and 43 flies (21 females and 22 males) from Paula Cândido were used in the respirometry bioassay.

1.2.5 *Statistical analysis*

The Data of potential-host screening and effect of fruit damage level were subjected to univariate analysis of variance (ANOVA) or a Kruskal-Wallis one-way ANOVA on ranks, when the assumptions of normality and homoscedasticity were not satisfied. Data of development traits were submitted to univariate ANOVA and averages were tested by a t test at 0.05 probability. Regression analyses were performed to analyze the daily emergence for each substrate (*i.e.*, fruits of Barbados cherry, strawberry or artificial diet) used through time. Regression analysis was performed using the curve-fitting procedure of Sigma Plot 12.0. The regression model was chosen based on parsimony, lower standard errors, and steep increases in R^2 with model complexity. The regression models for each treatment were considered different from each other if the confidence limits of their parameters did not overlap. The respiration data were subjected to a three-way analysis of variance and Tukey's honestly significant difference (HSD) test ($P < 0.05$) when appropriate (PROC GLM,SAS). The assumptions of normality and homogeneity of variance were tested for all parameters, and no data transformations were necessary (PROC UNIVARIATE, SAS Institute Inc., Cary, NC, USA).

1.3 Results

1.3.1 *Potential host screening bioassays*

When offered fruits from different species, the females of *D. suzukii* flies showed significantly different oviposition behavior (F= 15.5; *df* = 3; *P* <0.001). The average number of eggs was the highest when the females were offered Barbados cherries (*M. emarginata*) as substrate for oviposition and reached 15.52 ± 1.8 eggs per day (Figure 1.1). In contrast, lower rates of oviposition were found when the female flies were offered coffee berries, *C. arabica*, (2.56 ± 0.86 eggs per day), jabuticaba berries, *P. cauliflora*, (1 ± 0.27 eggs per day) and bonnet pepper fruits, *Ca. chinese*,(0.48 ± 0.2 eggs per day). Interestingly, while the majority of the eggs were oviposited inside the Barbados cherries, all the eggs were found in the surface or in the wounds of the fruits when the female flies were offered coffee, jabuticaba and bonnet pepper fruits (Figure 1.2). Furthermore, the insect was capable to complete its life-cycle and reach the adult phase only when female flies were offered Barbados cherries.

In Barbados cherries, the peak for daily emergence occurred earlier compared to artificial diet and strawberry fruits (Figure 1.3, Table 1.1). The total number of flies per female that emerged from Barbados cherries was significantly lower (*t* = 23.85; *df* = 8; *P* = 0.01) than the number of flies emerging from strawberry fruits (Figure 1.4A). Moreover, the flies mean developmental time significantly (F= 23.85; *df* =14; *P* < 0.001) differed between Barbados cherry and strawberry fruits (Figure 1.4B). In fact, to complete their life cycle, it took for the flies in average 10.33 ± 0.16 days in the Barbados fruit, 9.99 ± 0.09 days in the artificial diet and 11.78 ± 0.26 days in strawberry fruits. In addition, similar significant differences (F= 4.61; *df* = 14; *P* = 0.03) were found between the female sex-ratios of the progeny of flies emerging from Barbados cherries (56.7 ± 2.2) and strawberry fruits (45.8 ±1.2) (Figure 1.4C).

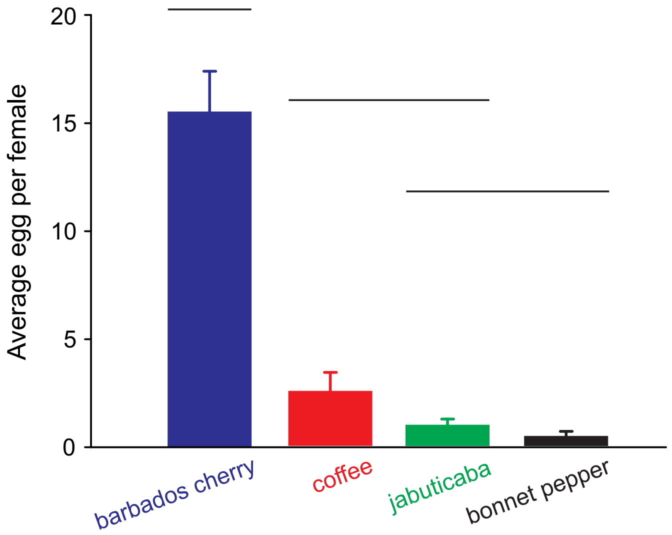

Figure 1.1: Average eggs laid per female of *Drosophila suzukii* on fruits four Neotropical species: Barbados cherry, *Malpighia emarginata*, jabuticaba, *Plinia cauliflora*, bonnet pepper, *Capsicum chinese* and coffee berries, *Coffea arabica*. Each bar corresponds to the mean of five independent assays (±SE). Treatments under the same horizontal bar do not differ statistically (*P*<0.05).

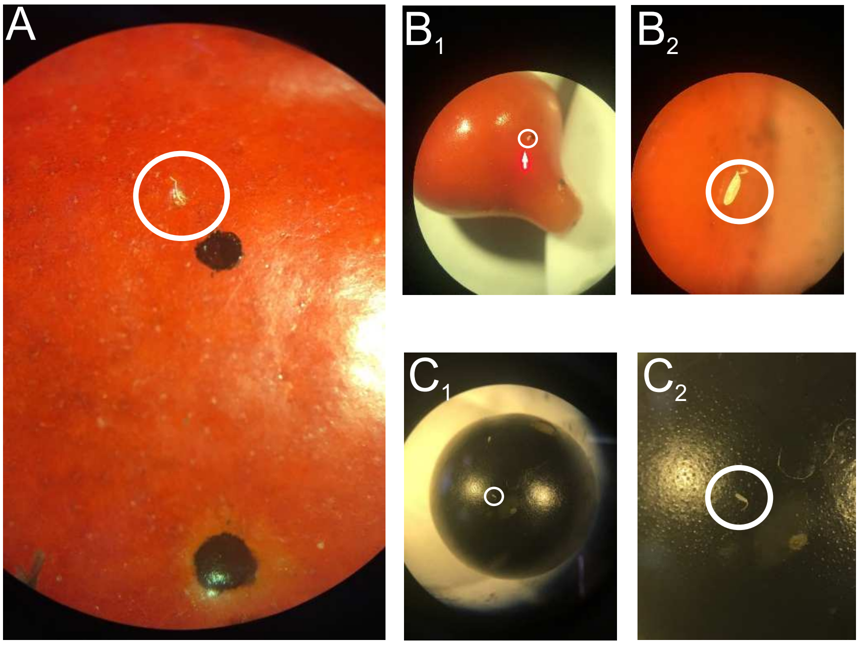

Figure 1.2: *Drosophila suzukii* egg laid on the fruit of Neotropical native hosts. (A) Egg of *D. suzuki*i inside of Barbabdo cherry. (B_1)and (B_2) Egg of *D suzukii* in the surface of bonnet pepper. (C_1)and (C_2)Egg of *D. suzukii* in the surface of jabuticaba.

Table 1.1: Summary of non-linear regression analyses of emergence parameters (shown in Figure 1.3).

	Estimated parameters (SE)						
Food source	a	b	x_0	df_{error}	F	P_{value}	R^2
Artificial diet	3.58 (3.48 - 3.68) a	0.65 (0.63 - 0.67) a	10.76 (10.74 - 10.78) a	7	451.5	<0.0001	0.99
Barbados cherries	2.59 (2.30 - 2.88) b	0.82 (0.72 - 0.92) b	10.17 (10.10 - 10.28) b	7	225.01	<0.0001	0.99
Strawberries	3.84 (3.45 - 4.23) a	0.84 (0.74 - 0.95) b	11.64 (11.54 - 11.73) c	7	296.4	<0.0001	0.99

Model: $y = a*\exp(0.5*((x-x0)/b)^2)$

Values followed with the same letter in the column do not differ significantly.

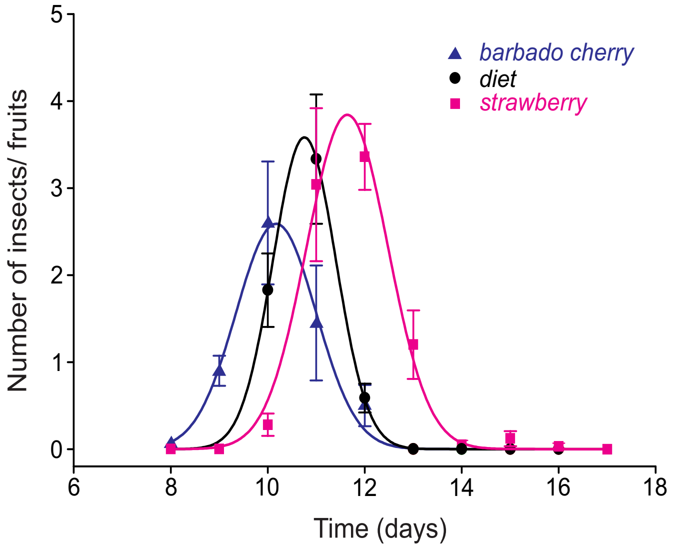

Figure 1.3: Daily emergence of *Drosophila suzukii* flies that emerged from Barbados cherry, *Malpighia emarginata*, Strawberries (*Fragaria ananassa* Duch) and artificial diet. Each point corresponds to the mean of five independent assays (±SE).

1.3.2 *Field survey*

The number of the different drosophilids flies (88% of Z. indianus, 3 % of *D. suzukii* and 9% of other species) that emerged from the field collected Barbados cherries depended on the damages levels in these fruits.

Significantly higher number of *D. suzukii* flies emerged from undamaged fruits compared with the heavily damaged ones (H = 12.2; *df* = 2; *P* = 0.002) (Figure 1.5A). In the undamaged fruits category, flies emerged from 80% of the samples collected while in the heavily damaged fruits, flies emerged only from 36% of the fruits. No statistical differences (H = 0.89; *df* = 2; *P* = 0.35) were found between the numbers of females and males emerging from the fruits of any of the three categories.

In addition to *D. suzukii*, other drosophilids emerged from the collected fruits. Among these drosophilids, *Z. indianus* was the most frequent (more than 88%). Contrarily to SWD, a significantly (H = 85.44; *df* = 2; *P* = 0.014) higher number of *Z. indianus* flies emerged from heavily damaged fruits compared with the undamaged ones (Figure 5B). A similar trend was observed for the other drosophilids (not identified at species level in the present work) with significantly higher number (H = 8.03; *df* = 2; *P* = 0.02) of flies emerging from heavily damaged fruits compared with the undamaged ones.

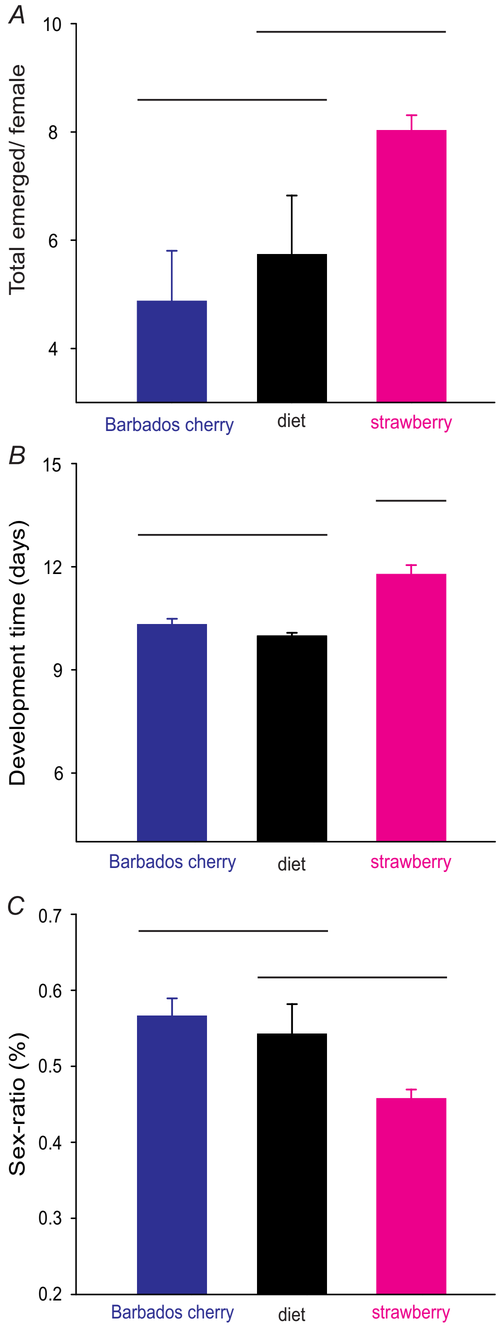

Figure 1.4: (A)The total number of flies per female, (B) Developmental time (days) and (C) The sex-ratio of *Drosophila suzukii* that emerged from Barbados cherry, *Malpighia emarginata*, Strawberries (*Fragaria ananassa* Duch) and artificial diet. Each bar corresponds to the mean of five independent assays (±SE). Treatments under the same horizontal bar do not differ statistically ($P<0.05$).

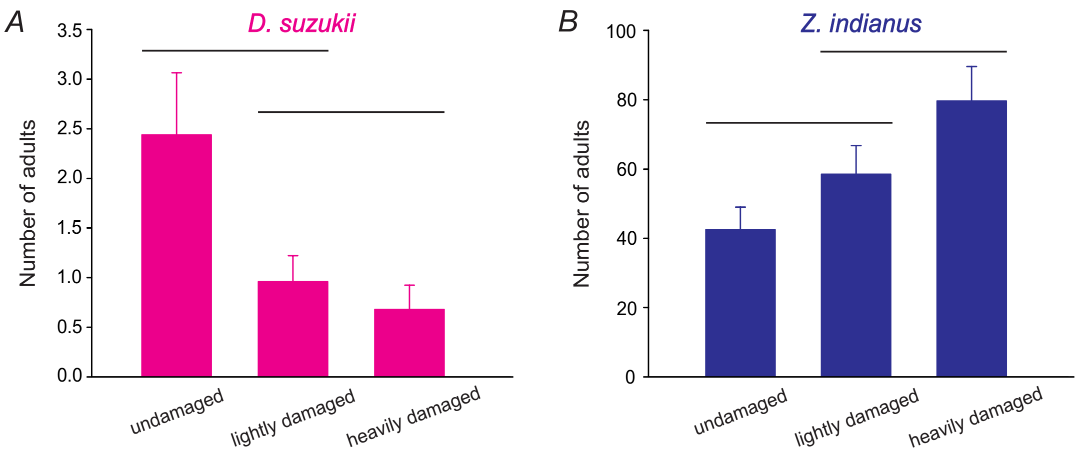

Figure 1.5: Number of *Drosophila suzukii* (A) and *Zaprionus indianus* (B) flies that emerged from undamaged, lightly and heavily damaged field collected fruits. Each bar corresponds to the mean of three independent assays (±SE). Treatments under the same horizontal bar do not differ statistically (P<0.05).

1.3.3 *Respirometry bioassay*

The ANOVA analysis showed that the factor population (F = 5.15; df = 2; P = 0.007) and interaction between population and sex (F = 7.80; df = 1; P < 0.001) did have a significant effect on the respiration rates of adult flies (Table 1.2). While the flies from the stock colony showed similar (F = 0.53; df = 1; P = 0.47) rates of respiration between sexes, female flies from the population of Ervália presented lower rates of respiration than male flies (F = 4.6; df = 1; P = 0.038). The opposite was found in the population of Paula Cândido as females flies had higher respiration rates compared to the males (F = 15.22; df = 1; P < 0.001) (Figure 1.6).

The feeding substrates (e.i., artificial diet and Barbados cherries *M. emarginata*) as well as the interactions: population x feeding substrate, sex x feeding substrate and population x feeding substrate x sex, did not significantly (P > 0.05) affect the respiration rates of adult flies.

Table 1.2: Analysis of variance values of the respiration rates of *D. suzukii* male and female flies from three populations, reared in either artificial diet or the Barbados cherry.

Source of variation	$d.f$	F	P_{value}
Population (P)	2	5.15	0.007*
Feeding Substrate (FS)	1	1.51	0.22
Sex (S)	1	0.001	0.97
P x FS	2	0.01	0.99
P x S	2	7.8	<0,001*
FS x S	1	1.06	0.31
P x FS x S	2	0.29	0.75
Residual	117	-	-

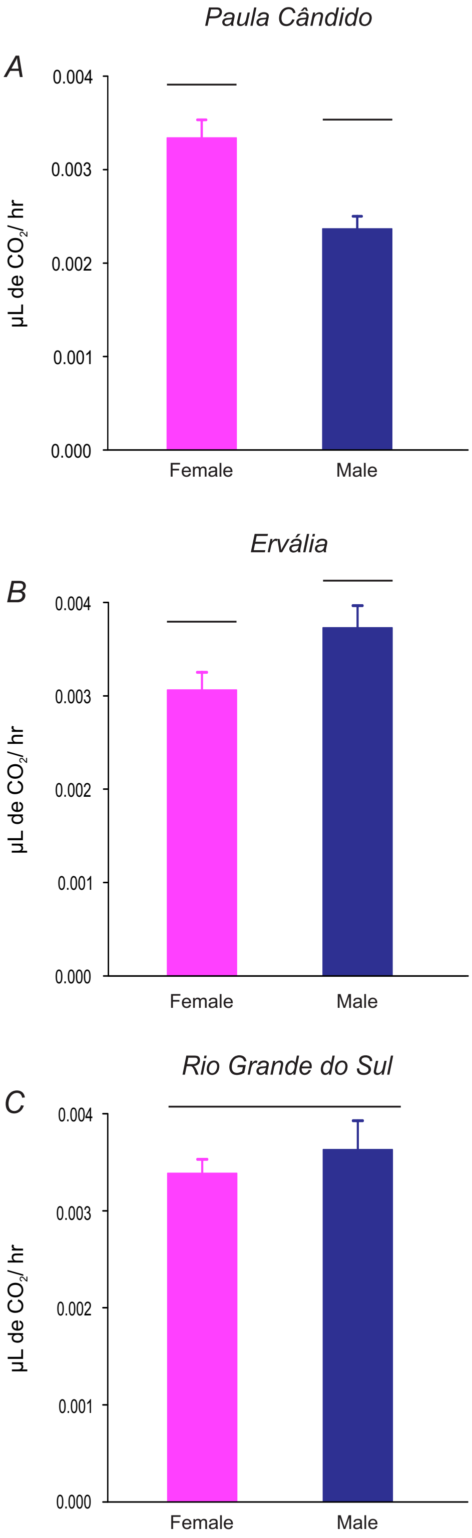

Figure 1.6: Respiration rates (µL de CO_2 /hr) of males and females of *Drosophila suzukii* flies field collected from Paula Cândido (A) and Ervalia (B) and Rio Grande do Sul (C). Bars correspond to the mean of femaless (21 from Paula Cândido, 23 from Ervália and 19 Rio Grande do Sul) and males (22 Paula Cândido, from Ervália and 20 from Rio Grande do Sul 24).Treatments under the same horizontal bar do not differ statistically (P<0.05).

1.4 Discussion

The present study reveals the first evidence that Barbados cherry, fruits of the Neotropical plant *M.emarginata*, are suitable hosts for *D. suzukii* in which the flies of this species can oviposit, develop and complete their life cycle. In addition, the fruits' infestation occurred naturally by this invasive species coincidently with other drosophilids such as *Z. indianus*. Furthermore, the biological traits such as development time, emergence and sex-ratio were different in this new host compared to strawberries fruits which are considered a preferred host (Lee et al. 2011).

The fruits of four different Neotropical plant species including jabuticaba, *P. cauliflora*, Barbados cherry, *M. emarginata*, bonnet pepper, *Ca. chinese* and coffee, *C. arabica*, were tested for their suitability as food and oviposition sources for SWD. Although the oviposition occurred on the fruit surface of the four species, the completion of life cycle was achieved only on Barbados cherries. Both suitable species and less or not suitable ones for *D. suzukii* may exist in some plant families suggesting that the host's phylogeny is of partial importance compared to the fruit parameters (Poyet et al. 2015). In fact, host suitability depends on several fruit traits and the fruits of different plant species are not similarly suitable to the different life stages of *D. suzukii* (Poyet et al. 2015). For example, fruits with waxy texture like (*Ruscus aculeatus*, *Rosa canina*, *Berberis thunbergii*) have been reported to be less attractive to *D. suzukii* (Koch and Ensikat 2008) which may explain the results obtained with the waxy fruits of bonnet pepper. Furthermore, although the fruit-penetrating ovipositor is a key morphological characteristic of *D. suzukii*, the hard skin of some fruits like the coffee berries may constitute a physical barrier for this fly (Cini et al. 2014). In addition, fruits with fibrous flesh such as jabuticaba may reduce the movement of larvae and decrease their efficiency of food intake hampering their attractiveness as oviposition support and feeding substrate for the larval stage.

Drosophila suzukii development is dependent on host nutritional quality and its development on various fruit hosts varies significantly with fruit type (Lee et al. 2011; 2015; Bellamy et al. 2013; Burrack et al. 2013; Tochen et al. 2014; Hardin et al. 2015; Jaramillo et al. 2014). The development of the *D.suzukii* on Barbados cherries and on the artificial diet was similar while it differed from the development on the preferred host (i.e.; strawberry). The fruit size, measured by its diameter and weight, is an important trait associated with the success of the different *D. suzukii* life stages as larger fruits increase the number of emerging *D. suzukii* adults (Poyet et al. 2015). Thus, the differences between the normal average size of Barbados cherries (1–4 cm diameter and 2-15g weight) (Delva and Schneider 2013) and fruit size of available commercial cultivars of strawberries resulting from breeding and selection programs (10-33g) (de Oliveira and Scivittaro 2009) may partially support the better performance (total emerged adults per female) in strawberry fruits. In addition, various other factors linked with fruit physiology such as volatile compounds, pH, shape, structure, firmness, colour… may also act as either limiting or favoring parameters of *D. suzukii* development (Bellamy et al. 2013; Burrack et al. 2012; Lee et al. 2011). Indeed, food source quality (artificial or natural source) affects development time and adult emergence rates (Tochen et al. 2014; Hardin et al. 2015). Difference in these parameters may explain the higher total number of emerged insect per female on strawberries compared to Barbados cherries and artificial diet as well as the anticipated emergence in the later fruit type. Overall, the differences in development times recorded here between the fruit types are similar to the ones previously reported in literature (Hamby et al. 2016).

The spotted wing drosophila has a very wide host range, including fruits of many cultivated, wild and ornamental host plants (Lee et al. 2015; Poyet et al. 2015). The wide polyphagy of *D. suzukii* can trigger alterations in physiological and metabolic processes in order to adapt and benefit from available food resources in newly invaded areas. Hence, the environmental variability may explain the differences in the respiration activities observed here between field collected populations (natural infestations) and the stock colony reared for many generations under controlled environment providing homogeneous conditions.

The plasticity of *D. suzukii* in host preferences and nutritional requirements is considered a key factor of its remarkable invasive success (Poyet et al. 2015). The first record of *D. suzukii* in South America occurred in 2013, in the South of Brazil (Deprá et al. 2014). Subsequently, it was recorded in the southeastern and central regions of the country approximately 1700 km to the north from the location of its first record (Paula et al. 2014; Andreazza et al. 2015). In these regions, the main cultivated host that is susceptible to important economic losses is strawberries (dos Santos et al. 2017). However, many of other crops cultivated in the southern Neotropical region and specifically in Brazil are also known as hosts of *D. suzukii*. These crops include blackberries, blueberries, cherries, grapes, peaches, plums and raspberries (Andreazza et al. 2017). Furthermore, in this region natural infestations were reported in other native fruits such as the Surinam cherry, *Eugenia uniflor*a L. (Myrtaceae), and *Cattley guava*, *Psidium cattleyanum* Sabine (Myrtaceae) (Müller and Nava 2014; Santadino et al. 2015). Modeling studies of distribution indicate potential extension of SWD presence to areas across the eastern extension of the Atlantic coast, including northeastern regions of Brazil (dos Santos et al. 2017). New infestation of native species like Barbados cherry as documented here will certainly play a crucial role in the expansion of the geographical range of the SWD to the Northern regions of Brazil and probably throughout the Central America. Barbados cherry plants can be found from South Texas, through Mexico and Central America to northern South America and the Caribbean. However, Brazil with a total area exceeding 10,000 ha; localized mainly in the Northern regions; is considered currently the world's leading producer, consumer, and exporter of these fruits (de Assis et al. 2008; Delva and Schneider 2013; Júnior et al. 2017).

The results from the field survey confirmed the preference of the *D. suzukii* to oviposit in healthy fruits of Barbados cherry in detriment of fruit already damaged. The presence of serrated ovipositor explains the ability of *D. suzukii* females to lay eggs on healthy ripe fruit (Sasaki and Sato 1995; Mitsui et al. 2006; Goodhue et al. 2011). Furthermore, injuries caused by external piercing and/or oviposition allow secondary pests such as *Z. indianus* to use damaged fruits and thus increases the losses (Renkema et al. 2013; Fartyal 2014; Joshi et al. 2014; Lasa and Tadeo 2015). The increase of *Z. indianus* oviposition associated with previous *D. suzukii* oviposition in Barbados cherry has been described in other fruits types known hosts of SWD (Bernardi et al. 2016). Barbados cherries have high agro-industrial potential and represent an appealing economic opportunity for local growers (Delva and Schneider 2013; Júnior et al. 2017). However, one of the problems faced by Barbados cherry producers is the great sensitivity of the mature fruits during picking, packing, processing, and/or distribution. Such concerns will be magnified with the presence of the spotted wing drosophila and other associated drosophilids in producing areas.

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Chapter 2

Density mediated stress affecting biological traits of the invasive pest, *Drosophila suzukii*

Density mediated stress affecting biological traits of the invasive pest, *Drosophila suzukii*

Abstract

It is known that the environment can influence the development of insects and potentially generate an outcome in their offspring. Based on the hypothesis that different densities in a generation may induce carry-over effects in the fitness of insects in the following generation, a laboratory study was conducted to understand the transgenerational effect of the density in the biology of the *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). The effect of density stress was measured by the exposure of insects to two densities (i.e. high and low) to test the impact of this stress in the susceptibility of adults exposed to increasing concentrations of deltamethrin. Also, the sexual fitness of the insect and its survival was tested after a sublethal exposure (LC_1 and LC_5) associated with parental density effect. Considering only the effect of density, it was possible to notice that insects kept under high-density parents' treatment showed a significant reduction in survival during the first generation (F_0), the density effect did not affect the toxicity but for sub-lethal exposure, it was possible to notice a hormetic effect, where insects even after the exposure to insecticide showed higher cumulative fecundity. The results obtained in this study suggest that the previous generation in high density, as what happens in lab conditions, can affect the fitness of the insects and consequently the output of experiments carried using them.

Keywords: Spotted wings Drosophila, rearing stress, transgenerational.

2.1 Introduction

The environment where the individual will develop is crucial to development and the fitness during the adult life history (Nylin and Gotthard 1998; Dufty Jr et al. 2002; West-Eberhard 2003). The density of the population is one of the main characteristic that contribute for the social environmental which can influence the develpoment in general, like the biology, physiology and the life history parameters like body mass, reproductive success, as well as individual´s behavior (Iba et al. 1995; Lihoreau et al. 2009; Kasumovic et al. 2012).

In the field conditions (natural conditions of individual), the occurrence of high density (*i.e.* crowded conditions) can be a frequent phenomenon (Atkinson 1979; Griffiths 1997) that represents an important selection agent (Bakker 1962; Roper et al. 1996). High or low densities of insects' populations can impact either the juvenile or adult phases, affecting their developmental and fitness parameters in relation with the social complexity and life history strategy of the organism (Peters and Barbosa 1977; Goulson and Cory 1995; Lihoreau and Rivault 2008).

The impact of population density on insect biology has been widely researched for many years (Long 1953; Goulson and Cory 1995; Iba et al. 1995; Ishida 1952; Lewontin 1955; Lihoreau and Rivault 2008; Pearl and Parker 1922; Peters and Barbosa 1977). The density mediated stress can affect various parameters of the biology of an insect, such as survival, developmental rate, size, feeding behavior, dispersive and aggregative behavior, and even aggression have been shown to be density dependent in some way (Long 1953; Watt 1960; Peters and Barbosa 1977). The first report on the effect of density using *Drosophila melanogaster* as a model, measured the "mean population average days" and "imagoes per mated female per day". These results showed the effect of the density in the reproduction of the females, where, the rate of reproduction per mated female per day declines as density of population increases (Pearl and Parker 1922).

Commonly, a low-density environment is better for non-social species than a crowded one due to lesser competition for resources which subsequently will generate advantages during later phases of life, known as the "silver spoon" effect (Descamps et al. 2008). This effect occurs for individuals that had an advantage in adulthood with plentiful early developmental resources over those that experienced a competition situation with a poor early conditions (Clutton-Brock 1988; Descamps et al. 2008; Monaghan 2007). Evidences of the silver spoon effect occurring in an insect population were reported in relation to certain physiological and physical conditions (*e.g.*, nutrition and/ or temperature) (Hopwood et al. 2014; Wong and Kölliker 2014; Scharf et al. 2015).

Approximately forty percent of insect and mite that are pests in some agroecosystems are invasive (Pimentel 1993; Pimentel et al. 2005), and the invasive species are often the most economically damaging pests (Hamby et al. 2016). Among the current invasive pests, it is possible to highlight *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) (Cini et al. 2012). The spotted wing Drosophila (SWD) is a newly worldwide pest of small soft-skinned fruits, such as strawberries, blueberries and cherries, with its presence reported already in many continents (Lee et al. 2011; Calabria et al. 2012; Deprá et al. 2014; Kinjo et al. 2014). With short generation time coupled to high reproductive potential, SWD can cause an increasing pest pressure through the crop-ripening season (Wiman et al. 2014).

The study of a new invasive insect pest based on laboratory rearing and laboratory cage experiments have been providing important information about behavior and therefore the ecological function of pests. However, great care must be taken in the interpretation of results of laboratory experiments and their extrapolation to field conditions (Vereijssen and Goldson 2016). Besides, the laboratory-rearing can also have dramatic effects on insect biology due to adaptation to laboratory conditions, inbreeding depression or through direct rearing effects (*e.g.* artificial diets) (Sørensen et al. 2012). Thus, the objective of this study is to analyze the transgenerational effects of the density in the biology and toxicological responses of *D. suzukii*. The evaluation of the effect of the stress mediated by density and its association with a sublethal exposure to deltamethrin, was assessed on the survival and the average fecundity of this invasive pest during two generations.

2.2 Material and Methods

2.2.1 *Insects and density treatment*

The *D. suzukii* population used in the experiments was originally obtained from a laboratory stock colony at Embrapa Clima Temperado, in Pelotas - Rio Grande do Sul, Brazil. The larvae were fed with an artificial diet, changed every day, made with a mixture of water, brewer's yeast, corn flour, sugar, agar, propionic acid and methyl 4-hydroxybenzoate (NipaginTM) as descrived elsewhere (Andreazza et al. 2016). The adults were maintained in plastic cages of 10 liters (29 cm x 21 cm x 14 cm). The insect colony and all the experimental cages were kept in a room with controlled temperature and humidity (25 ± 2ºC, and RH = 50 ± 10%).

One generation prior to the bioassays to be performed, the insects from the stock colony were separated to be reared in two situations: high and low-density treatment. In the high-density (the standard stock colony condition), flies were reared at a density of 0.133 flies/cm^3 with approximately 50% sex ratio (approximately 1500 insects reared in a single experimental cage). In the low-density however, flies were reared in a density of 0.06 flies/cm^3 with also approximately 50% sex ratio (approximately 30 insects in cages of 0.5 liter). The generation, in which the density treatment was applied, was denoted as the initial generation (F_i). Two offspring generations (denoted F_0 and F_1) were then used in the transgenerational experiments and were always maintained in low-density conditions (Figure 2.1).

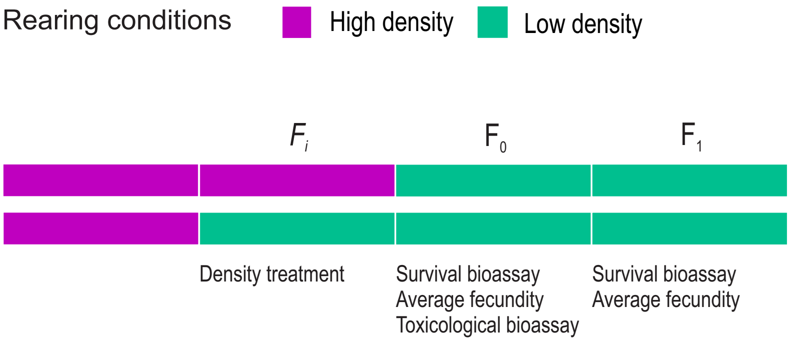

Figure 2.1: Schematic figure to demonstrate the chronology of the experiments with *D. suzukii* exposed to density stress.

2.2.2 *Parental-density effects on adult offspring survival*

The effect of density in the survival of the insects was evaluated in the two following generations (i.e., F_0 and F_1) to analyze the transgenerational effect of this stress. In the survival bioassay, initially, one day-old F_0 *D. suzukii* flies from both F_i groups (i.e., flies from parents reared in either, high or low-density) were separated into four replicates of 15 couples (30 flies) in plastic cages of 0.5 L closed with voile tissue. The density of flies in these cages corresponds to the low-density treatment (0.06 flies/cm^3). Oviposition and feeding substrate (*i.e.*, larvae artificial diet) and a solution of water and sugar (20%) were provided during the entire bioassay. Daily, the fly mortality was assessed, until the majority of the flies had died. From the oviposition substrate offered to the F_0 adults, it was reared the generation F_1 (*i.e.*, flies that had parents reared all in low-density, but grandparents reared in either, high or low-density) (Figure 2.1). These flies were then also submitted to the same survival bioassay, following the same procedures.

2.2.3 *Toxicological bioassay*

To evidence the effects of the parental-density on the susceptibility to an insecticide in *D. suzukii*, a concentration-response curve bioassay was performed. The insecticide used was the pyrethroid deltamethrin (Commercial product: Decis 25EC, DowAgroscience). For this, one day-old F_0 insects from either high or low-density parents were exposed by contact/feeding in exposition chambers following the IRAC protocol N° 26 recommended for bioassays with *Musca domestica* L. (Diptera: Muscidae) adults, with modifications. Each sample unit consisted of a 200 mL glass jar containing a 2cm long cotton wick imbibed with 1.8 mL of a solution containing the insecticide at one of different concentrations. The top of each jar was sealed with a foam plug. Inside the jar, were released 25 flies randomly collected from the rearing cage with the aid of a hand aspirator. The concentrations used were: 1, 2, 3, 4, 5, 7, 9 and 15 mg of a.i. L^{-1} each in four replicates. For all the dilutions and control, it a solution of water with sugar at 20% was used. After 24h of exposition, the flies were removed from the chambers and the mortality was assessed. Insect that did not show any kind of movement after a mechanical stimulus was considered dead.

2.2.4 *Parental-density effects on adult offspring responses to sub-lethal insecticide concentrations*

One day-old F_0 *D. suzukii* flies from either high or low-density parents were exposed to two sublethal concentrations and a control using the same methodology of exposure used to estimate the concentration-mortality curve. The sublethal concentrations of deltamethrin used were the ones estimated to cause 1 and 5% of flies' mortality (i.e., LC_1 and LC_5) for both parental-density treatments. After 24 hours of exposure the surviving F_0 insects were removed from the exposition chambers and separated in four groups (*i.e.*, 4 replicates) of 15 couples, for each parental-density treatment, inside 0.5 L plastic cages.

From this moment onward, the same methodology described in the initial survival bioassay was used, daily assessing not only the flies' mortality for the survival analysis, but also their daily cumulative fecundity. For this, the oviposition substrate that was changed daily until the death of all females in the cage, was assessed under a stereoscopic microscope and the eggs were counted. The number of eggs counted was divided by the number of living females in the cage when the substrate was offered, and therefore, the daily average number of eggs per female was used for the analysis. Similarly, to the initial survival bioassay, the eggs laid by the F_0 females (*i.e*, F_1 individuals) were reared to adult stage, when new cages containing 15 couples each for the next generation (F_1), from both treatments was also analyzed for adult survival (Oliveira et al. 2005; 2007; Guedes et al. 2006; Haddi et al. 2015). Cumulative fecundity were monitored until the death of females occurred.

2.2.5 *Statistical Analysis*

The data of flies' mortality along the time for both generations (*i.e.*, F_0 and F_1) were used to construct the Kaplan-Meier survival curves, and the log-rank test analysis and Holm-Sidak test in SigmaPlot 12.5 software (Systat Software, San Jose, USA) were used to identify significant differences between the curves. The concentration-mortality data were subjected to a probit analysis (PROC PROBIT, SAS Institute 2010) to estimate the lethal concentrations of deltamethrin. The cumulative fecundity was submitted to regression analysis using curve fit procedure SigmaPlot 12.5 software

(Systat Software, San Jose, USA), and the differences between the regression curves were based on the overlapping of the confidence intervals of their parameters.

2.3 Results

2.3.1 *Parental-density effects on adult offspring survival*

The effect of the density could be noticed in the first generation (*i.e.*, F_0) (Log-Rank χ^2= 93.292; *df* = 3; *P* < 0.001), once that flies from parents reared in high density had a survival time lower for females (LT_{50}=12.11 [10.73 - 13.48]) and male (LT_{50}=13.92 [12.5 - 15.32]) than flies from parents reared in low density with males with medium survival of 24.11 (20.15 - 28.07) days and females with survival time of 39.0 (33.80 - 44.20) days (Figure 2.2A).

The transgenerational effect of the density seems to disappear once that in the second generation (i.e., F_1) was not possible to notice difference between treatments (Log-Rank χ^2= 27.421; *df* = 3; *P* < 0.001), only between genders where males lived less when compared with females (Figure 2.2B). Males from parents reared in low density had a survival time of 28.52 (23.62 - 33.42) days and males from high density treatment had survival time of 26.83 (23.22 - 30.43). Females from low density treatment had LT_{50}= 38.67 (33.67 - 43.67) and females from parents reared in high density had LT_{50}= 34.50 (34.50 - 45.45).

The insects exposed to sublethal concentrations of deltamethrin were also submitted to survival analysis. For both treatments of high and low density, the insects were exposed to two sublethal concentrations of deltamethrin (1% and 5%). For the first generation (i.e., F_0), the females from high density treatment (Log-Rank χ^2= 1.046; *df* = 2; *P* = 0,593) and males from low density treatment (Log-Rank χ^2= 3.95; *df* = 2; *P*=0.139) do not differed from the two concentrations exposed and the control (Figure 2.4). The survival time for females from parents reared in high density were 13.92 (12.51 - 15.32); 13.43 (11.73 - 15.12) and 14.30 (12.63 - 16.16) for control insects, flies exposed to LC_1 and exposed to LC_5 respectively (Figure 2.4B). For males whose parents were reared in low density, the survival times were of 24.11 (20.15 - 28.07); 27.10 (23.28 - 30.93) and 19.17 (14.84 - 23.50) for control flies, flies exposed to LC_1 and exposed to LC_5 respectively (Figure 2.4C). For the females from parents reared in low density, the treatment of LC_1

exposure and LC_5 exposure do not differed from control (Log-Rank χ^2= 7.61; df = 2; P = 0.022). The average survival time for them was 39 (33.80 - 44.20) days for control insects, 40.98 (35.84 - 46.12) days to flies exposed to LC_1 and 29.5 (23.93 - 35.07) days for insects exposed to LC_5 (Figure 2.4D). *D. suzuki*i males from high density differed between treatments (Log-Rank χ^2= 50.172; df = 2; P<0.001). For them, insects from control treatment lived more (LT_{50}= 12.11 days; 10.73 - 13.48) and insects exposed to LC_5 were the one who lived less (LT_{50}=4.5 days; 3.69 - 5.32). The average survival time for insects from LC_5 treatment was 8.42 days (6.67 - 10.78) (Figure 2.4A). For the second generation (i.e., F_1), males from low density treatment (Log-Rank χ^2= 0.0711; df = 2; P=0.965) (Figure 2.5C), males (Log-Rank χ^2= 1.77; df = 1; P=0.183) (Figure 2.5A) and females (Log-Rank χ^2= 1.97; df = 1; P=0.657) (Figure 2.5B) from high density treatment do not differed statistically from the treatments. For the females from low density treatment the treatments differed statistically (Log-Rank χ^2= 11.62; df = 2; P=0.003). The average survival time was 33.77 (29.06 - 38.48) days for control insects, 37.39 (32.05 - 42.73) days for flies exposed to LC_1 and 46.17 (41.39 - 50.94) days to insects from LC_5 treatment (Figure 2.5D).

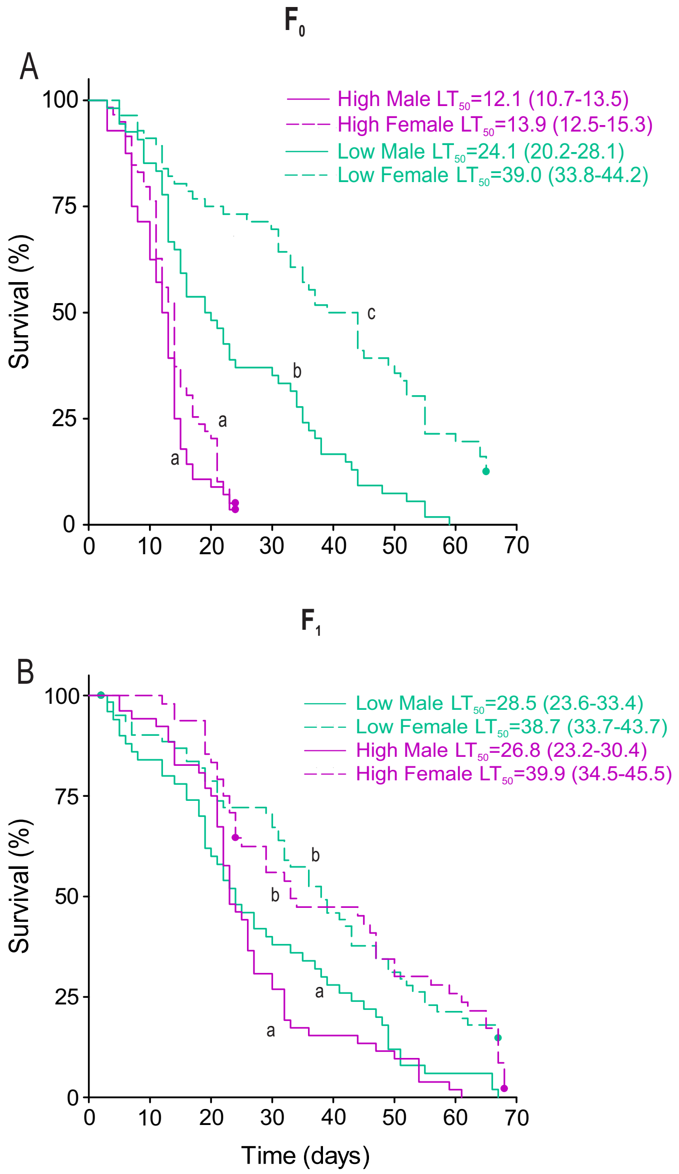

Figure 2.2: Survival curves of generation (A) F_0 and (B) F_1 of *D. suzukii* exposed to high and low density. Means followed by the same letter do not differ statistically by the Holm-Sidak test ($P < 0.05$).

2.3.2 *Toxicological bioassay*

The results for toxicological bioassay were submitted to Probit model showing adequate results for the concentration-mortality in *D. suzukii* (Figure 2.3). For insects under the high density, based on low values of χ^2 and high value of $P > 0.05$ ($P = 0.23$) it was obtained aLC_{50} = 4.84 (4.39 - 5.34) mg a.i / L. For insects under low density the results were basically the same once that under the same conditions of low values of χ^2 and high value of $P > 0.05$ ($P = 0.88$) it was obtained a LC_{50} = 4.77 (4.41 - 5.17) mg a.i/L.

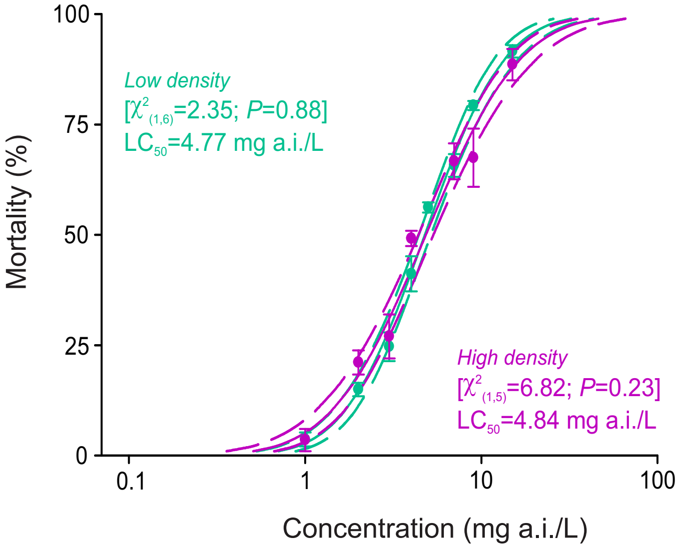

Figure 2.3: Toxicity of deltamethrin to *D. suzukii* from high and low density treatment. Lethal concentration (LC) values were estimated based in concentration–mortality bioassays using probit analysis. The symbols show the averaged mortality, and the vertical bars represent the standard error (±SE).

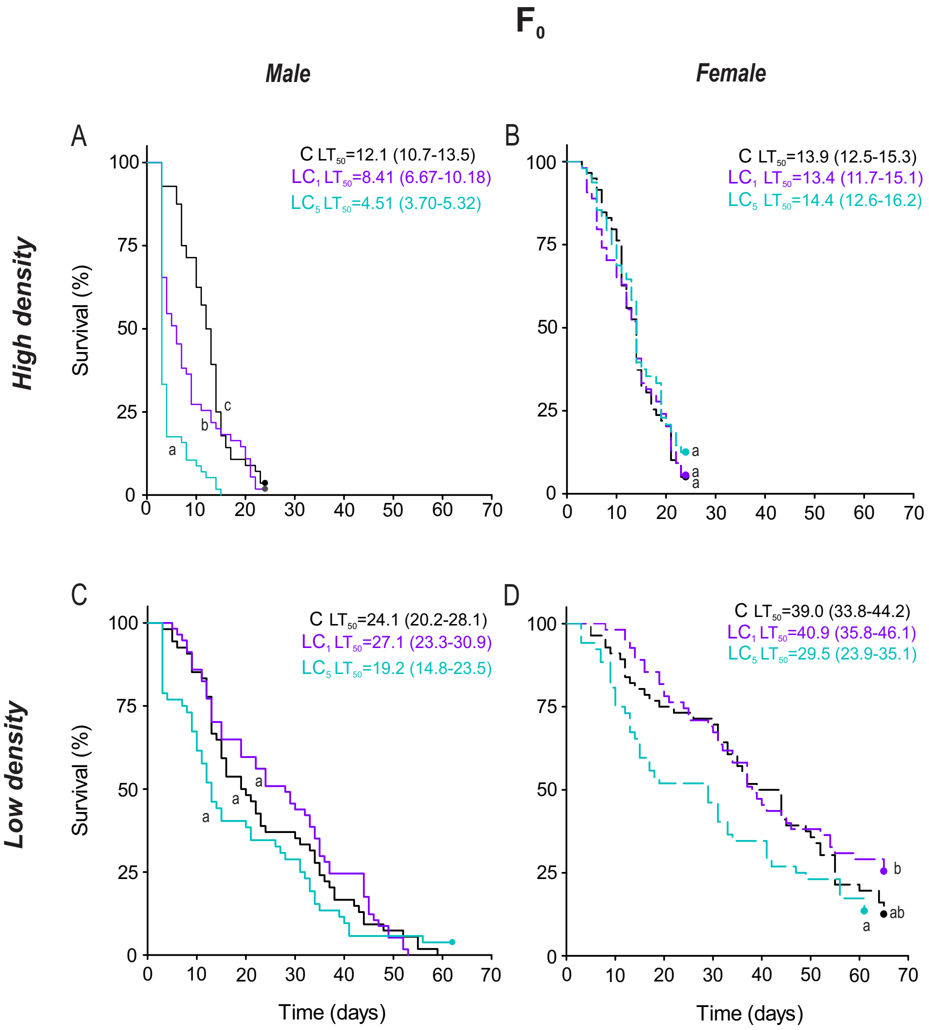

Figure 2.4: Survival curves of *D. suzukii* exposed to two sublethal concentrations and from parents reared at high or low density (Generation F_0). (A) males and (B) females of *D. suzukii* from high density treatment. (C) males and (D) females of *D suzukii* from low density treatment. Means followed by the same letter do not differ statistically by the Holm-Sidak test (*P* < 0,05).

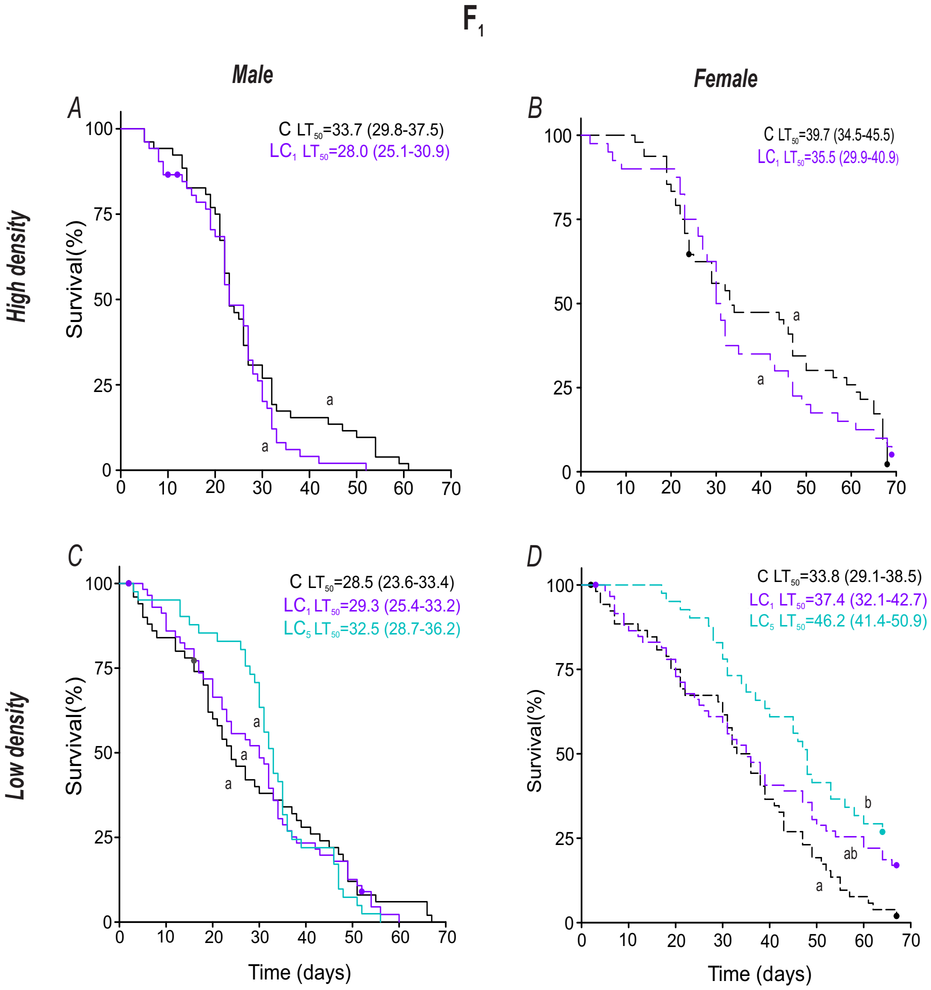

Figure 2.5: Survival curves of *D. suzukii* exposed to two sublethal concentrations and from second generation (F_1). (A) males and (B) females of *D. suzukii* from high density treatment. (C) males and (D) females of *D suzukii* from low density treatment. Means followed by the same letter do not differ statistically by the Holm-Sidak test ($P < 0.05$).

2.3.3 *Parental-density effects in cumulative fecundity in response to sublethal exposure*

Insects whose parents were reared in low density and exposed to sublethal doses exhibited high cumulative fecundity, where insects exposed to LC_1 showed the highest number of eggs per female if compared with control treatment (Figure 2.6C). In this same density, but in the second generation (i.e., F_1) the pattern change (Figure 2.6D). Insects that parents were exposed to LC_5, that had the lowest cumulative fecundity in the previous generation, shows now the highest peak, followed by insects with parents exposed to LC_1 and control insects. The average fecundity results fit well with the two-parameter Hyperbola (Table 2.1).

For insects whose parents were reared in high density, both that were exposed to sublethal doses (LC_1 and LC_5) exhibited higher cumulative fecundity than control treatment (Figure 2.6A). The average fecundity results fit well with the two-parameter Hyperbola (Table 2.1). For the same density, but in the second generation (i.e., F1) for cumulative fecundity (Figure 2.6B) the transgenerational effect of the sub-lethal doses seems to disappear once the control treatment does not differ statistically from insects whose parents were treated with LC_1.

Table 2.1: Summary of non-linear regression analyses of daily emergence (Figure 2.6)

		a	b	df_{total}	F	P_{value}	R^2
Low density F_0	Control	35.39 (34.38 - 36.40) a	19.56 (18.03 - 21.09) a	63	5976.22	<0.0001	0.99
	LC_1	47.40 (44.45 - 50.35) b	24.86 (21.08 - 28.63) a	63	1715.14	<0.0001	0.98
	LC_5	151.6 (79.31 - 223.9) c	266.4 (118.5 - 414.3) b	60	2270.8	<0.0001	0.98
Low density F_1	Control	94.61 (87.11 - 102.1) a	68.31 (59.51 - 77.12) a	65	5715.9	<0.0001	0.99
	LC_1	211.08 (149.6 - 272.5) b	156.9 (97.86 - 216.0) b	65	1738.1	<0,0001	0.98
	LC_5	122.3 (111.1 - 133.6) c	43.79 (36.20 - 51.37) c	62	2015.6	<0.0001	0.98
High density F_0	Control	9.61 (8.29 - 10.93) a	11.61 (8.18 - 15.03) a	22	726.8	<0.0001	0.98
	LC_1	21.77 (20.32 - 23.21) b	8.76 (7.34 - 10.18) a	22	2102.9	<0.0001	0.99
	LC_5	27.95 (19.99 - 35.92) b	31.56 (18.25 - 44.87) b	22	1052.3	<0.0001	0.99
High density F_1	Control	106.8 (103.4 - 110.2) a	29.55 (27.34 - 31.76) a	69	8556.8	<0.0001	0.99
	LC_1	106.6 (102.9 - 110.3) a	32.56 (30.02 - 35.10) a	69	8476.6	<0.0001	0.99

Model: $y = a*x/(b+x)$

Values followed with the same letter in each generation column do not differ significantly.

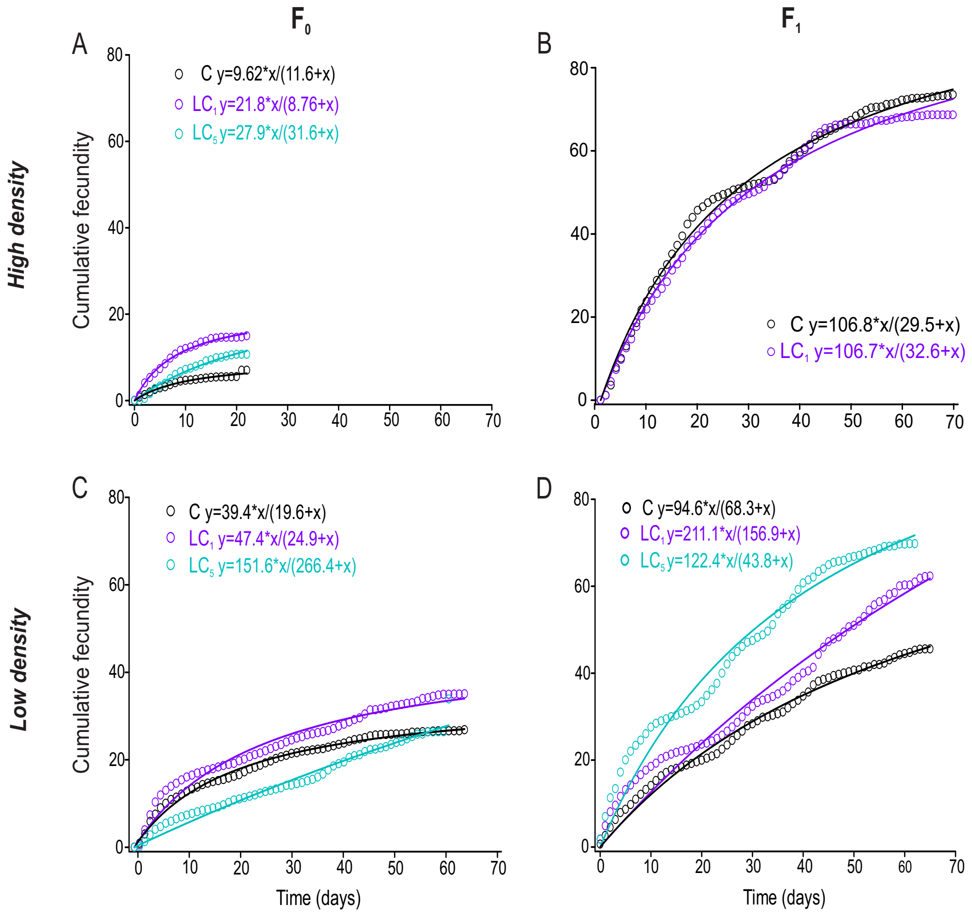

Figure 2.6: Cumulative fecundity of *D. suzukii* exposed to sublehtal doses of deltametrin under density treatment in two generations. (A) generation F_0 and (B) second generation F_1 from high density. (C) generation F_0 and (D) generation F_1 from low densty treatment. The symbols represent the mean observed data, and the lines represent the predicted trend of the best models selected

2.4 Discussion

In the present study, it was possible to elucidate the effect of the stress of density associated with sublethal exposure and its transgenerational effect in the biology of *D. suzukii*. From the results, it was clear to notice the parental-density effects on adult offspring survival. As well, for insects that suffered one kind of stress, (*i.e.* insects under the sublethal exposure and low density) the hormetic effect in the fecundity remained during the generations while insects under two stresses (*i.e.* insects exposed to sublethal doses and high density) showed the effect of its stress only during F_0, recovering in the following generation.

Lewontin (1955), showed that the organism's ability to pass its genes to future generations is dependent on the environment in which it is inserted to develop and reproduce. This ability may then influence their competence of carry over effect. It is consistent with our results where the stress of density suffered by the parents affected the survival of next generation of *D. suzukii*. The recovering from the transgenerational effect of stress occurs only in the second generation (*i.e.* F_1). This result can be interpreted as silver spoon effect (Clutton-Brock 1988; Monaghan 2007), because the low density conditions, considered beneficial for a non-social insect, resulted in fitness advantage for *D. suzukii*, where in this situation the insects had higher survival if compared with insects from high density treatment.

The effect of density alone is capable to affect the survival of the insects, as showed by Ishida (1952), where the density acted different on the sexes with males decreasing its longevity with the increase of density. This pattern was different in our study once that when the flies were exposed only to density treatment, in the first generation (F_0), males and females did not differ in the survival.

The toxicological bioassay was not affected for insects under high or low density parents treatment. So, it was speculated the occurrence of an effect of sublethal exposure when in association with the density stress in the fitness of the flies. Studies have been reporting the capacity of environmental stressors, like insecticides, causing stimulatory effect when at low concentration (Cutler 2013; Guedes and Cutler 2013; Guedes et al. 2016). This fitness gain occurs, despite the toxic effect expected when in high level, as a result of hormesis (Haddi et al. 2016). The literature brings examples of insects sub lethally exposed to insecticides exhibiting compensatory effects, acting in the fitness of them, like females showing higher reproductive rates if compared with control insects and also insects with effect in the survival when exposed to sublethal doses of insecticides. (Mallqui et al. 2014; Santos et al. 2016; Tan et al. 2012). Similar results were found in this study when the parental effect of density was combined with sublethal exposure of LC_1 and LC_5 with females showing hormesis in the cumulative fecundity.

In general, this study has highlighted the effect of laboratory rearing on populations of *D. suzukii* and how this can lead to erroneous conclusions about population density effects in the survival and toxicology of the insect showing the importance of care for the extrapolation in the interpretation of lab experiments to field conditions.

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Chapter 3

The use of ozone as a control alternative for *Drosophila suzukii* Matsumura (Diptera: Drosophilidae)

The use of ozone as a control alternative for *Drosophila suzukii* Matsumura (Diptera: Drosophilidae)

Abstract

Ozone is an unstable gas with a short half-life that decompose to oxygen. Its use has been proposed in different fields such as in post-harvest to degrade agrochemicals and to increase fruit's shelf-life, in water treatment, as antimicrobial and as insecticidal agent. It has already been approved as GRAS (generally recognized as safe) by the FDA (Food and Drug Administration) what make easy the use of this gas in the food industry. At the same time, the pest *Drosophila suzukii* has grown its importance in the field of fruits of thin skin. Despite being a polyphagous insect, the *D. suzukii* shows a preference of occurrence in strawberry fruits, where organic practices being commonplace, what makes necessary the search for new alternatives to control this pest. In this way, it was proposed to test the toxicity of ozone to egg phase of *D. suzukii*. Fifteen strawberry fruits for each treatment and ten fruits for control were exposed for 24 hours to 10 couples for oviposition. After, the strawberry fruits were exposed to four increasing concentrations of ozone (0.2, 1.2, 2.3, 3 mg/L) for 30 minutes at a continuous flow of 5 liters/min. It was possible to notice emergence even in the higher concentration of ozone used (*i.e.* 3 mg/L). Nevertheless, the average time of emergence and the distribution of the emergency in time were affected by ozone treatment. Thus, it is speculated that the time of exposure is more important than the increase of ozone concentration and also that the phase used in this experiment difficulted the efficacy of the treatment once that eggs can have lower respiration rates and can have a physical barrier to ozone penetration.

Keywords: Spotted wings Drosophila, strawberry, insect control.

3.1 Introduction

The gas ozone (O_3) was discovered in 1785 by van Marum who observed its formation through a spark of electric discharge in oxygen (O_2) (Chiattone et al. 2008). It is a colorless gas with a characteristic and pungent odor (Coke 1993). The highest oxidation potential (2.07 mV) of ozone makes it a powerful oxidizing agent (Guzel-Seydim et al. 2004; Da Silva 2011) and allows its use to oxidize contaminants in air and water. Owing to this characteristic, the ozone has been suggested as disinfectant agent in water treatment and in different applications in the food industry (Pérez et al. 1999).

Indeed, in agriculture field, the ozone is used as an antimicrobial (Gabler et al. 2010; Kasler and Yousef 2018) acting against nematoid (Qiu et al. 2009), fungus such as green mold (Boonkorn et al. 2012), inactivating bacteriophages (Kim et al. 1980) and as insecticidal agent (Ebihara et al. 2016). Another application of ozone gas is its use to increase fruit's shelf life (Guzel-Seydim et al. 2004; Heleno et al. 2014; Sousa et al. 2008; Pérez et al. 1999; Salvador et al. 2006), mainly because of its effective antimicrobial activity, even at room temperature (Clark 2004), once that the ozone can increase the durability of vegetables by the reduction of surface microbial count (Pandiselvam et al. 2017). The concerns in the safety about what is applied in the food have been growing up with the time, what raised the caution to look for alternative ways of degrading possible pesticides present (Pandiselvam et al. 2019). So, an alternative is the use of ozone gas in the post-harvest for degradation of chemical substances in food and consequently,to minimize the risk to health(Heleno et al. 2014; Savi et al. 2015).

A highlight in the use of ozone is its insecticidal activity. The ozone insecticidal mode of action is via oxidative stress causing damage in cell membranes or triggering cell death (Hollingsworth and Armstrong 2005). The literature brings reports of this activity in a variety of insects species such as in *Sithophilus zeamais* (Sousa et al. 2008; 2016), *Rhyzopertha dominic*a (Velasquez et al. 2017), *Lasioderma serricorne* (F.) (Amoah and Mahroof 2018), reducing the populations of *Ephestia kuehniella* Zeller and *Tribolium confusum* in different phases of their development (Işikber and Öztekin 2009), and also with susceptibility of *Tribolium castaneum*, *Plodia interpunctella*, and *Sitophilus oryzae* (McDonough et al. 2011).

Drosophila suzukii (Matsumura) or spotted wing Drosophila (SWD) is a new invasive worldwide pest of soft-skinned fruits (Deprá et al. 2014; Walsh et al. 2011). The damage caused by *D. suzukii* can be both, direct and indirect. The direct damage is due to the oviposition done by the female in the fruit, resulting in an egg where the larval will feed on the internal part of the fruit. The indirect effect occurs because of the exposure of the fruit to secondary pathogens (*e.g.*, bacteria and yeasts) and also to other drosophilid species (Walsh et al. 2011; Cini et al. 2012; Hamby et al. 2012; Ioriatti et al. 2015; Asplen et al. 2015b). The SWD has been causing economic losses in its native continent of Asia, the Americas, and Europe (Lee et al. 2011; Calabria et al. 2012; Deprá et al. 2014; Kinjo et al. 2014). A key feature of its rapid spread was the initial lack of regulation over the spread of any specie of drosophila associated with its capacity of quick translocation from a region to another, either flying or being passively introduced by the fruit trade (Cini et al. 2012).

For the control of *D. suzukii*, one of the main strategies is the use of chemical products (Haye et al. 2016). However, due to its occurrence mainly in fruits such as strawberries, where the use of organic practices being commonplace, the need arises for the search for compounds of insecticidal action alternative to synthetic ones that are compatible with the different production systems. The use of ozone was approved as GRAS (generally recognized as safe) by the FDA (Food and Drug Administration) (FDA 2001) in insect pest control. So, the objective of this study is to propose the use of ozone, which is unstable with a short half-life (20-50 min) and decomposes to oxygen not leaving residues in the food (Kells et al. 2001), as an alternative to control the invasive pest *D. suzukii*.

3.2 Material and Methods

3.2.1 *Insects rearing*

The *D. suzukii* population used in the experiments was originally obtained from a laboratory stock colony at Embrapa Clima Temperado, in Pelotas - Rio Grande do Sul, Brazil. The flies colony was kept in experimental plastic cages (29cm x 21cm x 14cm) that were kept under controlled temperature and humidity (25 ± 2ºC, and RH = 50 ± 10%). The larval stage was fed with an artificial diet made with a mixture of water, brewer’s yeast, corn flour, sugar, agar, propionic acid and methyl 4-hydroxybenzoate

(NipaginTM) as described by Andreazza et al. (2016).

3.2.2 *Ozone production*

The gas ozone was produced by an ozone generator developed by Ozone and Life (São José dos Campos, SP, Brazil), which uses compressed pure oxygen (99.99%) as input. For the generation of ozone, the oxygen flows through a refrigerator reactor where electric current is discharged through a dielectric barrier. The discharge is produced due a high-voltage current formed between two parallel electrodes, with a dielectric glass where the flow of oxygen is going to pass. The filamentous electric discharge is the responsible for the break of the oxygen molecule into atoms that rearrange into ozone (Sousa et al. 2008).

The quantification of the concentrations of ozone generated and introduced in the chamber, was based on an indirect titration using iodometric method. In brief, a beaker of 100 mL containing 50 mL of potassium iodide solution (KI) is manually directed to the flow from the ozone generator for 30 seconds. In this solution of gas bubbled is added 2.5 mL of sulfuric acid (H_2SO_4) 0.5 mol/L. This solution reacts with the KI solution to form iodine (I_2). The amount of I_2 formed, which is proportional to the bubbled ozone (equation a), is titrated with a standard solution of sodium thiosulfate ($Na_2S_2O_3$) 0.01 mol/L, using a starch suspension (1%) as indicator until the blue color disappear.

a) $O_{3(g)} + 2I^{-}_{(aq)} + H_2O_{(l)} \rightarrow I_{2(aq)} + 2OH^{-}_{(aq)} + O_{2(g)}$

3.2.3 *Strawberry fruits infestation and ozone toxicity bioassay*

To test the ozone toxicity to eggs of *D.suzukii*, the previously infested fruits of strawberry were exposed to four different concentrations of the gas. Fifteen fruits of strawberry (each fruit was a repetition) were separated individually in plastic cages of 0.5 L closed with voile tissue. In each cage, the strawberries were offered as oviposition substrate to five recently emerged (1-2 day) couples of *D. suzukii* for a 24 hours period before the ozone exposure.

The concentrations used were: 0.2, 1.2, 2.3 and 3.0 mg/L with a continuous flow of 5 liter/min. Achieving the desired ozone concentration as described above, the 15 fruits were placed in a plastic chamber of PVC hermetically sealed (20 centimeter of diameter and 65 centimeters of high) and exposed to the generated ozone for 30 minutes. After the exposure, the fruits were individualized in plastic cups of 100 mL covered by voile tissue until the emergence began.

The bioassay was divided into two days of exposure. In the first day, it was exposed 15 fruits to 1.2 and 2.3 mg/L and also 10 fruits that did not received the ozone treatment as a control. The second day it was exposed 15 fruits to 0.2 and 3.0 mg/L of ozone and 10 fruits as a control with no treatment. The insects that emerged were evaluated in the time of emergency and weighed on an analytical balance (Sartorius BP 210D, Germany) for comparison with insect from control treatment.

3.2.4 *Statistical analysis*

The weight of the insects was submitted to two way ANOVA (univariate analysis of variance) followed by a test t for comparison. The average daily emergence was submitted to regression analysis, and these models were compared by the overlapping of the confidence intervals of their parameters. All the data were submitted to the SigmaPlot 12.5 software (Systat Software, San Jose, USA).

3.3 Results

3.3.1 *Body mass of the insects*

After the emergence of *D. suzukii* that were exposed to different concentration of ozone when in egg phase, the insects were sexed and weighted. There was no statistical difference between the treatments, with only difference in the weight of sexes (t = 2.49; df = 0.14; P = 0.015) where the average weight of females and males were respectively 0.926 mg and 0.786 mg (Figure 3.1).

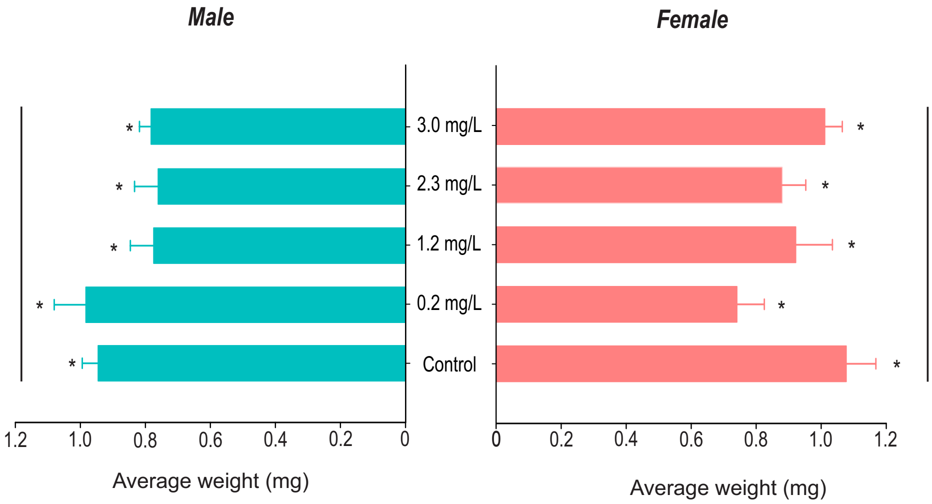

Figure 3.1: Body mass (mg) of newly emerged adult insects (24 h) of the five treatments (0.2, 1.2, 2.3, 3.0 and control) of *D. suzukii* females and males.

3.3.2 *Average daily emergence*

All the average daily emergence results fit well with the peak equation of three-parameter Gaussian model (Table 3.1). For the first day of exposure, all the parameters (i.e. a, b and X_0 from the model) of the insects exposed to 1.2 mg/L statistically differed from the control insects. This resulted in a delay of 0.33 day in the emergence of the insect exposed and a higher peak of emergency. The treatment of 2.3 mg/L of ozone showed no difference from the control (Figure 3.2) (Tabel 3.1).

The second day of exposure, eggs from the treatment of 3 mg/L of ozone showed a delay of 0.15 day in the emergence of the adults in comparison with control treatment. Also, in the second day of exposure, the treatment of 0.2 mg/L differed from the control in the peak of the average emergency, where the emergency occurred more distributed in the time for fruits that received the treatment (Figure 3.2) (Tabel 3.1).

Table 3.1: Summary of non-linear regression analyses of daily emergence (Figure 3.2).

	Estimated parameters (SE)						
	a	b		df_{error}	F	P_{value}	R^2
Control (1 day)	62.8 (59.8 - 65.7) a	0.61 (0.58 - 0.64) a	11.87 (11.83 - 11.92) a	7	1350.9	<0.0001	0.99
1.2 mg/L	72.5 (67.3 - 77.7) b	0.51 (0.46 - 0.55) b	12.02 (11.94 - 12.11) b	7	532.8	<0.0001	0.99
2.3 mg/L	64.4 (52.2 - 76.7) ab	0.52 (0.39 - 0.65) ab	12.08 (11.86 - 12.33) ab	7	77.69	0.0002	0.98
Control (2 day)	62.8 (59.8 - 65.7) A	0.61 (0.58 - 0.65) A	11.87 (11.83 - 11.92) A	7	1350.9	<0.0001	0.99
0.2 mg/L	46.6 (36.0 - 57.2) B	0.75 (0.57 - 0.94) A	11.90 (11.68 - 12.11) AB	7	50.16	0.0005	0.97
3.0 mg/L	61.6 (52.5 - 70.8) AB	0.61 (0.50 - 0.72) A	12.20 (12.08 - 12.32) B	7	165.61	<0.0001	0.99

Values followed with the same letter in the column do not differ significantly.

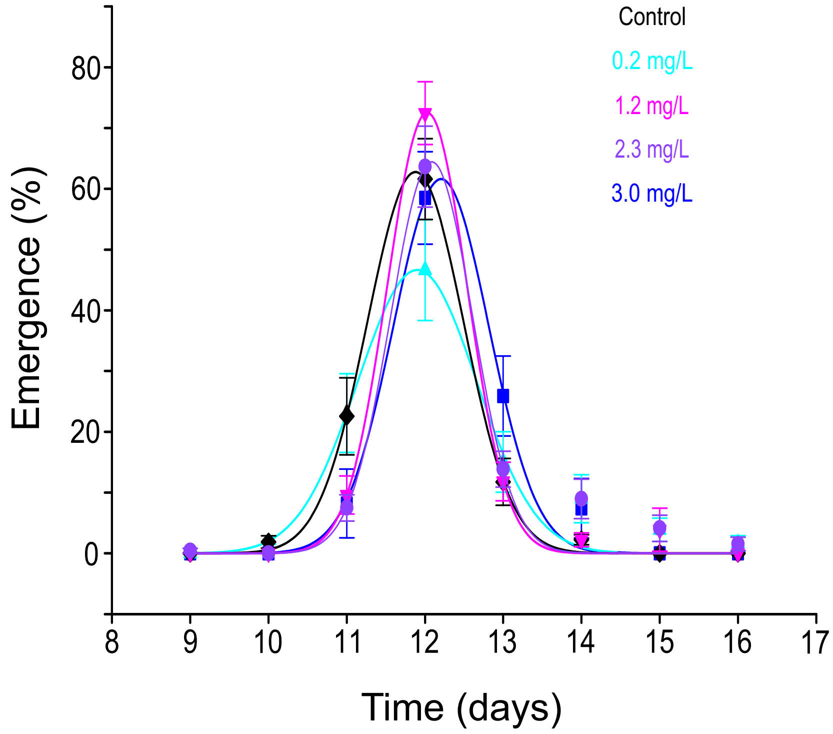

Figure 3.2: Daily emergence of *Drosophila suzuki*i flies that emerged from fruits of strawberry treated with increasing concentrations of ozone (0.2, 1.2, 2.3 and 3.0 mg/L). The symbols represent the mean observed data, and the lines represent the predicted trend of the best models selected.

3.4 Discussion

In this study, the use of ozone was proposed to be tested as an alternative way to control the egg phase of *D. suzukii* in fruits of strawberry by the exposure of six increasing concentrations of ozone to fruits previous infested with eggs of SWD. From these fruits it was possible to notice that even the higher concentration of ozone (*i.e.* 3 mg/L) used was not enough to control *D. suzukii* when in egg phase. Nevertheless, the average time of emergence was affected, causing a delay in the emergence for insects whose eggs were exposed to 3 and 1.2 mg/L. For the treatment of 0.2 mg/L the emergency occurred more distributed in time if compared with control treatment.

The toxicity of a substance used as an insecticide is related to the insect's ability to trigger biochemical defense mechanisms and these defenses may be dependent of high metabolic activity of the insect (Hostetler et al. 1994; Harak et al. 1999; Guedes et al. 2006). In this way, high body mass can be used as an indicator of high energy reserves that will result in rapid mobilization and response to detoxification caused for example by insecticide activity (Guedes et al. 2006). Therefore, the weight of the insects was measured one day after emergence to evaluate the impact of the ozone toxicity to the physiology of the *D. suzukii*, but the treatment had no effect in the mean insect body mass. Similar results were found by Sousa et al. (2008), where the conclusions go against the expected since it was not noticed a relation with weight and capacity to mobilize energy reserves for protection against ozone when compared to control treatment.

The effectiveness of exposure of ozone is highly dependent of the combination of an ideal concentration of ozone and an appropriate amount of time (Pandiselvam et al. 2019; McDonough et al. 2011). Boonkorn et al. (2012) used ozone fumigation to delay disease development in fruit of tangerine. Despite the experiment was realized to control a microorganism, they highlighted that the impact of the treatment was dependent of duration of exposure. The fumigation with ozone was made for 0, 2, 4 and 6 hours and these treatments led to incidence of disease of 83.33, 80.0, 10.0, and 10.0 % respectively (Boonkorn et al. 2012). Also, Amoah and Mahroof (2018), reached similar conclusions when they exposed *Lasioderma serricorne* into two situations: first the exposure was made to 100 - 400 ppm ozone for 1 hour and second exposure to 100 ppm ozone for 1 - 6 hours. They showed that to achieve greater toxicity effects, the duration of exposure is more relevant than the use of higher concentrations. These experiments reinforce the argumentation about our results where it is speculated that the exposure of strawberry fruits to ozone for higher periods of time would be more efficient than increase the concentration maintaining a short period of exposure.

McDonough et al. (2011), realized an experiment where they tested the susceptibility of all phases of *Tribolium castaneum* and *Plodia interpunctella* to high concentrations of ozone. The insects were treated with six ozone concentrations between 50 and 1800 ppm and as a result they showed that the most ozone-tolerant stages were eggs and pupae of *T. castaneum* and eggs of *P. interpunctella*, requiring 180 min of exposure at 1800 ppm ozone to achieve 100% mortality. Işikber and Öztekin (2009) also observed in their experiments, more tolerance of egg phase to ozone exposure if compared to sensitivity of larvae and pupae of *Ephestia kuehniella* and *Tribolium confusum*. A speculation was done about the composition of the eggs and pupae providing a physical barrier to ozone penetration (McDonough et al. 2011). The mode of action of a toxic gas will depend of the gas exchange system of insects. In this way, the efficacy of the control is related to amount of gas uptake by the insect by its respirations rate (Cotton 1932). So, if the ozone insecticidal activity depends of respiratory system, higher mobility of the insect would lead to higher respiration rates (Tiwari et al. 2010; Amoah and Mahroof 2018). Therefore, an explanation of emergence of the adults in our experiment can be result of lower respiration rate of egg in this life phase (Hoback and Stanley, 2001) what made difficult the action of ozone treatment.

As a conclusion, in this study it was possible to demostrate that ozone was not capable to control the egg phase of *D. suzukii* in the conditions used in this experiments. So, for future considerations further studies are nedded for a more efficient exposure methodology.

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General conclusions

In all the new potential hosts tested in these experiment, the oviposition of *Drosophila suzukii* occured on the surface of the species, but only in the fruits of Barbados cherry the fly could successfully oviposit, develop and complete their life cycle. Also, the field occurence of the *D. suzukii* in this new host was confirmed. When compared with already known hosts, the Barbados cherry showed that biological traits such as development time, emergence rates and sex-ratio differed from flies that developed in strawberry fruits.

Great care must be taken to extrapolate the interpretation of laboratory experiments to field conditions once that lab rearing condition can expose the insect to stress conditions. As showed in our studies, the effect of density can affect the biology of the pest *D. suzukii,* acting in its survival and fecundity.

Finally, the combinations of ozone concentration and time used in this study were not enough to control the egg phase of *D. suzukii*. New methodologies need to be prospected to evaluate the toxicity of this gas to other phases of development of the fly.