

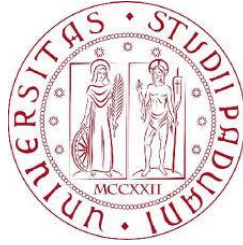
Università degli Studi di Padova

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Interactions between temperature and environmental variables in *Drosophila suzukii*



PhD degree in Crop Science



University of Padova

**Department of Food, Natural Resources, Animals and Environment
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Cycle XXIX**

Interactions between temperature and environmental variables in *Drosophila suzukii*

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Padova, 2nd October 2017

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Table of Contents

Abstract.....	vi
Riassunto.....	vii

Chapter 1: Introduction

Invasive species and <i>Drosophila suzukii</i>	9
Identification and life history.....	11
Trend of <i>Drosophila suzukii</i> with relation to Temperature and <i>Wolbachia</i>	14
Pest status and impacts on fruit production.....	18
Management.....	19
Aims of the work.....	22

Chapter 2: Combined effect of temperature and *Wolbachia* infection on the fitness of *Drosophila suzukii*

Abstract.....	24
Introduction.....	25
Material and Methods.....	27
Results.....	30
Discussion.....	32

Chapter 3: Temperature alters the response to insecticides in *Drosophila suzukii*

Abstract.....	43
Introduction.....	43
Material and Methods.....	45
Results.....	47
Discussion.....	49

Chapter 4

Effect of short cold temperature treatment on *Drosophila suzukii* fruit damage

Abstract.....	61
Introduction.....	61

Material and Methods.....	63
Results.....	65
Discussion.....	65
 Chapter 5:	
Conclusions.....	73
 Chapter 6:	
References.....	76
 Acknowledgements.....	98

Abstract

Drosophila suzukii Matsumura (Diptera: Drosophilidae) is an invasive species that became a pest of soft and stone fruits since its appearance in Southern Europe in 2008. Within *Drosophila* species, *D. suzukii* is the main cause of economic damage in fruit production. This is because the female, thanks to a serrated type of ovipositor, lays eggs in ripening fruit of many plant species, and the larvae rapidly consume the fruit. The PhD programme is aimed at providing concrete answers to farmers regarding the response of *D. suzukii* to temperature, in order to improve the IPM strategy against this pest. The study is based on the performance of *D. suzukii* with respect to *Wolbachia* infection, pesticides efficacy, and fruit infestation under variable temperature.

The dissertation consists of four parts.

The first part (chapter 1) introduces the background knowledge on *D. suzukii*, in relation to its spread, biology, pest status, and management options.

The second part (chapter 2) assesses the impact of temperature and *Wolbachia* infection on the fitness of *D. suzukii*, with special attention to nutritional stress, survival, fecundity, cytoplasmic incompatibility and viability.

The third part (chapter 3) investigates the effect of temperature on the efficacy of four groups of pesticides on the mortality of *D. suzukii* and its performance on different fruits under field and laboratory conditions.

The fourth part (chapter 4) examines the influence of short cold temperature stress on the development of *D. suzukii* in different fruits.

All together, the findings provide information for the improvement of the management of *D. suzukii* in fruit orchards of north-eastern Italy, emphasizing the role of some environmental variables such as temperature.

Riassunto

Drosophila suzukii Matsumura (Diptera: Drosophilidae) è una specie invasiva che ha rapidamente colonizzato piccoli frutti coltivati e selvatici in Europa meridionale dopo la sua introduzione nel 2008. Essa rappresenta la specie di drosofila con il maggiore impatto sulla produzione di frutta, in particolare per la possibilità, conferita da un particolare ovopositore di tipo serrato, di deporre uova in frutti in via di maturazione. In seguito le larve utilizzano la polpa del frutto rendendolo invendibile. Il programma del dottorato è mirato all'individuazione di strategie di gestione dell'insetto basate sulla comprensione della sua risposta a varie condizioni ambientali, con specifico riferimento alla temperatura. La tesi si compone di quattro parti.

La prima parte è costituita da una introduzione generale sulla specie, in relazione alla dispersione, allo sviluppo, e alle tecniche di contenimento.

La seconda parte è volta a comprendere l'effetto della temperatura sull'infezione dell'insetto da parte del batterio *Wolbachia*, con particolare riferimento all'effetto di stress nutrizionali e termici sulla performance e sull'incompatibilità citoplasmatica.

La terza parte è diretta allo studio delle relazioni tra quattro gruppi di molecole insetticide e la temperatura, valutando la mortalità dell'insetto in condizioni diverse di campo e di laboratorio.

La quarta parte è indirizzata a mettere in evidenza gli effetti di brevi esposizioni a basse temperature sull'attacco di piccoli frutti.

Complessivamente il lavoro fornisce informazioni su come la temperatura svolga un ruolo fondamentale nello sviluppo dell'insetto e nelle interazioni con altri fattori che ne possono regolare la riproduzione, sia naturali sia antropici. Alcune di queste risultano essere importanti per l'applicazione della lotta nei confronti di questo temibile parassita.

Chapter 1



Introduction

Species that have been introduced outside their native range by the direct or indirect intervention of humans are known as “alien species” (DAISIE, 2009; Kirkendall and Faccoli, 2010). Invasive alien species (IAS) are in particular one of the leading threats to native wildlife, human health and food safety, with associated economic impacts estimated in hundreds of US\$ billion (Pysek and Richardson, 2010). A first key step in the comprehension and management of biological invasions is to understand the pathways of introduction, the spreading and the spatio-temporal dynamics of colonization. In particular, the identification of those invaded areas that were colonized first, or more likely allowed the first successful establishment of the IAS, is needed to both prevent recurrent introductions of the same or other IAS and to identify possible idiosyncratic features that facilitated the invasion (Cini *et al.*, 2014).

The knowledge of the invasion processes is truly needed for management purposes of invasive species (Kenis *et al.*, 2007) as well as for conservation issues. Projects such as DAISIE (Delivering Alien Invasive Species Inventories for Europe) have investigated the European “aliodiversity” and developed strategies to face biological invasions (Genovesi *et al.*, 2004). It has been shown that over 90% of alien terrestrial invertebrates in Europe are arthropods, with the large majority being insects (Roques *et al.* 2009). This dominance is due to the high species richness of the class Insecta, as well as to the numerical abundance of individuals, the small body size, and some characteristics of their biology, including short generation time, flexible life-cycle patterns, and feeding habits, combined with their wide adaptability and strong association with humans. It has been also acknowledged that numerous IAS can disrupt the structure and functioning of ecosystems and largely contribute to biodiversity loss worldwide (Kenis *et al.*, 2009; Kenis and Branco, 2010).

Since the last few years, a new IAS dramatically threatening agriculture in European countries is *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) (also known as Spotted Wing Drosophila, SWD), a highly polyphagous pest which mainly infests thin-skinned fruits (Cini *et al.*, 2012). Thanks to its serrated ovipositor, SWD lays eggs in healthy, unwounded fruits (Sasaki and Sato, 1995) and larval feeding and development on fruit flesh make fruits unmarketable (Rota-Stabelli *et al.*, 2013). The impact of SWD on fruit production is therefore enormous, exacerbated by the high number of generations per year, the high fecundity of females

(Mitsui *et al.*, 2006; Walsh *et al.*, 2011) and by the possible secondary damages caused by other insects, fungi and bacteria after SWD attack (Goodhue *et al.*, 2011). The first known damage to commercial small fruits in Europe was found in Italy, Trento Province, during 2009 (Grassi *et al.*, 2009). Characterized by a high dispersal rate, a wide host range, an ecological pre-adaption to temperate climates and a deep impact, the invasion of this pest has a few precedents (Rota-Stabelli *et al.*, 2013; Ometto *et al.*, 2013), and SWD is quickly becoming a model for research on invasion biology and pest management (Dreves, 2011).

History of *Drosophila suzukii*

Drosophila suzukii was firstly described in 1916 originated as maggots from cherry and later named Cherry Drosophila in Japan. Since 1930, the great influence of *D. suzukii* was reported in the production of fruit in the region of Japan and also in safe grape varieties (Kanzawa, 1935). Subsequently, it was considered as an economic and invasive pest for small and stone fruits. In 1970, dispersal of *D. suzukii* occurred in the whole Asia (Delfinado and Hardy, 1977), and in 1980 in the Hawaii (Kaneshiro, 1983). In North America it was recorded in 2008 on raspberries in California and in 2009 in Oregon, Washington, British Columbia and Florida, while spreading through other states in following years (Hauser *et al.*, 2009); recently was found in south America as well, where it could have been introduced earlier (Deprá *et al.*, 2014). Incursion was also reported in Veracruz, Mexico during the survey in 2014 (Lasa and Tadeo, 2015). In 2014 to 2015, economy of Brasil suffered great damage to strawberry and damage continued to increase with the arrival of another drosophilid, *Zaprionus indianus* (Coquillett) (Diptera: Drosophilidae) (Bernardi *et al.*, 2016). Approximately all countries in the United States have noticed the incidence of *D. suzukii*, but not all observed crop damage (National Agricultural Pest Information System [NAPIS] 2013). Europe has not been secured to this fly and perceived its existence by 2008 in Spain and France. It was recorded in 2009 in the areas of north-eastern Italy (Trentino) (Cini *et al.*, 2012) and in Veneto official reporting in 2010 with severe damage (Griffo *et al.*, 2012). Since 2011, *D. suzukii* has spread all over the other European countries (eppo.int, 2017) (Fig 1.1).

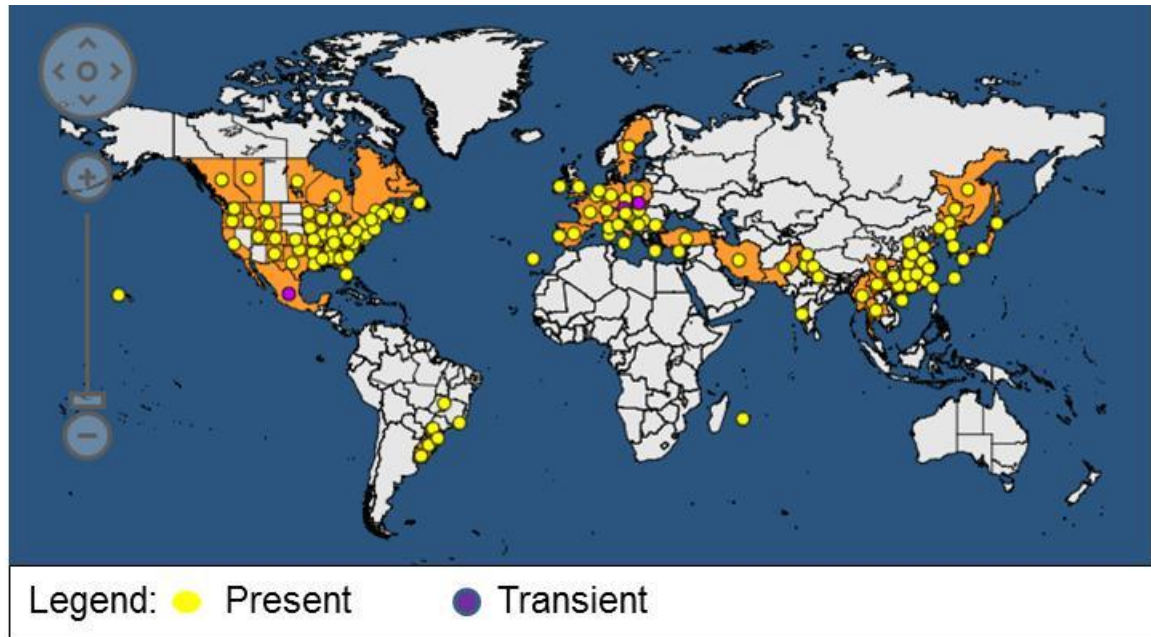


Fig 1.1- Current occurrence of *Drosophila suzukii* in the world (eppo.int, 2017)

Identification features

Two foremost characteristic features are usually considered to distinguish SWD from other drosophilids. First, one black spot on the angle of the leading edge of male *D. suzukii* wing and two sex combs organized on the segments of first and second tarsi; second, females have a modified form of ovipositor that is creased with sclerotized teeth, causing in a serrated edge that permits them to lay eggs inside fruit (Walsh *et al.*, 2011; Cini *et al.*, 2012; Fig 1.2). Body length is about 2.5 mm with dark, yellowish brown color (Kanzawa 1935). Disparities in look can be periodic with a large and dark phenotype seeming as temperature and photoperiod drop. Difference in fruit diets could be the reason in size variations (Kanzawa 1935).

Eggs of *D. suzukii* are milky white, oblong in shape with two projections string like which obtrude out from the fruit. Overall, eggs' width is about 0.5 mm and length of projections nearby 0.4 mm.

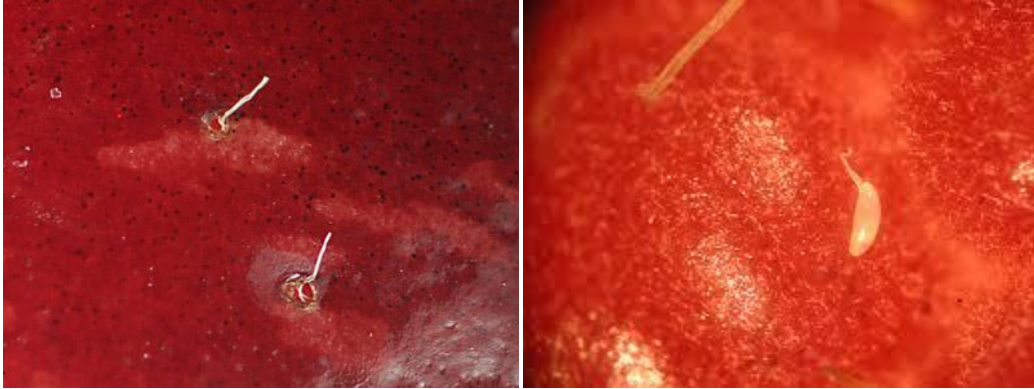


Fig 1.2- Eggs on cherry and raspberry (Photo by Hannah Burrack and Peter J Jentsch)

Small white larvae experience three ages until they are 5.5 mm long and 0.8 mm wide. The larvae have black shawls, as in the fruit fly found inward bending. Larvae feed in the fruit flesh and cause economic damage (Fig 1.3).



Fig 1.3- Larvae (Photo by Joseph Moisan-De Serres)

Oblong shape, red brown color pupae are 3.5 mm long and 1.2 mm wide, with two horns like protrusions that distribute into 7 to 8 tipped stars at the distal side (Fig 1.4). Protrusions are on the posterior end of the pupal case and overlapping with the respiratory organs of the larvae on the posterior side (Kanzawa, 1935).



Fig 1.4- Pupae (Photo by E Beers)

Life history

Flies have high reproductive potential. In the optimal environments, the life cycle can be as short as two weeks (Tochen *et al.*, 2014), and the fertility about 600 eggs per female with an average of 400 eggs (Kanzawa, 1939; Mitsui *et al.*, 2006; Walsh *et al.*, 2011). Oviposition rate can be more than 25 eggs per day, liable to the temperature (Kinjo *et al.*, 2014) (Fig 1.5).

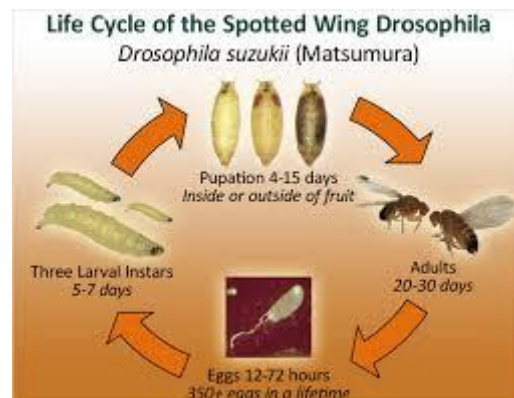


Fig 1.5- Life cycle of *D. suzukii* (Photo by Washington State University)

As with other insects, the developmental rate is totally dependent on temperature and the entire duration from eggs to adult is 10 to 79 days (Kanzawa, 1939; Lee *et al.*, 2011a; Tochen *et al.*, 2014). According to weather conditions, total 13 generations can be observed in a year (Kanzawa, 1939; Tochen *et al.*, 2014). Short generation time plus great reproductive potential lead to rapid population growth and put high force through crop-ripening season (Wiman *et al.*, 2014). Cherry at 22°C records the peak net reproduction and allows the highest intrinsic rate of population growth (Tochen *et al.*, 2014).

The life cycle from egg hatching to adult emergence ranges from about 9-10 days to 21-25 days at 25°C and 15°C, respectively (Kanzawa, 1939). Recent laboratory observations recorded the development from egg to egg laying female as ranging from about a week to 12-15 days at 21.1°C and at 18.3°C, respectively (Walsh *et al.*, 2011). Some adults (males and females) overwinter under extended suboptimal cold conditions (Dalton *et al.*, 2011). The lifespan of overwintering adults is considerably longer than non-overwintering adults and many survive until next May or June (Kanzawa, 1939). Females are more likely to overwinter than males. *D. suzukii* becomes moveable above 5°C, and if the average temperature rises beyond 10°C it starts to become energetic. If the temperature is high enough during the day, *D. suzukii* starts to oviposit. It is the most active between 20° and 25°C, but not very active when the temperature reaches 30°C (Kanzawa, 1939; Hamby *et al.*, 2013). Hamby *et al.* (2013) reported dawn and dusk as the most active periods.

Trend of *D. suzukii* seasonal phenology under fluctuating temperature

Temperature as key factor is playing a crucial role on the population dynamics of *D. suzukii* (Tochen *et al.*, 2014; Shearer *et al.*, 2016) and the access of vital food resources (Kimura, 2004; Mitsui *et al.*, 2006, 2007; Lee *et al.*, 2015). Under normal circumstances, *Drosophila* experiences cold shock before they are overcooled. For instance, *Drosophila* produces cold shock at -7°C for 1.5 h (Chen and Walker, 1994). Chill coma usually happens at constant 0°C and recovery is promising but highly movable trait (Gibert *et al.*, 2001). In general, it is difficult to make any sweep statements greeted the cold resistance of *Drosophila* because it is extremely flexible. This may indicate that *Drosophila* has some substitute for overwintering approaches and cannot be concluded separately from their cold resistance studies. Crowd of population is less during beginning of the growing season continues to increase throughout the season and at the peak stage during harvest (Sakai, 2005) and these fluctuating trends are determined by the recently established model from the work of Wiman *et al.* (2014), which helps to explain the *D. suzukii* seasonal phenology under different climatic conditions. *D. suzukii* does not seem to be able to survive at extreme low temperatures for a long time (Dalton *et al.*, 2011). Kanzawa (1939) also observed the growth and generation duration of *D. suzukii*. Though, this research has been conducted to the surrounding temperature and humidity as well as other temperature regimes. Sakai (2005) explored the work by finding reduction in the developmental time of *D. suzukii* as

the temperature increases to 25°C. Oviposition was not noticed above 32°C and below 10°C (Tonina et al. 2016). *D. suzukii* was expected to be moderately cold impatient as associated to other species of *Drosophila* (Mitsui et al., 2010). *D. suzukii* is predicted to have a lower thermal threshold level of around 5.98°C, and upper thermal threshold at around 29°C with developmental rate ping to zero at around 31.5°C. The temperature based mortality ratio of *D. suzukii* can be assessed from the survival rate of earlier issued numbers by Dalton et al. (2011) used for adults from -2 to 10°C, and numbers from Kinjo et al. (2014). Considerable deviation in upper limits of thermal amongst species negatively connected through annual precipitation by the dominant point of their spreading and with the contact between precipitation and extreme temperature, displaying that resistance of heat is a significant cause of scattering of *Drosophila* species (Kellermann et al., 2012). Tochen et al. (2014) also estimated in the range of 10-30°C as the upper limit of the range is much higher. It has also been suggested that the elderly population of the SWD may be the better flames to keep the susceptible fruit more stressful than other drosophilids (Tochen et al., 2014; Wiman et al., 2014). Various other temperature-base studies were also conducted by considering all stages of *D. suzukii* on development, survival and reproduction (Emiljanowicz et al., 2014; Hardin et al., 2015; Jaramillo et al., 2015). Asplen et al. (2015) used *D. suzukii* physiological records, including nonlinear sub-models to catch temperature-based development and survival rates. *D. suzukii* is also considered as a chill intolerant while morph of winter adult is highly cold-tolerant stage of life (Stephens et al., 2015a). Enhanced stress of freeze tolerance was also assessed after fast cold hardening or acclimation in numerous species of *Drosophila* sp. including *D. suzukii* (Bauerfeind et al., 2014; Jakobs et al., 2015; Stephens, 2015b). In many reported findings, survival under cold conditions was measured by succumbing insects one or the other in a single lower temperature with altered intervals (Jakobs et al., 2015; Toxopeus et al., 2016) and to a chains of lower temperatures having single period of exposure (Kimura, 2004; Ryan et al., 2016; Wallingford and Loeb, 2016). Generally, existence of *D. suzukii* in heat and cold environments based on the intensity of stress and length and methodologically used approaches which totally depend on thermal tolerance sites (Enriquez and Colinet, 2016). Tonina et al. (2016) also proved that *D. suzukii* behaved well at low temperature in the mountains, where it exploits a large number of native hosts.

Impact of *Wolbachia* on life history under fluctuating temperature

Symbionts can significantly change the behavior of the host. Such behavioral amendments can improve the spread and transmission of symbionts, while also influencing the fitness and life biology of their hosts (Hoffman and Truelli, 1997; Hughes *et al.*, 2012). *Wolbachia* is a particularly common symbiont of arthropods, infecting two-thirds of the species of insects (Hilgenboecker *et al.*, 2008). Endo-symbionts are transferred vertically from the mothers to the offspring and greatly manipulated reproduction in various ways according to the species of host (Werren, 1997; Miller *et al.*, 2010). *Wolbachia* can also alter the sex ratio by feminization of males (Moreau *et al.*, 2001; Engelstädter and Hurst, 2009), killing of males during growth (Randerson *et al.*, 2000; Sullivan and Jaenike, 2006), or induced female parthenogenesis (Schilthuizen and Stouthamer, 1997; Werren *et al.*, 2008). As *Wolbachia* is only spread by females, these reproductive manipulations are used to increase the spread of *Wolbachia* in the host population. While hundreds of articles on *Wolbachia* have seemed over the past decade, few have been evaluated regarding interaction of host and *Wolbachia*. For less than 20 species, estimates relevant to the contamination frequency of numerous population, exploring transmission proficiency in the nature, reproductive influences and other phenotypic analysis which may elucidate the *Wolbachia* perseverance and occurrence (Chrostek *et al.*, 2014).

In addition to the genetic effects, environmental aspects can also put a great impact on the symbionts. Among these aspects, the temperature is particularly essential in the ectotherms, and its impact on the interaction of the host and symbionts in common, as well as in the special *Wolbachia* symbiosis which has been noted earlier (Thomas and Blanford, 2003; Mouton *et al.*, 2006, 2007). The grouping of genetic and environmental issues can cause to difficult interaction of genotype and environment as described in other symbiotic schemes (Greub *et al.*, 2003). The performance of *Wolbachia* under different temperature conditions of was poorly verified (Clancy and Hoffman, 1998; Hurst *et al.*, 2000), and performance of each *Wolbachia* strain was not studied in several hosts of infected species to environmental conditions. Though, it is well known that symbiosis collapse at both elevated and low temperatures (Stouthamer *et al.*, 1990; Perrot-Minnot *et al.*, 1996; Johanowicz and Hoy, 1998; Van Opijnen and Breeuwer, 1999) and that heat decreases the transfer power of *Wolbachia* (Hurst *et al.*, 2001), demonstrating that temperature has a significant influence in symbiotic population. One reference to this research is that curative temperatures differ among species of host. Furthermore, no literature is available on how

temperature impacts the host reproduction having *Wolbachia*, in spite of abundant studies emphasizing the significance of *Wolbachia* for the appearance of Cytoplasmic Incompatibility (Boyle *et al.*, 1993; Breeuwer and Werren, 1993; Sinkins *et al.*, 1995; Bourtzis *et al.*, 1996, 1998; Poinso *et al.*, 1998; Noda *et al.*, 2001; Ikeda *et al.*, 2003; Veneti *et al.*, 2003).

Impact of nutritional stress

The worth of dissimilar hosts for the development of larvae is flexible and combined effect of host plants and quality of diet upsets the time of development, life span, size and fecund power (Krainacker *et al.*, 1987; Kaspi *et al.*, 2002), as already explained in *D. suzukii* by Bellamy *et al.* (2013). The capability of flies to manage with the stress of nutrition executed via low protein hosts seems to be facilitated through bacterial symbionts (Jurkevitch, 2010). Fitness of flies is improved by the existence of microbes in the gut when the quality of diet is poor (Ben-Yosef *et al.*, 2010). Positioned to poor protein food is estimated to outcome in evolutionary cooperation as nutritional situations of larvae disturb life history of adults (Boggs and Freeman, 2005; Hahn, 2005; Boggs, 2009; Colasurdo *et al.*, 2009). Nutritional protein in growth is identified as a vital component in traits of life history of adults. Poor protein accessibility during insect development is linked to reduce the fitness through series of traits for instance short body size, less growth rate and fecundity suppressed (Sentinella *et al.*, 2013; Tu and Tatar, 2003). Jaramillo *et al.* (2015) also assessed parameters of life history on different types of diets in *D. suzukii* and found fluctuation in development time, through the blueberry-feeding developing as adults earlier than the artificial media, while other related fitness methods do not differ concerning the two diets. The most noticeable approach by which fluctuating environment may have strong influence on the situations of body is by nutritional effects causing from changeability in kind of food and its accessibility (Sisodia and Singh, 2012). Overall, diet influence can be categorized such as quantitative (access of food) or qualitative (composition of food). The quantitative impacts are apparent as animals get energy and additional nutritional necessities from food. Therefore, in a normal range of settings there is a possibility of positive relation in both food accessibility and body situation. Qualitative influences frequently are alienated into two group's namely nutritional lacks and inhibitory metabolites.

Pest status and impacts on fruit production

The damage can be direct and indirect. Internal feeding of larvae is the main source of direct damage resulting to atrophy of tissues of fruit. Besides, the oviposition of SWD exposes fruit to secondary infection by pathogens (Cini *et al.*, 2012; Hamby *et al.*, 2012; Ioriatti *et al.*, 2015). SWD fruit degradation can increase its susceptibility to other species of *Drosophila* (Walsh *et al.*, 2011). Destruction produced by *D. suzukii* larvae makes the fruit unmarketable (Bolda *et al.*, 2010). In Japan's SWD's economic losses are determined to cherries (Kanzawa, 1939; Yamakawa and Watanabe, 1991; Sasaki and Sato, 1995), while recently to blueberries (Shimizu, 2004; Kawase *et al.*, 2008). An economic impact assessment is relatively scarce, with the most emphasis on California (Bolda *et al.*, 2010; Goodhue *et al.*, 2011) or the European Trentino region (De Ros *et al.*, 2013). In 2008, economic losses in California, Oregon and Washington State (based on the largest reported loss of production) were assessed around 40% in blueberries, 50% in raspberry, 33% in cherries and 20% in strawberries. The production of these three states can maintain a loss of 511 million dollar per year due to *D. suzukii* (Bolda *et al.*, 2010). It is estimated that 37% of raspberries are reduced and 20% from managed strawberries due to the lack of management in California (Goodhue *et al.*, 2011). In 2009 late autumn, Washington and Oregon bared 20 to 40% losses in blueberries and caneberries (Gerdeman and Tanigoshi, 2011). SWD has grown in numerous small fruit crops throughout southern Europe to cause serious yield losses to sweet cherries, strawberries, raspberries, blackberries and blueberries. It is reported that there are extreme damage in northern Italy (Trentino) and France to grape vines, strawberries and sweet cherries is recorded up to 100% (Cini *et al.*, 2012; Weydert and Mandrin, 2013) (Fig 1.6). In France, it has also been documented in apples and peaches, although without economically significance (Weydert and Mandelin, 2013). De Ros *et al.* (2013) presented the first assessment of the impact of the European economy, although the work focused only on the region of Trento, Italy. It is also estimated that 400 hectares of soft fruit production area confronted losses 0.5 to 3 million euro in 2010-2011.



Fig 1.6- Damage on different fruits (Photo by A. Grassi)

Management

Monitoring

Kanzawa (1935) started initial investigation of *D. suzukii* during 1930. He captured flies with a mixture of various types including fruit wines, molasses, rice wines as well as acetic acid. The wine solution of rice and honey in a plastic bottle with a net was subsequently established and practiced to spot the incidence of flies (Sakai, 2005). Response of *D. suzukii* was also approved by Landolt et al. (2015) to the solution of vinegar and wine, indicating that the mixture of these two is more attractive than the single component. The mixture of volatile compounds (acetic acid, ethanol, acetoin and methionol) has also been recognized as attractive agent to flies in the field (Cha *et al.*, 2012). Traps with red and yellow color, high bait surface area (90 cm²) and the side entrance traps trapped more flies than traps with matching parts (Lee *et al.*, 2013). The monitoring of the presence of larvae in the fruit can be achieved by immersing the crushed fruit in a saline solution or a sugar aqueous solution, but the method is inefficient and time consuming and there is no known current level of sampling error (Wiman *et al.*, 2014). Further approaches have been discovered by sieves or syrups of boiled fruits to achieve altered successes. By using PCR-RFLP, molecular detection can now find all stages of *D.suzukii*, abolishing the need to eradicate larvae (Kim *et al.*, 2014). Yeast and microorganisms also play an important role in determining the volatile profile of attractants (Hamby and Becher, 2016). *D. suzukii* traps also proved to be most suitable option in consideration of the high selectivity, the early detection ability, the good attractiveness and the long persistence, for instance, Droskidrink considered for the availability and high attractiveness (Tonina *et al.*, 2017).

Cultural control

Several cultural control techniques are currently applied in Asia, Europe and North America based on the type of crop. Sanitation is among the most important cultural methods to combat SWD around the world (Koppler, 2014; Shi, 2015). In the ripening season, sanitary measures including clearing ground covering vegetation, removal of dropped and over-ripe fruits have been suggested (Lee *et al.*, 2011; Shi, 2015). Larvae inside fruits have been killed by solarisation, especially used for berry crops but its hard to apply to stone fruits. Furthermore, the utilization of mulches cause reduction in standing water can contribute to the decline of humidity in fruit orchards (Hoashi-Erhardt and Bixby-Brosi, 2014). In China, soil tillage is recommended to spoil overwintering habitats of the fly in winter, while in spring, cherry trees is supposed to be covered with black plastic fabric around ground, preventing from overwintered adults climbing up to the canopy of tree (Shi and Wang, 2015). Besides sanitary measures, the use of nets covering fruit is an effective complementary method to physically exclude SWD. Particularly in cherry crops, nets are among the essential control measures around the world (Grassi and Pallaoro, 2012; Gamper, 2015; Cormier *et al.*, 2015). Bagging cherry clusters in the early fruit stage with white semitransparent paper bags has also been recommended in China (Shi and Wang, 2015).

Biological control

Biological agents are playing a vital and active role in the IPM program (Daane and Johnson, 2010; Biondi *et al.*, 2018). Several research groups have been working in order to find effective parasitoid species suitable for using in *D. suzukii* biocontrol programs. A lot of research have been displayed on both SWD-coevolved (Nomano *et al.*, 2015; Daane *et al.*, 2016) and fortuitous parasitoids of the newly invaded areas (Cancino *et al.*, 2015; Gabarra *et al.*, 2015; Miller *et al.*, 2015; Mazzetto *et al.*, 2016; Wang *et al.*, 2016a). Hymenoptera parasitoids have been also explored *D. suzukii* (Cini *et al.*, 2012). *Pachycrepoideus vindemiae* has been recognized as *D. suzukii*'s parasites in Italy and Oregon (Rossi-Stacconi *et al.*, 2013). In laboratory environments, the populations of two common pupal parasitoids from France and Spain are effective for SWD (Chabert *et al.*, 2012; Gabarra *et al.*, 2015). Currently, the pupal parasitoid *Trichopria drosophilae* seems to be vital for implementing augmentative biocontrol programs against SWD.

In fact, this parasitoid has recently been shown to coexist with other SWD parasitoids (Daane *et al.*, 2016; Wang *et al.*, 2016c). *T. drosophilae* has the ability to parasitize *D. suzukii* on a wide temperature condition range, spanning from 15°C up to 30°C (Rossi Stacconi *et al.*, 2016). Moreover, this seems to be abundant in natural and semi-natural areas (Knöll *et al.*, 2017). In addition, the insect pathogenic fungus strain *Isaria fumosorosea* has the ability to offer 85% mortality in *D. suzukii* in laboratory (Naranjo-Lázaro *et al.*, 2014). In the case of sterile insect technology, preliminary work involving the genetically improved *D. suzukii* adults will result in female mortality during nurturing and male sterility (Schetelig and Handler, 2013). About predators, several species have been observed feeding on SWD juvenile stages (Woltz *et al.*, 2015) and their substantial contribute to biocontrol has been recently quantified by Woltz and Lee (2017). Trophic actions of several predators were also exposed by Cuthbertson *et al.* (2014).

Chemical control

Chemical control program is generally used and more formulations are available to growers in the fruit growing regions such as spinosyns, organophosphates, pyrethroids and neonicotinoids (Haye *et al.*, 2016). At present, public information on the level or extent of pesticide resistance in SWD is very limited. More information is available regarding *D. melanogaster* (Meigen) that has developed resistance to insecticides (Remnant *et al.*, 2014; Wan *et al.*, 2014). Hamby *et al.* (2013) observed that activity of detoxification gene in the SWD has a daily rhythm pattern while baseline testing of most important pesticide categories was done by Whitener and Beers (2015). Findings in Europe and the US also displayed good fallouts for organophosphate products or nonstop uses by pyrethrins and spinosyns (Beers *et al.*, 2011; Bruck *et al.*, 2011; Van Timmeren and Isaacs, 2013). Neonicotinoids have been used to a limited extent in control strategies because they are perceived to be less effective (Bruck *et al.*, 2011), and, if they are used in foliar sprays, are anticipated to have broad-spectrum effects and negative impacts to beneficial arthropods (He *et al.* 2012). Spinosyns proved to be an effective insecticide against SWD (Bruck *et al.*, 2011; Haviland and Beers, 2012; Zhang *et al.*, 2015). Spinetoram and dimethoate have also been screened for efficacy in Italian cherry orchards (Profaizer *et al.*, 2015). Insecticide screening trials by Cuthbertson *et al.* (2014) also confirmed the high efficacy of spinosad and chlorantraniliprole against SWD. Within the United States, a total of 18 insecticides are listed for use on blueberry, caneberry, strawberry, grape and stone fruit (Fruit Advisor, 2015). Cowles *et*

al. (2015) demonstrated that the addition of sucrose as a phagostimulant improved the activity of several insecticides to target SWD adults and as a result increased protection of fruit from infestation.

Aims of the work

The aim of the present PhD thesis was to assess the role of environmental variables, and in particular temperature, on the relationships between SWD, its *Wolbachia* symbiont, and the pest management. The results may provide concrete answers to farmers regarding the action to take against the pest, in the perspective to improve the IPM strategy. A first objective of this study is to elucidate whether temperature and *Wolbachia* infection affected the fitness of *D. suzukii*. A second objective is to understand how temperature stress modifies the response of different insecticides used against *D. suzukii*. A third objective is to test the effects of a short exposure to low temperature on the development of *D. suzukii* and decay of infested fruits.

Chapter 2

Combined effect of temperature and *Wolbachia* infection on the fitness of *Drosophila suzukii*

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Combined effect of temperature and *Wolbachia* infection on the fitness of *Drosophila suzukii*

Abstract

Maternally inherited *Wolbachia* is prevalent among insects and is often linked with modifications of host fitness. The result and evolution of host symbiont interaction depend on environmental limits but are difficult to predict since they arise from complex interactions among host, parasite and environment. In our study, we evaluated whether fitness traits of *Wolbachia*-free and *Wolbachia*-infected individuals of *Drosophila suzukii* (Matsumura) (Diptera Drosophilidae) were affected when flies were exposed to temperature stress (29°C versus 22°C) as well as to nutritional and starvation stress. Two lines of *D. suzukii* were prepared for the experiments: a *Wolbachia*-free line, treated with antibiotics, and a *Wolbachia*-infected line, hosting the endosymbiont. Survival and fecundity of the two lines were assessed as well as the effect of nutritional stress on developmental time (days) and starvation stress on survival (hours). Our results showed that fecundity differed between temperatures and infection status of flies. No difference was found in egg hatch rates for crosses involving *Wolbachia*-free females at 22°C while there was a noticeable variation in hatch rates due to change in fertility at 29°C. Interestingly, at 29°C, *Wolbachia*-free flies survived longer than *Wolbachia*-infected flies while no variation was observed for *Wolbachia*-free and *Wolbachia*-infected flies regarding nutritional and starvation stress, except for temperature impact on survival (*Wolbachia*-infected flies had shortened survival compared to *Wolbachia*-free) and time of development (difference between males and females). It is concluded that the combined effect of high temperature and *Wolbachia* may be useful to understand the performance of *D. suzukii*-*Wolbachia*-host-symbiont interaction in nature.

Key words: temperature, nutrition, cytoplasmic incompatibility, survival, performance

Introduction

Drosophila suzukii (Matsumura) (Diptera Drosophilidae) is an invasive species that threatens soft fruit industries in North America and Europe (Cini *et al.*, 2014; Walsh *et al.*, 2011) through feeding on unripe and undamaged fruits (Lee *et al.*, 2011; Atallah *et al.*, 2014). It has become the 8th pest of this genus introduced into Europe and is considered a highly damaging pest of agricultural areas (Asplen *et al.*, 2015), as it attacks various ornamental and wild fruits (Poyet *et al.*, 2015). The species is spreading rapidly, becoming a significant pest and a cause for concern to fruit handling industries, but existing traditional control methods (chemical, cultural, monitoring) have been neither sustainable nor efficient. Combined bio-control strategies building on knowledge of the pest ecology and biology might provide an answer to the problem. Among these bio-control strategies (including entomopathogens, parasitoids, parasites), the endosymbiont *Wolbachia* might play an important role. *Wolbachia* (Rickettsiaceae) are obligate intracellular symbionts that have been recorded in the reproductive systems of 20 to 76% of insects (Hilgenboecker *et al.*, 2008; Zug and Hammerstein *et al.*, 2015) and transmitted from infected females to their offspring. Some benefits of *Wolbachia*-infection include increased fecundity, survival, nutritional supply (Zug and Hammerstein, 2015), protection against pathogens (Cattell *et al.*, 2016; Martinez *et al.*, 2017) and thermal tolerance (Chen *et al.*, 2000; Montllor *et al.*, 2002).

Wolbachia-infection also linked to a variety of reproductive abnormalities in the host (Carrington *et al.*, 2010; Guruprasad *et al.*, 2011). These abnormalities include male-killing (Hurst *et al.*, 1999), feminization (Rigaud *et al.*, 1991), parthenogenesis (Stouthamer *et al.*, 1990), and cytoplasmic incompatibility (CI) (Hoffmann *et al.*, 1990; Werren *et al.*, 2008). CI is a sperm-egg incompatibility that occurs in crosses involving a male that harbors at least one *Wolbachia* strain that the female lacks, all other crosses being fertile (Poinsot *et al.*, 2003).

The conservation of *Wolbachia* in wild populations is elucidated by positive impacts upon fitness traits for instance a longer survival of *Wolbachia*-infected flies (Fry and Rand, 2002). In the mosquito *Aedes albopictus* Skuse, *Wolbachia*-infected females survive longer, produce more eggs and have higher hatching rates than *Wolbachia*-free females (Dobson *et al.*, 2002). Unfavorable effects include a decrease in lifespan for the wMelPop strain in *Drosophila melanogaster* (Chrostek and Teixeira, 2015). This phenotype provides indirect fitness benefits to

Wolbachia infected females and allows *Wolbachia* to take over a host population (Turelli and Hoffmann, 1995).

Wolbachia was also found to be associated with *D. suzukii* by Cordaux *et al.* (2008), who first sequenced the *Wolbachia* genome and found that it was a distinctive strain, and by Siozios *et al.* (2013). These findings were confirmed by Mazzetto *et al.* (2015), who found mutualistic association between *Wolbachia* and *D. suzukii* through increased fecundity. A few later studies have documented some beneficial effects of *Wolbachia* (Tochen *et al.*, 2014; Mazzetto *et al.*, 2015) and its effects regarding protection against viruses in *D. suzukii* (Cattel *et al.*, 2016), whereas polymorphic infection in *D. suzukii* caused weak CI or reproductive manipulation (Hamm *et al.*, 2014).

The transmission efficiency of *Wolbachia* is reduced at high temperature (Hurst *et al.*, 2001), demonstrating that this has a main impact on its population. In Australian lines of *D. melanogaster*, the frequency of *Wolbachia* suggested that the endosymbiont endows fitness benefits that totally depend on the environment (Hoffmann *et al.*, 1994, 1998). Several findings have confirmed that temperature leads to major impacts on the effects of associations between host and microorganism remarkably by disturbing the virulence of microorganisms (Thomas and Blanford, 2003). This is why *Wolbachia* becomes the main cause of complete CI at extreme temperatures (Bordenstein and Bordenstein, 2011). Indeed, temperature effects on a wide variety of heritable symbionts have recently been studied by Corbin *et al.* (2017).

Furthermore, novel metabolic ways provided to hosts, such as when under nutrition (food media prepared with less yeast) and starvation stress (without food), have also been offered as a main route to endosymbiosis of bacteria (Douglas, 1994). Protein deficiency decreases fecundity and development in *D. melanogaster* (Wang, 1995). In contrast, diet limit or mild starvation can enhance longevity as well as tolerance to stressors such as heat (Wenzel, 2006; Smith *et al.*, 2007), indicating the complication of organismal nutrient acquirement and utilization.

As there is no indication that *Wolbachia* can induce strong reproductive effects, such as CI, in relation to temperature in European populations of *D. suzukii*, we explored whether *Wolbachia* provides temperature-linked fitness benefits. The effects of temperature on *Wolbachia*-free and *Wolbachia*-infected lines of *D. suzukii* were assessed by considering survival, fecundity, CI and

viability whereas nutritional and starvation stress was examined by observing developmental time (days) and survival (hours) under temperature stress for both lines.

Materials and methods

Insect culture

The *Wolbachia*-infected culture of *D. sukuzii* used for the study was obtained from Department DAFNAE-Entomology, from adults collected from cherries and strawberry organic orchards of Verona province, (North-Eastern Italy) in the autumn 2014. The flies were nurtured in plastic vials (Falcon type of 50 ml capacity, 30 mm diameter, 115 mm length) with *D. sukuzii* specific rearing medium prepared according to Bloomington Drosophila Stock Center (Indiana University, 2014) and Tonina *et al.* (2016). The medium contained 75 g cornmeal, 17 g yeast, 15 g sucrose, 12 g soybean meal, 5.6 g agar, 5 ml propionic acid and water to 1000 ml. All ingredients were mixed and heated for about 25 min at 100°C, excluding propionic acid that was added at a temperature below 50°C before pouring 15 ml of medium into vials. Insects were reared in climatic chamber at 23±2°C and 70-80% relative humidity with a photoperiod of 16L:8D.

DNA isolation and *Wolbachia* detection

Wolbachia incidence in the population was assessed on 30 adults (15 males and 15 females) by specific PCR assays. The microbial DNA was extracted following the protocol described in Palmano *et al.* (2000). Detection of *Wolbachia* was accomplished by amplifying 16S rDNA gene using specific primers 16S-F (TTGTAGC(C/T)TGCTATGGTATAACT) and 16S-R (GAATAGGTATGATTTTCATGT) (O'Neill *et al.*, 1992). All PCR reactions were conducted in a 20µl volume containing 4µl PCR of 5x colorless GoTaq Flexi Buffer (Promega), 2.5 mM MgCl₂, 0.1 mM dNTPs, 0.5 µM of each primer, 1U of GoTaq Flexi DNA polymerase (Promega) and 2µl of extracted DNA. Cycling conditions were an initial denaturation step at 94°C for 5 min followed by 35 cycles with a denaturation step at 95°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min.

Preparation of *Wolbachia*-free lines

After maintaining 1,000 flies culture on normal rearing media, two lines of flies were created; *Wolbachia*-free line (hereafter: WF) following a commonly used antibiotic procedure (Poinsot and Mercot, 1997; Bordenstein and Werren, 1998) and *Wolbachia*-infected line (hereafter: WI) on the medium without antibiotic. We created WF lines adding at the medium mentioned above tetracycline [final concentration 0.2 mg ml^{-1} (0.02%)] (Min and Benzer, 1997). This procedure was continued for four discrete generations to ensure that *Wolbachia* were totally removed. After tetracycline treatments, *Wolbachia* elimination was confirmed by PCR analysis. Consecutively, the new descendants were nurtured for two discrete generations on the normal food medium without antibiotic before the beginning of crossing experiments, to avoid any possible effect of the antibiotic on the flies' fitness (Fry *et al.*, 2004). The WI lines received identical food and environment as the WF lines except for tetracycline. The presence of *Wolbachia* was confirmed by PCR assessment.

The preparation and maintenance of WF and WI lines were conducted at two different temperatures, $22\pm 1^\circ\text{C}$ and $29\pm 1^\circ\text{C}$, 70–80% relative humidity and under a photoperiod of 16L:8D.

Fecundity

At the start of the seventh generation, four crosses were made between *Wolbachia*-free (WF) and *Wolbachia*-infected (WI) flies as follows: (1) $\text{WI}_{\text{♂}} \times \text{WI}_{\text{♀}}$, (2) $\text{WF}_{\text{♂}} \times \text{WI}_{\text{♀}}$, (3) $\text{WI}_{\text{♂}} \times \text{WF}_{\text{♀}}$, (4) $\text{WF}_{\text{♂}} \times \text{WF}_{\text{♀}}$. Each cross was replicated five times. For each cross, a newly enclosed single female and male (3 day old) were placed in food vials to mate and lay eggs.. The flies were transferred to fresh vials containing 15 ml of food on each day and the number of eggs deposited during the previous 24 h in the food vials was recorded. Fecundity was checked every day for up to 12 days. Vials were maintained at two temperatures ($22\pm 1^\circ\text{C}$ and $29\pm 1^\circ\text{C}$).

Cytoplasmic incompatibility and viability

CI occurs when the ratio of eggs hatching from the incompatible cross (3) $\text{WI} \times \text{WF}$ is less than compatible crosses (1) $\text{WI} \times \text{WI}$ and (2) $\text{WF} \times \text{WI}$. CI was estimated by holding the egg-counting vials from the fecundity assays at both temperatures for an additional 35-40 h, when the number

of unhatched eggs in each vial was recorded and compared with the previous egg counts. This was repeated five times for all vials containing eggs during 12 days from the fecundity assay (Mazzetto *et al.*, 2015). Larval to adult viability was assessed from eggs deposited in vials during a 24-h period. The protocol was to hold food vials containing the eggs of each day for 14 days after laying and count the number of emerging adults from the vials set at the two temperatures. The number of emerging adults was then compared with the number of hatched eggs recorded previously for that vial.

Survival

At the start of the seventh generation, survival of *Wolbachia*-free (WF) and *Wolbachia*-infected (WI) lines was measured by placing 30 adults (15 males and 15 females) in three replicate plastic boxes (20×20×20 cm). The three boxes were placed in climate chambers separately for each temperatures (22±1°C and 29±1°C), 70-80 % relative humidity, and 16L:8LD photoperiod. The food media was replaced each 1-2 days as indicated by Strunov *et al.* (2013); dead flies were removed and both sexes counted daily for both WF and WI flies. The boxes were monitored in this way until all the flies had died.

Nutritional stress

At the start of the seventh generation, the impact of nutrition was investigated by rearing larvae of *Wolbachia*-free (WF) and *Wolbachia*-infected (WI) flies on yeast poor food media. Nutritional stress was imposed using a lower amount of yeast (0.17 g yeast, 1%) as poor medium compared to the normal medium (17 g yeast, 100%) (Harcombe and Hoffmann, 2004). Five vials were set up for both media (poor and normal) and WF and WI lines. Twenty eggs were placed in each vial ("8×6") containing 20 ml food media. The trial was carried out at 22±1°C and 29±1°C. To determine nutritional effects, the time necessary for the flies to emerge was assessed.

Starvation stress

At the start of seventh generation, starvation tolerance was determined by putting 30 flies (15 males and 15 females) into 40 ml glass vials with no food. Humidity in vials was kept high by using cotton wool soaked with 10 ml of water (Service *et al.*, 1985). Five replicates were tested at each temperatures ($22\pm 1^\circ\text{C}$ and $29\pm 1^\circ\text{C}$) for WF and WI lines. Mortality was assessed twice a day.

Statistical analysis

All experimental procedures showed a normal distribution and equal variability. Uni-variate analysis using General Linear Model (SPSS 10.1) was performed to determine the influence of temperature on fecundity, CI, and viability for both WF and WI lines. Survival data were analyzed with Kaplan-Meier (KM) log rank tests, to check the mortality rates for each temperature of both lines on the basis of sex. Three way ANOVA was also used to compare the survival at both temperature on the basis of days and sex for both lines. Nutritional and starvation stress was checked by using Uni-variate analysis with relation to temperature stress on developmental time (days) and survival (hours) for both lines. For the comparisons of tests, means were separated by Tukey-Kramer (HSD) test at 5% significance level (Sokal and Rohlf, 1995).

Results

Wolbachia was proven to consistently infect our field collected *D. suzukii* population. PCR assays showed that *Wolbachia* was present in maintained culture with 93.8% average infection rate, 87.5% in males and 100% in females. No *Wolbachia* was detected in treated (WF) specimens.

Fecundity

Significant variation in fecundity among crosses ($F_{3, 480} = 50.82$, $P < 0.01$), temperatures ($F_{1, 480} = 1595.89$, $P < 0.01$), and time ($F_{11, 480} = 38.9$, $P < 0.01$) was found (figure 1). Crosses 3 ($WI_{\sigma} \times WF_{\phi}$) and 4 ($WF_{\sigma} \times WF_{\phi}$) showed a significant decrease in fecundity after *Wolbachia*

removal. Fewer eggs were laid at 29°C than at 22°C. The significance of time effect is due to the increase of day by day of the number of eggs laid.

The interactions temperature × crosses and temperature × days were significant ($F_{3, 480} = 4.22$, $P < 0.01$; $F_{11, 480} = 20.23$, $P < 0.01$ respectively), while the interactions crosses × days ($F_{33, 480} = 0.30$, $P = 1.00$) and temperature × days × crosses ($F_{33, 480} = 0.34$, $P = 1.00$) were not.

In short, the higher temperature drastically reduced the ability of both *Wolbachia*-free and *Wolbachia*-infected lines to lay eggs, although crosses 3 (WI♂×WF♀) and 4 (WF♂×WF♀) at 29°C produced fewer eggs than crosses 1 (WI♂×WI♀) and 2 (WF♂×WI♀).

Cytoplasmic incompatibility and viability

The combined effect of temperature and antibiotic treatment affected the CI and viability. All crosses (1-4) did not significantly differ at 22°C ($F_{3, 240} = 0.10$, $P = 0.95$) whereas they did at 29°C ($F_{3, 240} = 16.98$, $P < 0.01$). A reduction in hatching rate was found in crosses 3 (WI♂×WF♀) and 4 (WF♂×WF♀) at 29°C (figure 2).

No major changes were recorded in viability of adults at 22°C ($F_{3, 240} = 1.02$, $P = 0.38$), while at 29°C fewer adults were obtained from the crosses having *Wolbachia*-free females compared to *Wolbachia*-infected ones with significant differences ($F_{3, 240} = 13.16$, $P < 0.01$) (figure 3).

Survival

As main effect, temperature significantly affected the longevity of both male and female flies ($F = 319.58$; $df = 1$; $P < 0.01$). At 22°C *D. sukuzii* adults survived longer (20-30 days) than 29°C (figure 4A, 4B). About the influence of bacterial infection at 22°C, infection status did not display any difference in the survival ($F = 0.48$, $df = 1$, 408; $P = 0.47$) while it was noticed at 29°C ($F = 4.93$, $df = 1, 408$; $P = 0.02$) (figure 4A, 4B and 5). There was a significant interaction between temperature × infection status ($F = 4.8$; $df = 1, 816$; $P = 0.02$).

According to Kaplan Meier analyses, temperature significantly affected the survival ($\chi^2 = 96.45$, $df = 1$, $P < 0.01$) At 22°C, infection status did not impact the survival ($\chi^2 = 0.70$, $df = 1$, P

= 0.78) while it did at 29°C ($\chi^2 = 3.35$, $df = 1$, $P = 0.05$) (figure 5). There was significant influence between temperatures for the survival of both *Wolbachia*-infected and *Wolbachia*-free populations ($\chi^2 = 62.68$, $df = 1$, $P < 0.01$ and $\chi^2 = 35.89$, $df = 1$, $P < 0.01$, respectively). No difference was observed for the survival of both males and females in *Wolbachia*-free and *Wolbachia*-infected lines at 22°C ($\chi^2 = 0.49$, $df = 1$, $P = 0.48$ and $\chi^2 = 0.38$, $df = 1$, $P = 0.53$, respectively) (figure 4A) and 29°C ($\chi^2 = 1.89$, $df = 1$, $P = 0.16$ and $\chi^2 = 1.45$, $df = 1$, $P = 0.22$, respectively) (figure 4B).

Nutritional stress

Nutritional effects assays showed that, food media ($F_{1,40} = 0.10$, $P = 0.74$ and infection status ($F_{1,40} = 0.43$, $P = 0.52$) did not influence the adult developmental time. Only temperature affected developmental time ($F_{1,40} = 62.27$, $P < 0.01$) (figure 6).

No significant interaction was found between media \times infection status ($F_{1,40} = 0.00$, $P = 1.00$), media \times temperature ($F_{1,40} = 0.00$, $P = 1.00$), infection status \times temperature ($F_{1,40} = 0.10$, $P = 0.74$) and media \times infection status \times temperature ($F_{1,40} = 0.10$, $P = 0.74$).

Starvation stress

Infection status did not impact the starvation resistance ($F_{1,40} = 3.13$, $P = 0.09$) (Figure 7) but temperature did ($F_{1,40} = 696.15$, $P < 0.01$), as both *Wolbachia*-free and *Wolbachia*-infected flies died earlier at 29°C than at 22°C. However, sex had an impact on starvation ($F_{1,40} = 7.25$, $P = 0.01$) (figure 7), as females lived longer than males. There was no interaction between infection status \times sex ($F_{1,40} = 0.29$, $P = 0.59$), infection status \times temperature ($F_{1,40} = 3.41$, $P = 0.09$), sex \times temperature ($F_{1,40} = 1.0$, $P = 0.33$) and infection status \times sex \times temperature ($F_{1,40} = 0.15$, $P = 0.70$).

Discussion

Findings of the present study elucidate the combined impact of temperature and *Wolbachia* infection on the fitness of *D. sukuzii*. We found that fecundity was negatively affected by high temperature for both *Wolbachia*-free and *Wolbachia*-infected flies. Due to change in fertility, reduction in hatch rate (CI) and viability was also observed at high temperature for *Wolbachia*-free females. Under the temperature stress of 29°C, *Wolbachia*-free flies lived longer than *Wolbachia*-infected flies. No significant effect was detected due to nutritional and starvation stress, irrespective of temperature on *Wolbachia*-free and *Wolbachia*-infected flies.

Fecundity

Our experiments showed that fecundity dropped in crosses with *Wolbachia*-free females. High temperature also decreased the number of eggs but the pattern of egg increase and decrease was the same in all crosses at both temperatures (figure 1). Tochen *et al.* (2014) also obtained fewer eggs at 30°C in *D. sukuzii* infected with *Wolbachia*. Evans *et al.* (2017) suggested that *D. sukuzii* prefers microclimate with mild temperatures and high humidity for reproduction and does not perform well when exposed to direct sunlight with extreme heat. Our data thus suggest that female fecundity was beneficially influenced by *Wolbachia*. Mutualistic links where *Wolbachia* had positive impacts on fecundity are known for many insects (Dobson *et al.*, 2002; Fry *et al.*, 2004; Mazzetto *et al.*, 2015) and in filarial nematodes (Bandi *et al.*, 1999). The higher fecundity may be one of the reasons for *Wolbachia* persistence in the wild (Fenton *et al.*, 2011), as well as in *D. sukuzii* populations from North America and Europe (Cattel *et al.*, 2016).

Cytoplasmic incompatibility and viability

In addition, our results indicate that the change in fertility may result from the combined effect of temperature and antibiotic treatment. No CI was recorded at 22°C in all crosses having *Wolbachia*-free and *Wolbachia*-infected lines whereas CI could have occurred at 29°C in crosses 3 and 4 (figure 2). The reason for this is that heat shock and the consequent endogenous rise in heat shock proteins played a major role in influencing male sterility and CI (Snook *et al.*, 2000). This has been documented in other insect orders (Rinehart *et al.*, 2000; Chakir *et al.*,

2002; Vollmer *et al.*, 2004). Trpis *et al.* (1981) indicated CI at high temperature in numerous species. Hoffmann *et al.* (1986) reported parallel effects to our study that placing larvae at high temperature could suppress the incompatibility of *D. simulans* males. It is clear from our findings that flies having *Wolbachia* performed well under a temperature of 29°C but when they were *Wolbachia*-free. However, a *Wolbachia* decrease was also reported in hosts nurtured at a temperature above 30°C (Van Opijnen and Breeuwer, 1999; Zhukova *et al.*, 2008) and below 13°C (Pintureau *et al.*, 2003). These two elements (temperature and antibiotic) help in understanding the species performance in a fluctuating environment, as explained by Araripe *et al.* (2004).

As in CI, we did not observe any indication of viability difference between *Wolbachia*-free and *Wolbachia*-infected flies at 22°C as found at 29°C (figure 3). Similar to our outcome, Fry *et al.* (2004) proved that the viability of some strains of *D. melanogaster* caused harmful modifications in fitness. Overall, a disparity between *Wolbachia*-free and *Wolbachia*-infected flies was observed in relation to high temperature, which induced survival, CI and viability of *Wolbachia*-free flies whereas at 22°C *Wolbachia* had a negative influence on all parameters.

Survival

An ectothermic animal's survival greatly relies on temperature conditions (Cossins and Bowler, 1987). In our findings, when the temperature rose from 22±1°C to 29±1°C, *Wolbachia*-free females survived longer (28 days) than *Wolbachia*-infected females (19 days) (figure 4B, 5), as previously detected by Tochen *et al.* (2014). This result confirms that obtained by Min and Benzer (1997) with the strain wMelPop of *D. melanogaster*, who found that all *Wolbachia*-infected flies kept at 29°C died within 14 days and *Wolbachia*-free flies within 28-30 days. However, when survival was evaluated at 22±1°C, *Wolbachia*-free and *Wolbachia*-infected flies maintained the same differences in lifespan (figure 4A). These findings show that the virulence of *Wolbachia* depends on temperature (Carrington *et al.*, 2009), which could play a vital role in host-*Wolbachia* interactions (Mouton *et al.*, 2006; Kusmintarsih, 2012). Our study extended these findings to *D. suzukii*. The effects were also observed in strains of *D. melanogaster*, although the influence on fitness associated with the removal of *Wolbachia* varied according to the strain (Fry and

Rand, 2002; Fry *et al.*, 2004). Two main causes were suggested by Strunov (2013) to explain the early death of *Wolbachia*-infected flies. First, the multiple obliteration of brain cells and the evacuation of replicating bacteria were seen at high temperature (Rasgon *et al.*, 2006). Second, the increase in proportion of clusters of bacteria may be the main cause of steady deterioration (Strunov, 2013).

Nutritional and starvation stress

Energy and protein resources are imperative in adult *Drosophila* as they hunt for food (Harcombe and Hoffmann, 2004; Brownlie, 2009; Hoffmann *et al.*, 2011). The prevalence of nutrition supplementation in other endosymbiotic systems suggested that *Wolbachia* might also influence nutrition (Harcombe and Hoffman, 2004). In this study, both nutrition (figure 6) and starvation (figure 7) assays did not detect any changes between infected and cured flies. This suggests that *Wolbachia* does not offer *D. suzukii* any fitness benefit linked to nutritional tolerance. *Wolbachia* does not therefore seem to exploit unique metabolic ways to maintain a mutualism with this fly. Variability was only observed at high temperature and concerned developmental days (figure 6). Current research indicates that *Wolbachia* can confer a compensatory effect during conditions of nutritional stress in *D. melanogaster* (Brownlie *et al.*, 2009). In our results, negligible deviations between *Wolbachia*-free and *Wolbachia*-infected could be perceived for tolerance to starvation, but temperature had a significant impact on male and female survival under starvation stress. Our findings also correlated with results documented by Hangartner and Hoffman (2016), who found an increase in *D. melanogaster* survival of about 4 h at 23°C. Some studies showed contrary findings to our results, as they observed up to twofold difference between females previously fed yeast than those given none (Burger *et al.*, 2007). It has also been argued that starvation effects can increase the level of stochasticity and thereby artificially lowering estimates of adaptive capacities for upper thermal tolerance (Santos *et al.*, 2011; Santos *et al.*, 2012).

Conclusions

We conclude that the effect of temperature is a key element influencing *D. sukii*-*Wolbachia* strains and its lower performance also mediated by the effects on its symbiont. So, testing the association of *Wolbachia* with *D. sukii* under different fluctuating temperature regimes may open the way to a better understanding of the varying performance of the pest when exposed to seasonal temperature variation.

Acknowledgements

Thanks to Fabio Mazzetto, Omar Rota-Stabelli and Myron Zalucki for their comments and suggestions on the ms. The work benefited of a grant from the University of Padua.

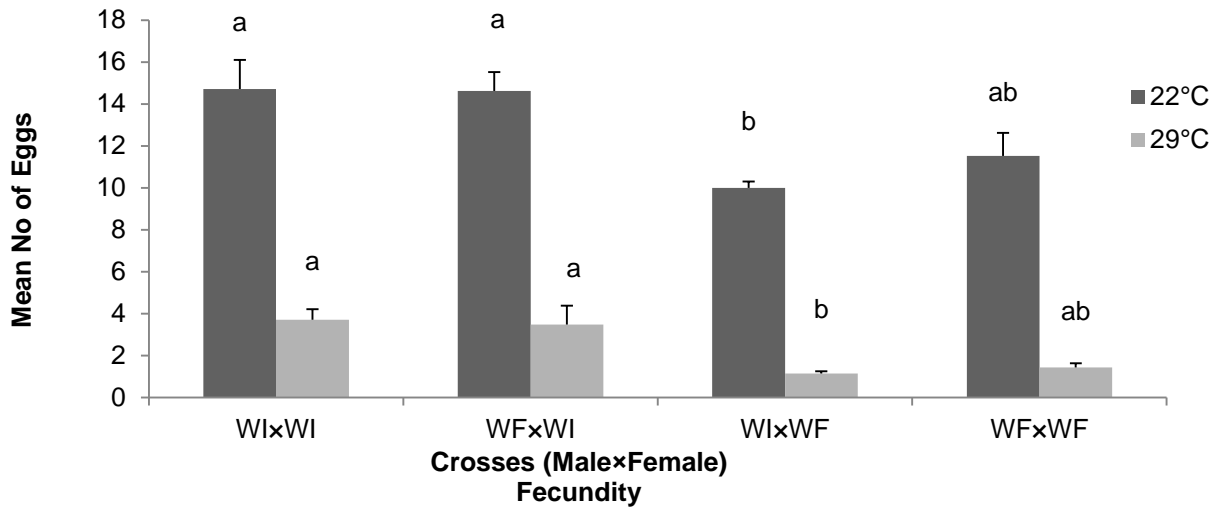


Figure 1. Mean number (\pm SE) of eggs of *D. sukuzii* females during 1-12 days in the four crosses (1) WI δ × WI ϕ : *Wolbachia*-infected male × *Wolbachia*-infected female; (2) WF δ × WI ϕ : *Wolbachia*-free male × *Wolbachia*-infected female; (3) WI δ × WF ϕ : *Wolbachia*-infected male × *Wolbachia* free-female; (4) WF δ × WF ϕ : *Wolbachia*-free male × *Wolbachia*-free female under two temperatures (22 and 29°C). Different letters above histogram bars indicate significant differences ($P < 0.05$) in pairwise comparisons within the same temperature.

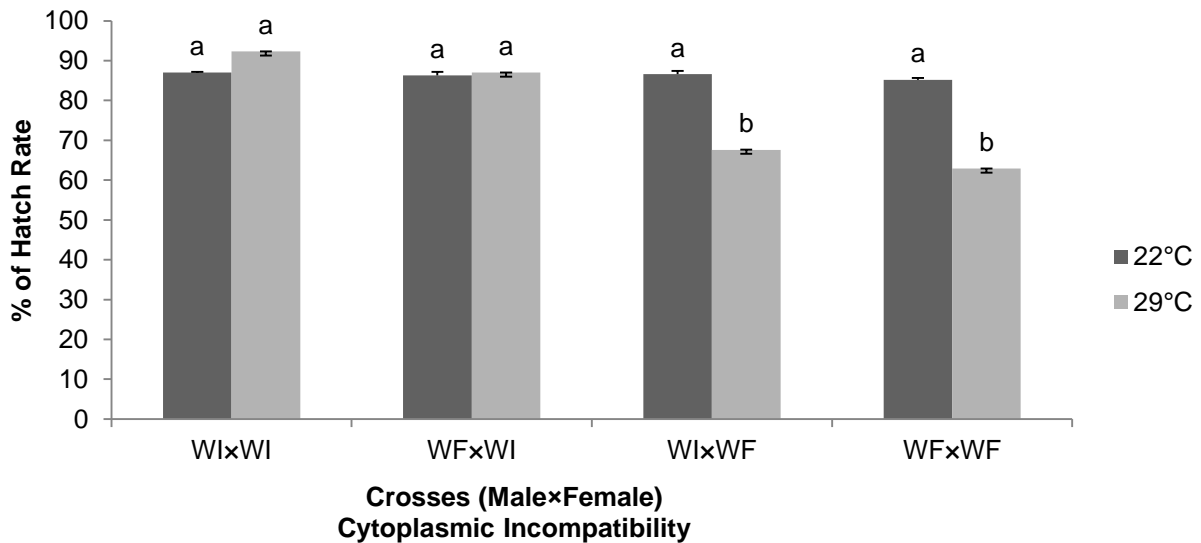


Figure 2. Mean (\pm SE) percentage of eggs hatching from each of four crosses under two temperatures (22 and 29°C). Different letters above histogram bars indicate significant differences ($P < 0.05$) in pairwise comparisons within the same temperature.

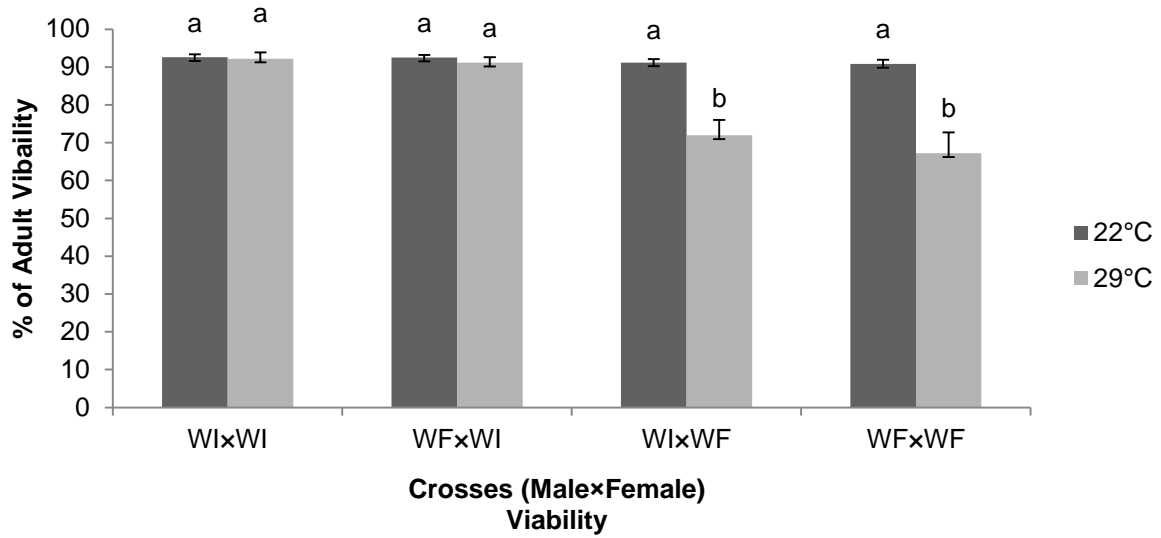


Figure 3. Mean (\pm SE) percentage of adult *D. sukuzii* emergence from each cross under two temperature regimes (22 and 29°C). Different letters above histogram bars indicate significant differences ($P < 0.05$) in pairwise comparison within the same temperature.

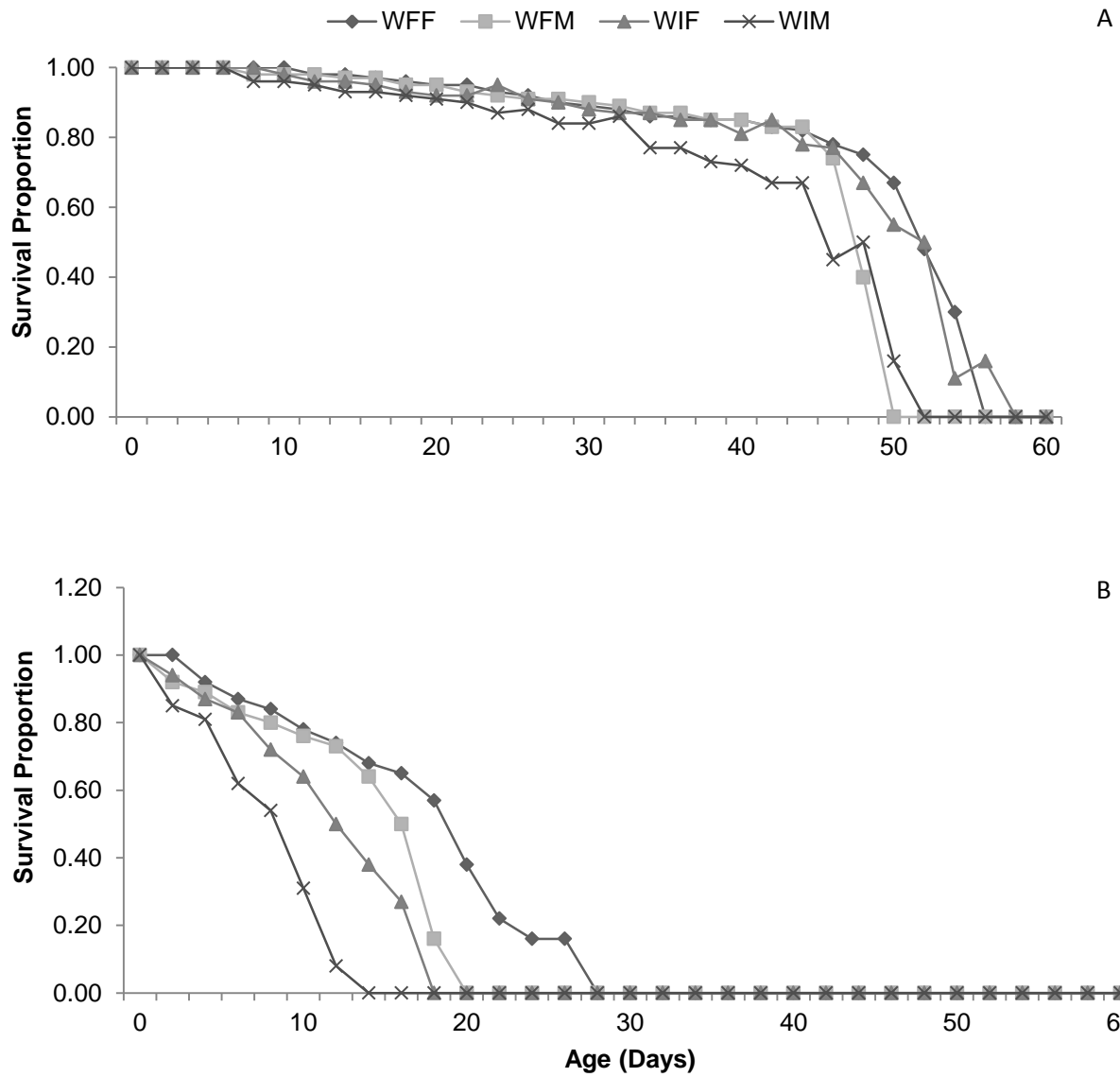


Figure 4. Proportion of survival for both *Wolbachia*-free (WF) and *Wolbachia*-infected (WI) lines of *D. sukuzii* male (M) and female (F) at 22 (A) and 29°C (B).

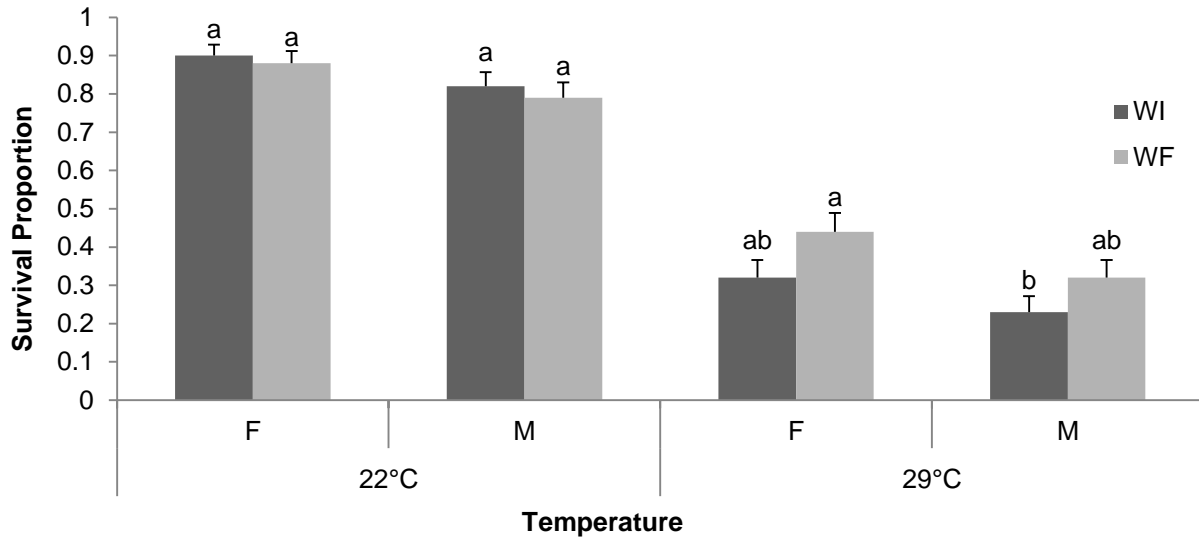


Figure 5. Average of survival proportion for both *Wolbachia*-free (WF) and *Wolbachia*-infected (WI) lines of *D. sukuzii* male (M) and female (F) at 22 and 29°C. Different letters above histogram bars indicate significant differences ($P < 0.05$) within each temperature among sex and infection status.

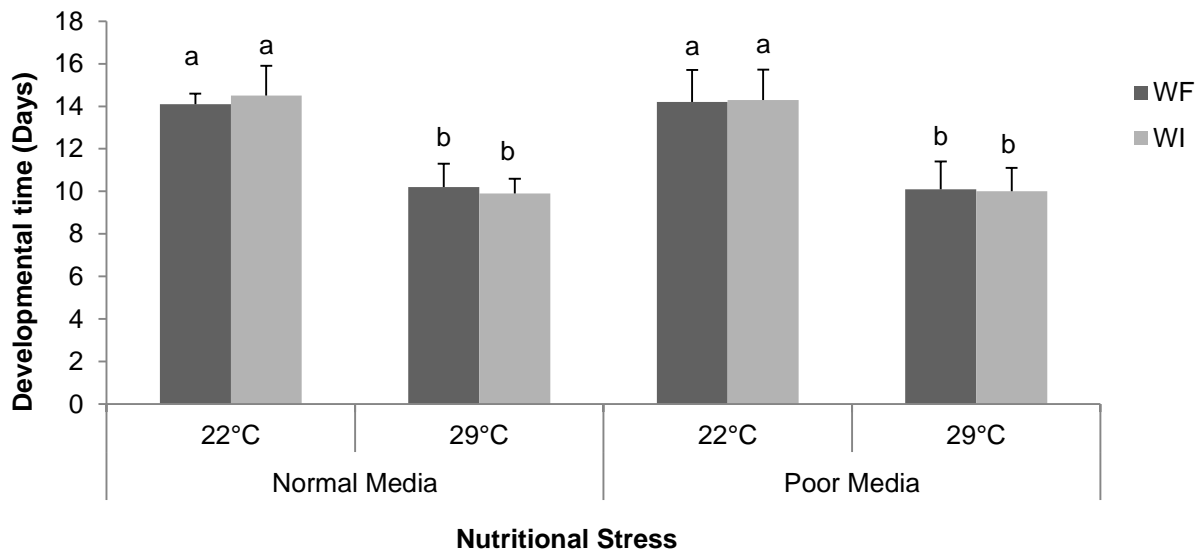


Figure 6. Mean number of developmental time (±SE) for *Wolbachia*-infected (WI) and *Wolbachia*-free (WF) lines under nutritional stress (normal and poor (low yeast) media). Different letters above histogram bars indicate significant differences ($P < 0.05$) in pairwise comparison within each food between temperature and infection status.



Figure 7. Survival time (hour) (mean \pm SE) *Wolbachia*-infected (WI) and *Wolbachia*-free (WF) lines of *D. sukuzii* males and females under starvation stress. Different letters above histogram bars indicate significant differences ($P < 0.05$) in pairwise comparison within sex between temperature and infection status.

Chapter 3

Temperature alters the response to insecticides in *Drosophila suzukii*

This paper will be submitted soon in Pest Management Science.

Saeed N., Tonina L., Battisti A., Mori N. Temperature stress alters the response to insecticides in the invasive pest *Drosophila suzukii*.

Temperature alters the response to insecticides in *Drosophila suzukii*

Abstract

Invasive agricultural pests face new habitat and host plant conditions in the invaded range and challenge pest management practices. *Drosophila suzukii* has become an important pest in the soft fruit industry in Europe, partly because of low temperatures tolerances and build up to large populations in wild host fruits adjacent to commercial berry fields in mountain areas. Starting from field observations on the different efficacy of insecticides under variable temperature regimes, field and laboratory experiments were designed to test the efficacy of major types of insecticides available against this pest. Pyrethroids and spinosins proved to be the most effective under all temperature conditions in all assays. Organophosphates and neonicotinoids showed significantly lower efficacy at low temperatures, indicating that they are not suitable to protect crops under those conditions. The management of the pest in cold habitats, that are very suitable for the cultivation of high quality berries such as farming systems of the Alps, has to take such limitations into considerations. Our results show how invasion biology has unexpected implications that need to be thoroughly considered for the mitigation of the impacts of such species.

Keywords: Spotted Wing *Drosophila*, Soft fruits, Thermal response, Chemical control, Integrated Pest Management

Introduction

Temperature is one of the most important factors affecting geographic distribution and performance of insects, as well as their susceptibility to insecticides (Chown and Nicholson, 2004; Holmstrup *et al.*, 2010; Laskowski *et al.*, 2010; Zalucki *et al.*, 2017). The effect of temperature on insect susceptibility to pesticides has been studied for various insect pests (Boina *et al.*, 2009; Khan and Akram, 2014; Toth and Sparks, 1990; Abbes *et al.*, 2015). The influence of temperature on toxicity can be either positive or negative, depending on the mode of action of the insecticide, the insect species, and the route of exposure (ingestion or contact) (Scott 1995; Glunt *et al.*, 2013). Temperature can alter the efficacy of pesticides by modifying their

absorption, adsorption, persistence, and degradation (Harwood *et al.*, 2011). Most active ingredients showed synergistic effects with temperature and only in a few cases the highest toxicity was observed at intermediate temperature conditions (Amarasekare and Endelson, 2004; Biondi *et al.*, 2013).

Drosophila suzukii Matsumura (Diptera: Drosophilidae) is a highly polyphagous pest of small fruits in many parts of the world (Lee *et al.*, 2011; Cini *et al.*, 2012). Its host range is wide, including soft and stone fruits as well as various non-crop plants (Kenis *et al.*, 2016). Its range expansion has been followed by an increase in economic importance in both Americas (Goodhue *et al.*, 2011) and Europe (Cini *et al.*, 2012). The species is well adapted to a wide range of temperatures, with population build up observed at low (10-11°C) (Tochen *et al.*, 2014; Tonina *et al.*, 2016) as well as at high (28°C) temperatures (Kinjo *et al.*, 2014). Its adaptation to mountain climates has made it a threat to berry orchards in various parts of Europe (Kenis *et al.*, 2016; Tonina *et al.*, 2016).

Current control of *D. suzukii* relies primarily on the application of a range of insecticides such as pyrethroids, organophosphates, spinosyns and neonicotinoids (Van Timmeren and Isaacs, 2013, Cuthbertson *et al.*, 2014), but there is no evidence on how temperature, affect the toxicity of insecticides. This seems particularly important for those regions characterized by a strong temperature gradient, typically associated with conspicuous elevation differences over relatively short distances, which facilitates the migration of the pest among crops and wild hosts (Mitsui *et al.*, 2010; Tonina *et al.*, 2016).

This study investigates the role of temperature on the efficacy of insecticides against the invasive pest *D. suzukii* under both field and laboratory conditions. The study was prompted by field data on the effect of insecticides with different modes of action (MoA) recorded on strawberry grown under lowland and mountain climates, and it continued in the laboratory with assays, on four fruit types, of topical and residual effects at three temperatures. Our aim was to provide crop managers with better control options for this pest in relation to the different thermal conditions of small fruit crops.

Material and Methods

Field trials

The field experiments were set up in 2015 in two different strawberry-growing areas of the Veneto region (North-Eastern Italy): one farm was located in the plain (49 m, 45°222811 N - 10°582564 E, hereafter “plain-farm”) and one in a high hill location (1,095 m, 45°365580 N - 11°053017 E, hereafter “hill-farm”). The cultivar (Irma everbearing day neutral cultivar, registration number VR 95.42.03), the cultivation system (soilless cultivation under plastic tunnel) as well as agronomic practices (i.e, irrigation, fertilization, pruning, fungi control, and pest management) were the same in the two sites. Given that strawberry becomes attractive to the pest from when fruits are changing color (Lee *et al.*, 2011), insecticides were applied during the ripening period. We used formulations registered in Europe according to the Commission Implementing Regulation (EU) No. 1107/2009 and No. 540/2011, and authorized in Italy for commercial use on strawberry. We selected one insecticide for each of the three IRAC mode of action (MoA) (deltamethrin, acetamiprid and spinosad) (Table 1). Insecticides were applied at 12 and 4 days before harvest according to the local IPM program (Veneto Region - Phytosanitary service, 2015). The applications were performed on Sept. 17th and 25th and on Sept. 18th and 26th in plain and hill-orchard, respectively.

Trials were designed according to EPPO guideline (EPPO, 2013). Untreated plots were included in completely randomized blocks designs and each treatment comprised four replications (plots). Each plot consisted of 10 m² strawberry. A motorized sprayer with a disc core nozzle (Albuz, ATR 80 yellow) was used for the foliar applications, with 12 bar of pressure, and a water volume of 800 L/ha. During the trial period, the pest abundance was checked weekly using 2 red traps Drosotrap® baited with Droskydrink® (a mixture of apple cider vinegar, red wine and brown sugar according to Tonina *et al.*, 2017) for each orchard. Fruit infestation and number of larvae were evaluated at first and second fruit harvest on Sept. 29th and Oct. 2nd and on Sept. 30th and Oct. 3rd in plain and hill-orchard, respectively (corresponding to 4 and 7 days after last application). At each sampling, 100 randomly selected ripe strawberries per plot were collected in the field and observed under a dissecting microscope. The percentage of infested fruits by *D. suzukii* larvae was recorded. After that, the larvae were extracted from the fruits by immersing the strawberries in a 10% NaCl solution for 6 hours and the number of larvae in 100 fruits was recorded.

Temperature of the two orchards was obtained from daily data recorded at 2 m above ground by Veneto Region Weather Station (http://www.arpa.veneto.it/bollettini/meteo60gg/Staz_127.htm for “plane-orchard”; http://www.arpa.veneto.it/bollettini/meteo60gg/Staz_251.htm for “hill-orchard”).

Laboratory trials

The population of *D. suzukii* used in the experiments originated from wild individuals collected in the autumn of 2014 in northern Italy. Male and female adults were placed in 50 mL plastic culture vials (diameter 30 mm, length 115 mm) with ~15 ml specific rearing medium (Bloomington Drosophila Stock Center, Indiana University) prepared according to Tonina et al. (2016). Cultures were maintained in climate chambers at 23±1°C, 70±10% RH and 16:8 h L:D regime. Wild *D. suzukii* adults were introduced into the colony on multiple occasions in 2015 and 2016 to ensure that the genetic make-up of the individuals screened in the laboratory was representative of the field populations. In addition to the three insecticides tested in the field, an organophosphate was added (phosmet) and all insecticides were tested for topical and residual toxicity.

Topical exposure was on newly emerged adults. Experiments were organized using five replicates, each containing five males and five females. Distilled water served as control. Adults were immobilized with the help of portable CO₂ dispenser and positioned on a Petri dish. A solution of insecticide was applied topically (2 µl) under a stereomicroscope (Yee and Alston, 2006). After application, the flies were individually shifted back into new Petri dishes with a small quantity of sugar solution. Dishes were kept in a cabinet at 16:8 L:D photoperiod, 65±5% RH and three different temperatures, i.e. 14, 22, and 30°C, for 24 hours. After this period, the mortality was checked. Flies were considered dead when they did not react to a mechanical stimulation with a soft brush.

Residual toxicity of each insecticide was tested using bioassays with four fruit or berry types: sweet cherry (*Prunus avium*, cultivar Kordia), blueberry (*Vaccinium corymbosum* cultivar Elliot), strawberry (*Fragaria x ananassa* cultivar Irma) and red grape (*Vitis vinifera* cultivar Corvina). Insect-free fruits were collected from plants covered with insect-proof nets (mesh size 0.6x0.8 mm) in orchards located in Trento and Verona provinces, North-Eastern Italy, and not subjected to pesticide applications. Fruits were first dipped for 2-3 sec in a solution of insecticide

according to the rates used in the field (Table 1) and then air dried for 2 h. Fruits were placed on 100 ml plastic condiment-cups with ventilated lids (5 fruits per cup) (see previous study Bruck *et al.*, 2011; Cuthbertson *et al.*, 2014 for details). Ten newly emerged (5 d old) *D. suzukii* adults (5 males and 5 females) were introduced into the condiment-cups. Five replicates were considered per each formulation and fruits dipped in distilled water acted as control. The dishes were maintained in the same cabinet and conditions used for topical toxicity. Mortality of adults and number of eggs laid on each fruit were recorded 24 hours after exposure under microscope. The fruits were maintained under the same conditions for 10 additional days and the number of emerging adults was determined at the end of this period.

Data analysis

In the field experiment, the percentage of infested fruit and the number of larvae per fruit were analyzed using linear model (LM) for each experimental site and fruit picking date. Percentage data were arcsine square root transformed. Interactions among factors were tested using a linear model with insecticide and site as fixed factors. The laboratory data were analyzed by multiple ANOVA using GLM with insect mortality as response variable. Temperature, insecticide, and gender were main effects for topical toxicity, and fruit type was added for residual toxicity. Number of eggs laid in fruits was transformed to $\log(x+1)$ and multiple ANOVA was performed to check for the effect of temperature and fruit type as main effects. For all comparisons, means were separated by Tukey-Kramer (HSD) test at 1% significance level (Sokal and Rohlf, 1995). Abbott's formula (Abbott 1925) was used to correct for control mortality. Analyses were carried out with R (The R Foundation for Statistical Computing <http://www.R-project.org>).

Results

In the field, the daily temperature ranged from 10.5 to 29.4°C (average 18.9°C) in plain-orchard and from 4.6 to 20.5°C (average 12.1°C) in hill-orchard, as expected based on the different elevations (Figure 1). The total catch of *D. suzukii* adults was about 5-time higher in the hill- than in plain-orchard (579/675 and 114/138, respectively) (Figure 1). The infestation rate in the control plots was about 7% in plain- and 15% in hill-orchard at the first fruit picking and it increased to 13% and 26%, respectively, at the second fruit picking. Two of the three insecticides

(deltamethrin and spinosad) reduced infestation significantly and in a similar way in both plain- and hill-orchards during the harvest period (Table 2). On the contrary, acetamiprid significantly reduce infestation levels only in the plain-orchard, where its action was inferior to that of the other insecticides especially 7 days after the last application. The number of larvae per 100 fruits followed the same trend (Supplementary material, Table 1S). The interaction of insecticide and site was significant ($P < 0.01$), at both 4 and 7 days after the last application, for the percentage of infested fruits as well as for the number of larvae per fruit (Tables 2 and 1S).

In the laboratory, topical toxicity was higher after exposure to deltamethrin and spinosad (mortality from 92 to 100%) than to acetamiprid and phosmet (mortality from 24 to 87%) (Figure 2). The mortality caused by acetamiprid ($F_{2,30} = 20.16$; $P < 0.01$) and phosmet ($F_{2,30} = 8.45$; $P < 0.01$) increased significantly with temperature while there was no change for deltamethrin ($F_{2,30} = 1.21$; $P = 0.31$), spinosad ($F_{2,30} = 0.0$; $P = 1.0$), and control treatments ($F_{2,30} = 2.43$; $P = 0.10$) (Fig. 2). Males suffered higher mortality than female flies but only at low and medium temperature (14°C : $F_{1,49} = 18.96$; $P < 0.01$; 22°C : $F_{1,49} = 11.87$; $P < 0.01$; 30°C : $F_{1,49} = 2.79$; $P = 0.09$).

Residual toxicity was higher for deltamethrin and spinosad than for acetamiprid and phosmet (Figure 3). In addition, mortality increased significantly with temperature for each formulation. In particular, as temperature increased, the Abbott mortality increased from 74% to 100% for deltamethrin ($F_{2,120} = 74.01$; $P < 0.01$), from 82% to 100% for spinosad ($F_{2,120} = 50.82$; $P < 0.01$), from 18% to 57% for acetamiprid ($F_{2,120} = 39.34$; $P < 0.01$), and from 44% to 78% for phosmet ($F_{2,120} = 40.39$; $P < 0.01$). The temperature affected the mortality of controls ($F_{2,120} = 11.84$; $P < 0.01$) but only in the case of blueberry. Mortality interacted significantly with fruit type; at 14°C it was higher in cherry (48%) and strawberry (47%) than in blueberry (44%) and grape (36%) (14°C : $F_{3,200} = 8.41$; $P < 0.01$; Suppl. Mat. Figure 1S). Male flies died more than female flies at 14°C and 22°C but not at 30°C (14°C : $F_{1,200} = 8.41$; $P < 0.01$; 22°C : $F_{1,200} = 6.46$; $P < 0.01$; 30°C : $F_{1,200} = 4.51$; $P = 0.03$). The effect of residual toxicity on the number of eggs laid in the laboratory trial was also affected significantly by insecticide and temperature, with a pattern reversed to that observed for adult mortality (Figure 4). The number of emerged adults confirmed the values recorded for oviposition, with the only exception of strawberry because of a mold infection that prevented eggs hatchin or larval development (Figure 2S).

Discussion

Temperature affected the response of *D. suzukii* to insecticides in both field and laboratory conditions. Deltamethrin and spinosad were the most effective under all conditions, confirming the observations reporting higher efficacy of pyrethroids and spinosad than neonicotinoids on *D. suzukii* and *Zaprionus indianus* (Gupta) (Diptera: Drosophilidae), in both topical and dip bioassay at 25°C (Andreazza *et al.*, 2017). Similar outcomes were recorded for pyrethroids and spinosad in laboratory (22°C) and field summer trials against *D. suzukii* (Beers *et al.*, 2011; Bruck *et al.*, 2011; Lee *et al.*, 2011).

We found a lower performance of the insecticides, and especially of organophosphates and neonicotinoids, at low temperature. This is particularly important in the case of *D. suzukii* because this species does perform well at low temperature, being capable of developing at temperatures as low as 10-12°C (Kinjo *et al.*, 2014; Tonina *et al.*, 2016). Berries are frequently grown in mountain conditions, where such temperatures are often registered during the growing season. Our results also suggest that the performance of the insecticides was the same irrespective of the density of the fly. The latter was much higher in the mountain-orchard, probably because the higher frequency of wild hosts in the vegetation surrounding the orchard (Kenis *et al.*, 2016).

There are several possible reasons for the lower performance of some insecticides at low temperature. Chemicals used against *D. suzukii* (pyrethroids, organophosphates, spinosyns, and neonicotinoids) lead to mortality by disrupting the normal functioning of nervous system (Khan and Akram, 2014). Different metabolic activities in insect body, responsible for the degradation of insecticides and normal functioning of the nervous system, are highly temperature dependent (Montgomery and McDonald, 1990). Since insects are generally ectotherms, environmental temperature can affect the effectiveness or toxicity of an insecticide (Glunt *et al.*, 2013). It is plausible that molecules respond differently to temperature and thus result in different effects on the target insect; for example, the potency of an insecticide declines at low temperature in a molecule-specific way due to different biotransformation processes (Harwood *et al.*, 2009). Furthermore, it is possible that the behavior of the target insects may affect their response to an insecticide, as evidenced in a study in field populations of *D. melanogaster* (Fournier-Level *et al.*, 2016).

The organophosphate phosmet showed a positive relationship with temperatures, although only in the laboratory because no formulations were available for field use on strawberry. This observation confirms the expectation of higher performance of organophosphates at elevated temperature (Glunt *et al.*, 2013). Toxicants that act on cellular enzymes, such as organophosphates that operate by binding to active sites of acetylcholinesterase, may increase their effectiveness with an increase of temperature (Harwood *et al.*, 2009; Hooper *et al.*, 2013). Our outcomes are in agreement with those obtained with other insect pests (Boina *et al.*, 2009; Norment and Chambers, 1970; Saleem *et al.*, 2008).

The neonicotinoid acetamiprid was the least effective in both field and laboratory trials. Our findings are in agreement with studies on *D. suzukii* (Bruck *et al.*, 2011; Andreatza *et al.*, 2017) and on a non-target predator (Mansoor *et al.*, 2015). However, the toxic effect of this insecticide should be better considered as some findings have displayed higher systemic action of acetamiprid after application against the cherry fly *Rhagoletis indifferens* (Diptera: Tephritidae) in direct spray and dried residue method (Yee and Alston, 2012). Like organophosphates, neonicotinoids resulted temperature dependent. A positive temperature correlation was found also for stored-product beetles (*Sitophilus zeamais*, *Oryzaephilus surinamensis*, *Tribolium castaneum*, and *Rhyzopertha dominica*), when exposed to thiamethoxam, imidacloprid, and acetamiprid at three constant temperatures (22, 27, and 32°C) (Arthur *et al.*, 2004). Probably the temperature may increase the uptake of toxicants (Jeschke and Nauen, 2008).

The pyrethroid tested in the present study showed high activity against *D. suzukii* irrespective of temperature. The toxicity of this molecule at low temperature agrees with findings in other insect species (Musser and Shelton, 2005; Saleem *et al.*, 2008; Khan and Akram, 2014). In contrast with organophosphates, pyrethroids toxicity decreases with increasing temperature (Harwood *et al.*, 2009). Being axonic poisons, pyrethroids control the movement of sodium ions during the movement of nerve impulse (Song *et al.*, 1996). The sensitivity of neurons increases when the temperatures goes from 25 to 15°C to, which results in repetitive nerve firing (Hooper *et al.*, 2013; Song *et al.*, 1996). In addition, sodium influx increases due to the stability of open sodium channels at low temperatures (Salgado *et al.*, 1989; Harwood *et al.*, 2009). On the

contrary, with temperatures higher than 30°C the neuronal sensitivity decreases reducing the pyrethroid activities (Hooper *et al.*, 2013).

Like pyrethroids, spinosad showed high activity against *D. suzukii*. The improved toxicity of spinosad at elevated temperatures could be linked to an increase in the uptake of the toxicants (Noyes *et al.*, 2009). Our results match with those obtained on grasshoppers (Amarasekare *et al.*, 2004), on grain pests (Pozidi-Metaxa and Athanasiou, 2013), and a non-target parasitoid (Abbes *et al.*, 2015). Still, additional research is required to understand the better efficacy of spinosad as temperature increases.

The increasing impact of *D. suzukii* in economically important fruit crops (Bellamy *et al.*, 2013; Lee *et al.*, 2011; Walsh *et al.*, 2011) makes the control of this invasive fly vital for reducing the damage to fruit production and alleviating stress levels for growers, traders, and consumers. The results demonstrate that the insecticide potency may vary with temperature in both field and laboratory conditions, indicating that some molecules should not be used in cold areas. In these situations, temperature should be added to factors considered in the decision-making process about registered formulations to be used to prevent fruit damage.

Table 1. Mode of Action, characteristics and rates of insecticide formulations used in field and laboratory bioassays. * Insecticides used in field trials according to Italian legislation.

IRAC MoA group	Active ingredient	Formulation	Trade name	Rate f.c. mL hL ⁻¹	Manufacturer
Organophosphates (1B)	phosmet	20% EC	Spada 200 EC®	375	Gowan
Pyrethroids (3A)	deltamethrin*	2.8% EW	Decis EVO®	50	Bayer CropScience
Neonicotinoids (4A)	acetamiprid *	5% SL	Epik SL®	150	Sipcam Italia
Spinosyns (5)	spinosad *	48% SC	Laser®	20	DOW- Agrosciences

Table 2. Percentage of infested strawberry observed in the orchards, 4 and 7 days after last application. Means within each column followed by the same latter are not significantly different (Tukey's LSD test; $P=0.01$).

Insecticide	Plain-Orchard		Hill-Orchard	
	4 days	7 days	4 days	7 days
control	7.0±1.8 A	14.8±2.7 A	13.3±3.3 A	25.8±3.6 A
deltamethrin	0.5±0.6 B	1.8±0.9 C	1.3±0.5 B	4.5±1.3 B
acetamiprid	3.3±0.9 B	8.0±2.2 B	8.8±1.7 A	21.0±3.6 A
spinosad	0.5±0.6 B	1.8±0.9 C	1.0±0.8 B	3.8±1.0 B
F	30.8	43.7	38.9	72.4
P	<0.01	<0.01	<0.01	<0.01

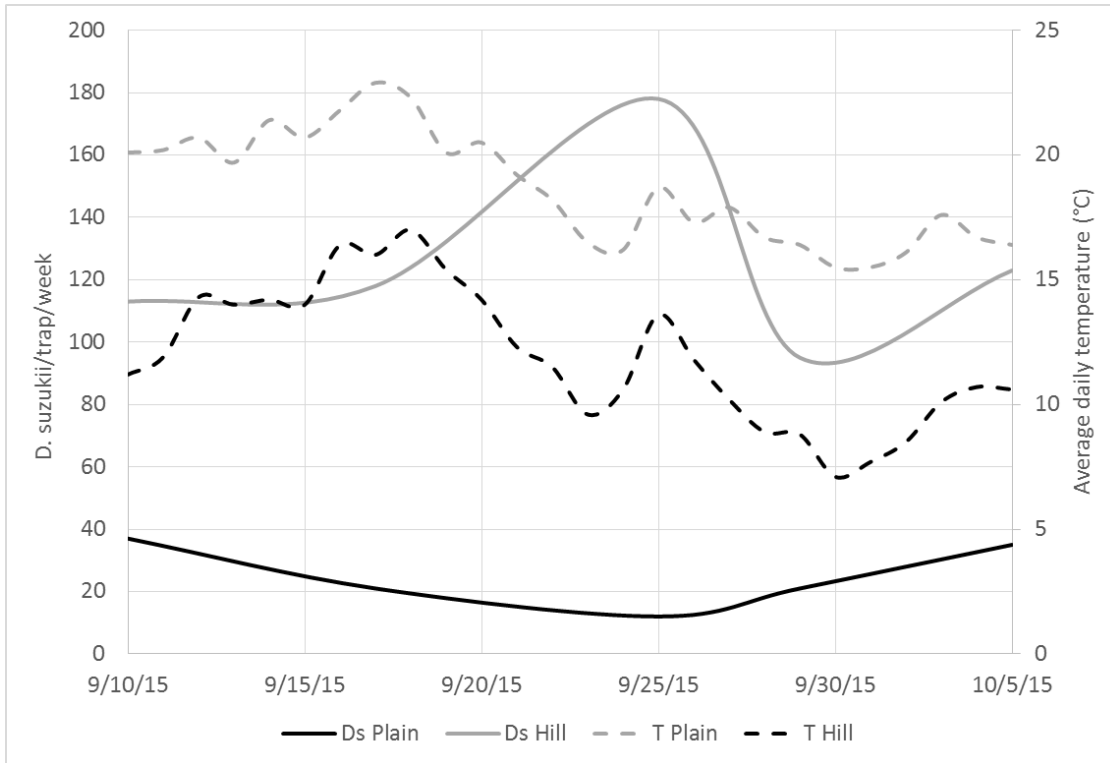


Figure 1. *Drosophila suzukii* captures and daily average temperature recorded in the two strawberry orchards in 2015.

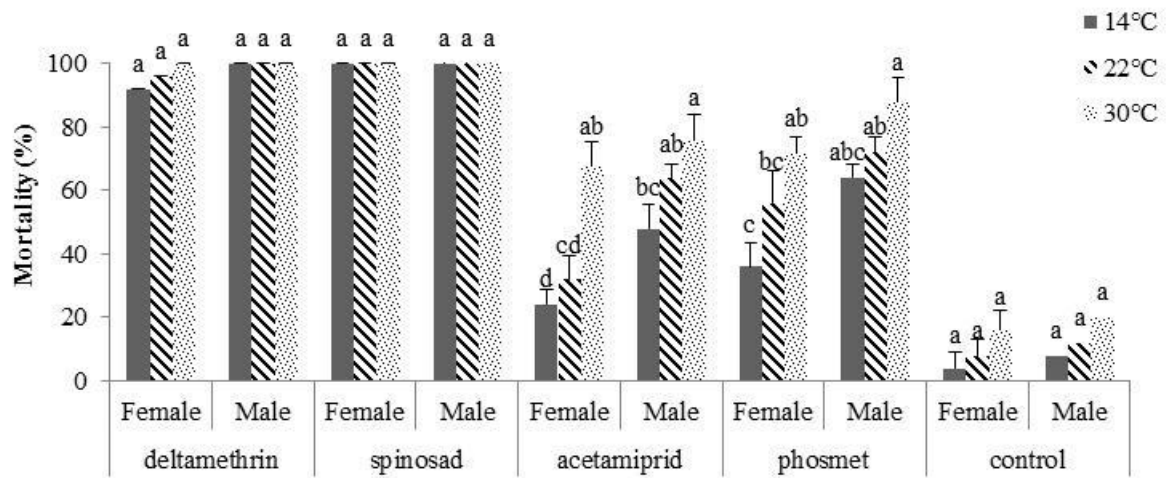


Figure 2. Adult mortality of *Drosophila suzukii* (mean \pm SE) topically treated with insecticides at three constant temperatures. Different letters above histograms showed significant differences at Tukey's HSD test; $P < 0.01$ within each treatment and gender.

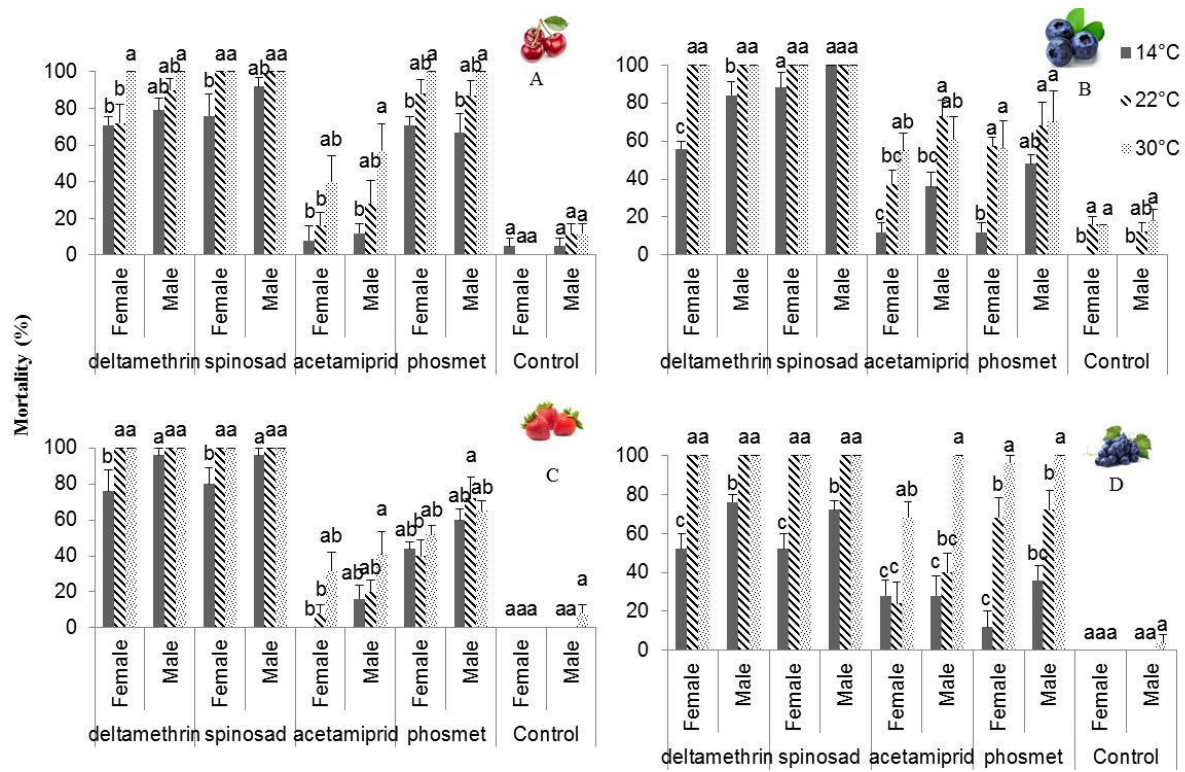


Figure 3. Adult mortality of *Drosophila suzukii* (mean ± SE) exposed for 24 h to four fruits (A: cherry; B: blueberry; C: strawberry; D: grape) treated with insecticides at three constant temperatures. Different letters above histograms showed significant differences at Tukey's HSD test; P < 0.01 within fruit and treatment.

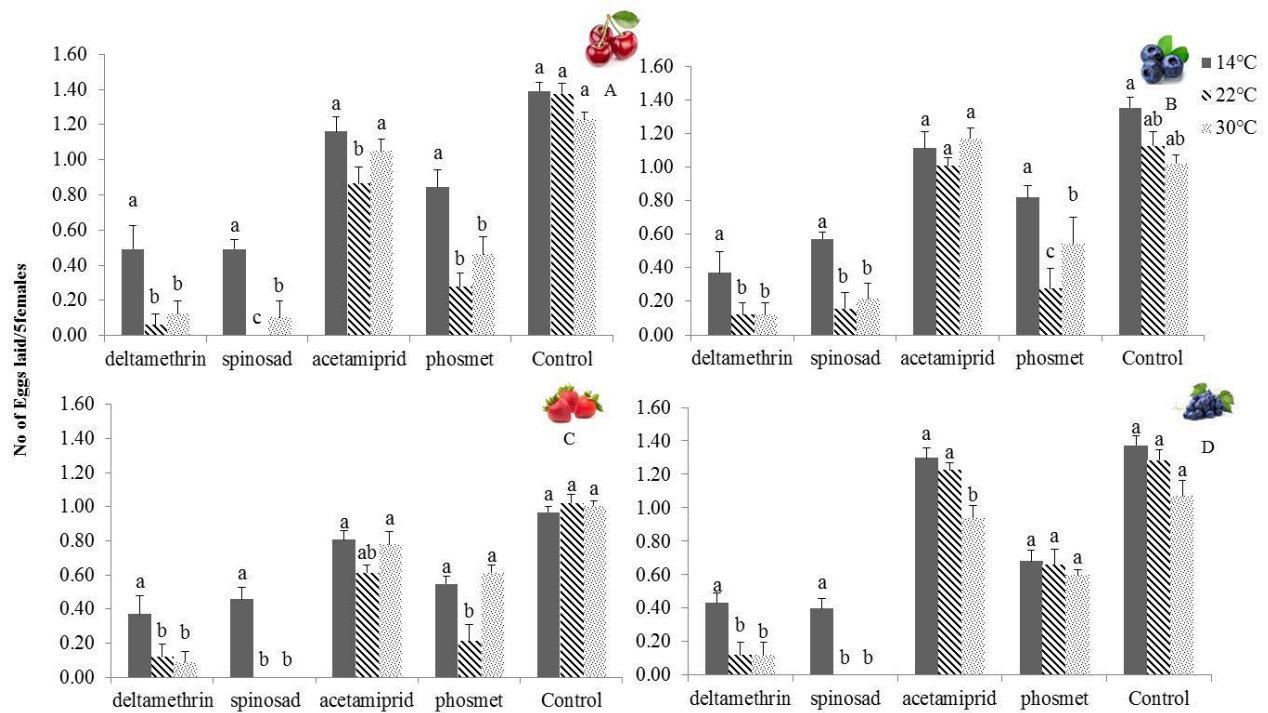


Figure 4. Number of eggs laid (mean \pm SE) by 5 females of *D. suzukii* exposed for 24 h to four types of fruits (A: cherry; B: blueberry; C: strawberry; D: grape) treated with insecticides at three constant temperatures. Different letters above histograms showed significant differences at Tukey's HSD test; P<0.01 within combination fruit and treatment.

Supplementary material

Table 1S. Number of larvae on 100 strawberry fruits observed in the orchards, 4 and 7 days after last application. Means within each column followed by the same letter are not significantly different (Tukey's LSD test; $p < 0.01$).

Insecticide	Plain-Orchard		Hill-Orchard	
	3 days	7 days	3 days	7 days
control	7.3±2.2 A	15.3±3.9 A	14.8±1.9 A	26.0±5.7 A
deltamethrin	0.5±0.6 B	1.5±1.3 C	1.3±1.0 B	5.0±2.5 B
acetamiprid	2.5±1.3 B	9.0±1.4 B	9.0±3.3 A	22.5±4.2 A
spinosad	0.3±0.5 B	1.3±1.3 C	0.8±0.5 B	4.0±1.8 B
F	23.5	35.7	46.7	35.5
P	<0.01	<0.01	<0.01	<0.01

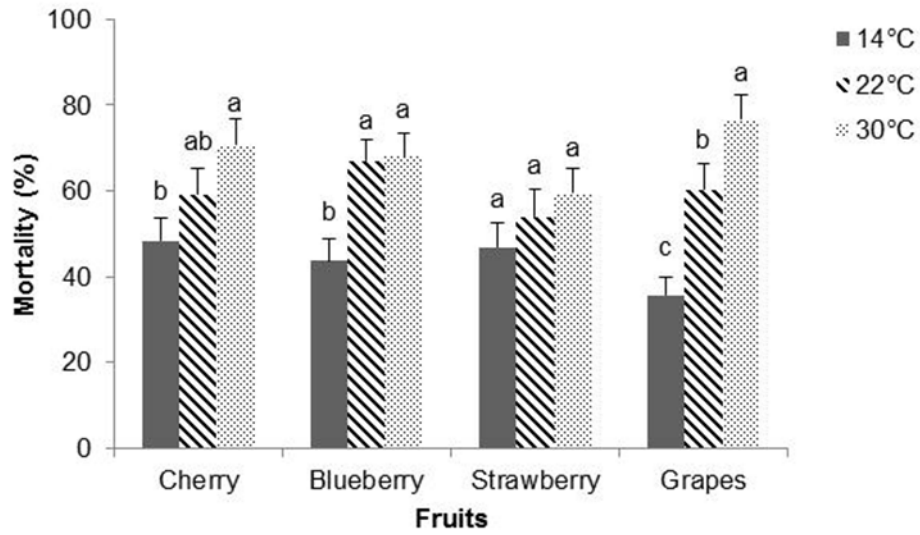


Figure 1S. Average adult mortality of *D. suzukii* (mean \pm SE) exposed for 24 h to treated fruits at three constant temperatures. Different letters above histograms showed significant differences at Tukey's HSD test; $P < 0.01$ within each fruit.

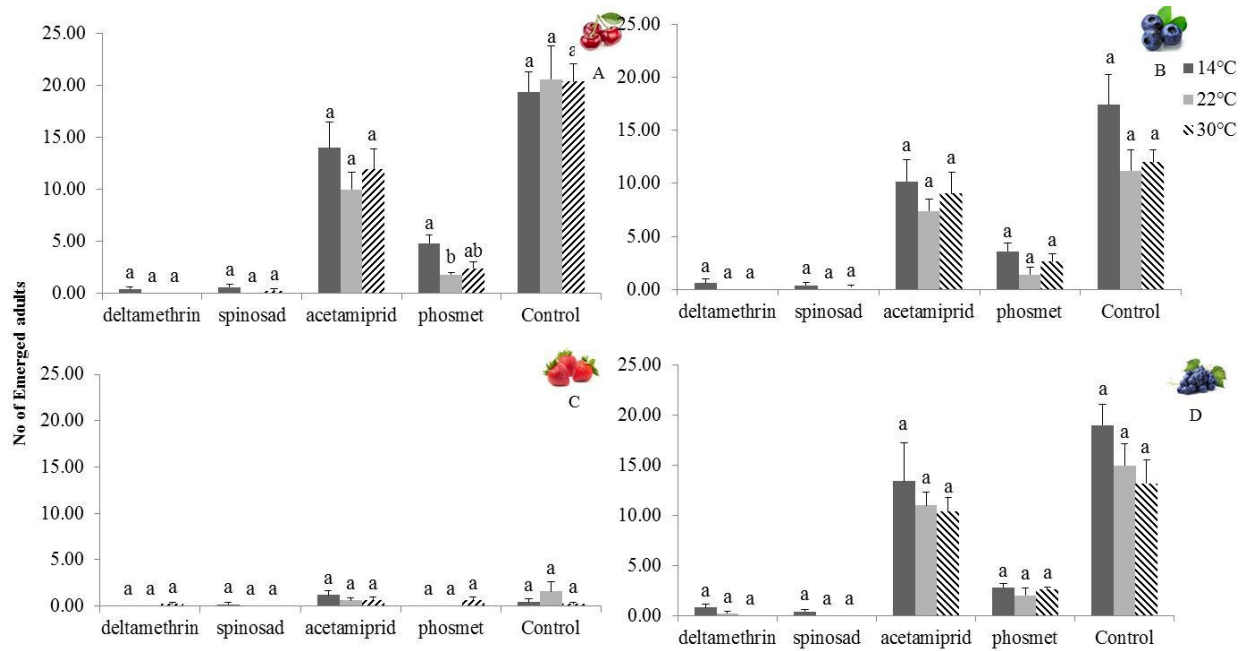


Figure 2S. Number of emerged *D. suzukii* adults (mean \pm SE) from four types of fruits (A: cherry; B: blueberry; C: strawberry; D: grape) treated with insecticides at three constant temperatures. Different letters above histograms showed significant differences at Tukey's HSD test; $P < 0.01$ within combination fruit and treatment.

Chapter 4

Effect of short cold temperature treatment on *Drosophila suzukii* fruit damage

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Saeed N., Tonina L., Battisti A., Mori N. Effect of short cold temperature treatment on *Drosophila suzukii* fruit damage.

Effect of short cold temperature treatment on *Drosophila suzukii* fruit damage

Abstract

Blueberry (*Vaccinium corymbosum*) and strawberry (*Fragaria ananassa*) are fruits heavily colonised by *Drosophila suzukii* in the field while damage increases considerably during the postharvest period. In order to reduce the damage, a short exposure to low temperature can be used to limit the survival of *D. suzukii* eggs and prevent the decline in quality of infested fruits. Berries were artificially infested using adults of *D. suzukii* and kept at 0.5 and 5.0°C for 10 and 24 hours. After the temperature treatment, berries were maintained at 23±2°C recording emergence of flies and damage was visually assessed at 3, 6, and 9 days after the treatment. Berries treated with 0.5°C for 24h had a higher reduction in emergence of adults in both blueberry (83%) and strawberry (59%) and lower fruit damage. The treatment prolonged the shelf life of infested fruits up to 6 days in blueberry and 3 days in strawberry, compared to untreated control. A direct relation was found between the number of emerging adults and the decay index. It is concluded that short cold temperature exposures would be helpful in order to constrain pest development and sustain berry marketability.

Keywords: Cold treatment; Blueberry; Strawberry; SWD; Fruit quality; Shelf life

Introduction

The Spotted Wing Drosophila (SWD) *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) is a dangerous pest due to its capability to lay eggs inside newly ripe fruit (Cini *et al.*, 2012; Lee *et al.*, 2015), its wide hosts range (Dreves *et al.*, 2009; Kenis *et al.*, 2016), rapid development rate (Tochen *et al.*, 2014), lower thermal threshold (Tonina *et al.*, 2016) and high dispersal ability. Its spread is rapidly increasing throughout North America, Europe (Asplen *et al.*, 2015; Lee *et al.*, 2015) and South America (Santos, 2014). Among soft fruits, blueberry, (*Vaccinium corymbosum*) and strawberry, (*Fragaria ananassa*) are species heavily damaged by *D. suzukii* (Goodhue *et al.*, 2011;; Santos, 2014; ; De Ros *et al.*, 2015; Ioriatti *et al.*, 2015; Lee *et al.*, 2015) because its presence makes infested fruit unmarketable, due to a zero tolerance for damage in fresh market fruit. Potential crop losses due to *D. suzukii* in strawberries, cultivated

blueberries, caneberries, and cherries are high, estimated at greater than \$700 million annually only in the USA (Asplen *et al.*, 2015). Individual growers found a drop of 5000 euros/year/farm in strawberry in the south part of France (Senat, 2012) and 3 million €/year for the whole Trento Province (North Italy; De Ros *et al.*, 2013). . As the recent outcomes from the early stages of this ongoing invasion results on economic impact, damages are expected to rise in the future leading to a severe drop of up to 37% in fruit producer's income (Goodhue *et al.*, 2011).

The primary *D. suzukii* management approaches are currently relying on the use of insecticides (Bruck *et al.*, 2011; Haviland and Beers 2012; Diepenbrock *et al.*, 2016). Chemical control can reduce infestation by killing adult flies and larvae present in the fruit (Wise *et al.*, 2015), although it is often not sufficient to prevent damage (Van Timmeren and Isaacs 2013). Trapping (Burrack *et al.*, 2015; Tonina *et al.*, 2017), edible fruit coating (Swoboda-Bhattarai *et al.* 2014), and sanitation (Zalom *et al.*, 2010) are also recommended to reduce the population density of the pest and decrease the number of insecticide applications (Klick *et al.*, 2016).

The control of *D. suzukii* on soft fruits is difficult due to the increase in susceptibility to attack close to harvest (Lee *et al.*, 2011; Swoboda-Bhattarai and Burrack, 2016) and the scalar fruit picking characterized by very restricted turns (De Ros *et al.*, 2013). In addition, eggs laid in ripe fruit are not easily detected by people involved in fruit picking, resulting in the presence of potentially infested fruits inside containers shipped to the market. This is a major problem as damage becomes evident during the marketing phase, and it is further complicated by the spread of mould attack from damaged to undamaged fruits (Grassi *et al.*, 2009; Baufeld *et al.*, 2010; EPPO, 2010). Due to the fact that the use of chemicals is not allowed during fruit storage, pesticide free procedures are necessary to control insect infestations, pathogen infections and sustain fruit quality (Olaimat, 2012). Postharvest cold treatments have been used as a control measure for pests, e.g. *Ceratitis capitata* (Wiedemann) (Lima *et al.*, 2011). Cold storage could be an effective physical measure to limit development and survival of *D. suzukii* as early work in Japan suggested that eggs and larvae in cherries die when held at temperature <2.2°C for at least 96h (Kanzawa 1939). Actually, Aly *et al.* (2017) showed that no eggs or young larvae survived at 1.1° C for 72 h and fewer older larvae survived after 72 h at 1.1, 3.9, and 5.0° C on different fruits in the USA. As soft fruits have a short shelf life (1-2 days according to El-Ramady *et al.* 2015), cold treatment may represent a good option to sustain the marketability.

This study was designed to better understand the effect of postharvest exposure to short periods of low temperature on strawberry and blueberry infested by *D. suzukii* eggs, and the associated decrease in fruit quality. This information would be helpful for further improvements in postharvest management of fruits affected by the pest.

Material and methods

Insect culture

The culture of *D. suzukii* used for the study was obtained from Department DAFNAE-Entomology, from adults collected from cherries and strawberry organic orchards of Verona province, (North-Eastern Italy) in the autumn 2013. The flies were nurtured in plastic vials (Falcon type of 50 ml capacity, 30 mm diameter, 115 mm length) with *D. suzukii* specific rearing medium prepared according to Fondazione Edmund Mach (modified from Bloomington Drosophila Stock Center, Indiana University, 2014) and described in Tonina *et al.* (2016). The medium contained 75 g cornmeal, 17 g yeast, 15 g sucrose, 12 g soybean meal, 5.6 g agar, 5 ml propionic acid and water to 1000 ml. All ingredients were mixed and heated for about 25 min at 100°C, excluding propionic acid that was added at a temperature below 50°C before pouring 15 ml of medium into vials. Insects were reared in climatic chamber at 23±2°C and 70-80% relative humidity with a photoperiod of 16L:8D.

Fruit source and preparation

Fresh insecticide free strawberries (*Fragaria ananassa* cv “Irma”) and blueberries, (*Vaccinium corymbosum* cv “Brigitta”) collected from insect-proof net covered orchards located in Velo Veronese (VR) and Borgo Valsugana (TN) were used for the experiments respectively. Fruits were collected with intact stems and retained in cold rooms at 4°C up to 3 hours before they were exposed to *D. suzukii*. Before the bio-assays, fruit were thoroughly observed under a stereo-microscope to discard those with the presence of eggs, larvae or damages.

Infestation of fruits with *D. suzukii*

The fruits were placed in plastic cages (20×20×20 cm) sealed with fine net. Adults of *D. suzukii*, 4-5 days old, were used for fruits infestation. To achieve enough number of eggs on berries, 200 female and 200 male were released into the cages containing 250 blueberries, 50 female and 50

male were confined in the cages containing 50 strawberries. The infestation was carried out at $23\pm 2^{\circ}\text{C}$ and $65\pm 5\%$ relative humidity with a photoperiod of 16L:8D. Twenty-four hours after the beginning of infestation, the flies were removed and eggs on each fruit were counted under stereo microscope.

Postharvest Cold storage treatments

The infested fruits were placed in 100 mL plastic glasses, one strawberry and five blueberries were used for each replication. The plastic glasses containing fruits were positioned at 0.5°C or 5°C for 10 or 24 h for both species. After cold durations, the glasses were shifted to the laboratory at $23\pm 2^{\circ}\text{C}$ and $65\pm 5\%$ relative humidity. Control was left at $23\pm 2^{\circ}\text{C}$ from the egg laying. After that, the fruits were daily assessed for 2 weeks to record fly emergence. The whole experiment was replicated ten times for each temperature/duration treatment and fruit species.

Measurement of fruit decay

The decay level was evaluated per each berry after 3, 6 and 9 days from cold stress treatment. Decay level of each fruit was usually determined on the basis of scale reported in Table 1 (Ayala-Zavala *et al.*, 2005). Fruit decay incidence was represented as the average of decay number which was obtained from 50 fruits of blueberry and 10 fruits of strawberry for each cold treatment.

Statistical analysis

One-way ANOVA was conducted to check the effect of cold temperature treatments on adult emergence for each fruit, two-way ANOVA was used to check decay of both fruits among treatments, durations, and their interactions. For all comparisons, means were separated by Tukey-Kramer (HSD) test at 5% significance level (Sokal & Rohlf 1995). Abbott's formula (Abbott, 1925) was used to find the corrected reduction in fly emergence. Tests were carried out using general linear models (SPSS 10.01). Correlations between fruit decay and adult emergence from infested fruits were evaluated using Pearson's correlation test on pooled treatments. Effects of single factors (emergence and treatment) and their interactions were evaluated for each storage length using a linear model. Tests were conducted with R (The R Foundation for Statistical Computing <http://www.R-project.org>).

Results

Effect of temperature and storage duration on reduction of adult's emergence

In blueberry, short cold stress showed an effective impact on fruits holding *D. suzukii* eggs ($F_{4, 50} = 15.70$; $P < 0.01$; Fig 1A). In particular, adult emergence was highly suppressed at $0.5^{\circ}\text{C}\times 24\text{h}$ (Abbott efficacy of 83%), $5^{\circ}\text{C}\times 24\text{h}$, $0.5^{\circ}\text{C}\times 10\text{h}$ and $5^{\circ}\text{C}\times 10\text{h}$ (31%) showed a good efficacy (79, 77, and 73% respectively, too).

Like blueberry, cold stress also affected the reduction in adult's emergence on strawberry ($F_{4, 50} = 9.64$, $P < 0.01$; Fig 1B). Inhibition of egg hatching was reported significantly higher at $0.5^{\circ}\text{C}\times 24\text{h}$ (Abbott efficacy of 59%) than $5^{\circ}\text{C}\times 10\text{h}$ with intermediate values at $5^{\circ}\times 24\text{h}$ and $0.5^{\circ}\text{C}\times 10\text{h}$ (50% and 44%, respectively).

Effect of temperature and storage duration on decay level of fruits

Larvae developed from eggs highly impacted the decay of both fruit species ($F_{4, 300} = 73.42$, $P < 0.01$) in all treatments. On blueberry (Fig 2A, 3A) after 3 days of cold stress the decay index was 1.4-1.7 with an average reduction of 60% compared with untreated. At 6 days of storage, only the $0.5^{\circ}\text{C}\times 24\text{h}$ and $5^{\circ}\text{C}\times 24\text{h}$ reduced the fruit decaying, after 9 days blueberry achieved 4-5 decay index in all treatments. In strawberry the decay index was 2.7-3.5 after 3 days of shelf life (Fig 2B, 3B) with a decay fruit reduction ranging to 62% ($0.5^{\circ}\text{C}\times 24\text{h}$) to 44% ($5.0^{\circ}\times 24\text{h}$). At 6 days of storage the strawberries were completely deteriorated.

A significant correlation between decay index and adult emergence was observed in blueberry (Figure 4A) at 6 days after treatments for pooled samples ($t = 4.37$, $df = 48$, $P < 0.01$, $r = 0.53$) and in strawberry (Figure 4B) at 3 days after treatment ($t = 6.32$, $df = 48$, $P < 0.01$, $r = 0.673844$). Interactions of adult emergence with fruit decay and treatments were not significant ($F_{4,40}=0.081$, $P=0.99$ and $F_{4,40}=0.705$, $P=0.59$ for blueberry and strawberry, respectively) and consequently not considered in the correlations.

Discussion

Due to the fact that soft fruit *D. suzukii* infestation cannot easily be detected before harvest, especially when a relatively low infestation is recorded in the field, growers have two alternatives: (i) they may decide not to sell their fruit or (ii) they may adopt different

combinations of cold treatments to keep damage to an undetectable level. Soft fruits are characterized by short storage life (El-Ramady *et al.*, 2015) and for that reason fruit handling between field and market must be faster. Our findings show that a short cold temperature exposure may reduce *D. suzukii* adult emergence and damage in infested blueberries and strawberries.

The treatment 0.5°Cx24h showed the best reduction in emergence of adults. This result is in agreement with the data on postharvest cold storage on the development and survival of immature *D. suzukii* at 1.1° – 5.0°C but with a longer duration (72h; Aly *et al.*, 2017). Three days of exposure to cold conditions may not be applicable in fast chains, where shorter periods at lower temperature are a possible alternative. Keeping temperature just above zero for some hours allows to reduce the time required to kill eggs and young larvae (Stephens *et al.*, 2015; Plantamp *et al.*, 2016).

The reduction in adult emergence, as a consequence of eggs and young larvae mortality, was correlated with the quality decay level. The short cold temperature exposures significantly prolonged the shelf life at room temperature of infested blueberries and strawberries. No defects were observed in blueberries kept for 3 days after cold treatment at 0.5 and 5.0°C for 24 h, while minor damage was seen for the 10h treatment. Due to different susceptibility of fruits to *D. suzukii* damage (Burrack *et al.*, 2013; Lee *et al.*, 2015), at the same storage conditions strawberries had higher decay level than blueberries.

Postharvest short-term cold storage of infested fruits at 0.5°Cx24h was found to be effective in reducing the emergence of adults and in maintaining fruit quality long enough to allow marketability. Both in the field and at market level, farmers and traders have been taking an interest in what happens when they transport fruit infested with *D. suzukii* eggs to the consumer using refrigerated trucks. Indeed, short cold duration would be helpful in reducing egg vitality and decrease damage. Further work is required to test the optimal conditions for integrating such a treatment in the soft fruit processing, packaging, delivery, and selling, within the general framework of reducing the impact of *D. suzukii* (Diepenbrock *et al.*, 2016).

Table 1. Scale for the measurement of quality decay level for fruits infested by *D. suzukii* (modified from Ayala-Zavala et al., 2005).

Decay level	Description	Overall quality index
0	No damage	excellent
1	intact fruit (slight imperfection, entry hole may be hidden)	good
2	less than 5% decay (sign of feeding; some frass; fruit less turgid)	acceptable
3	between 5-20% decay (feeding more evident; more frass; slight wrinkling)	bad
4	between 20-50% decay (much feeding and frass; tunnelling obvious; extensive wrinkling)	unacceptable
5	more than 50% decay (fruit surface open; flesh separation)	unacceptable

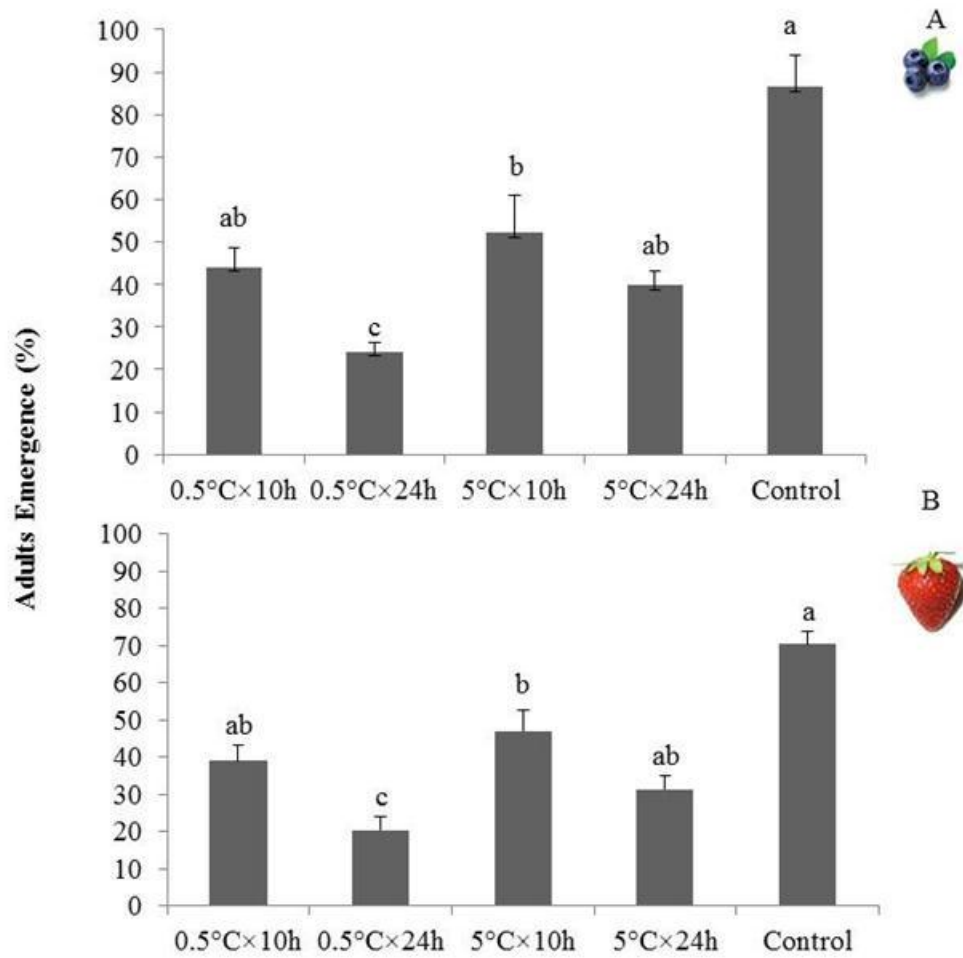


Figure 1. Adult emergence (percentage of decline of adults emergence in respect of eggs laid) under different treatments on (A) Blueberry and (B) Strawberry. Bars show standard error and different letters show significant differences at Tukey's HSD test; $p=0.05$.

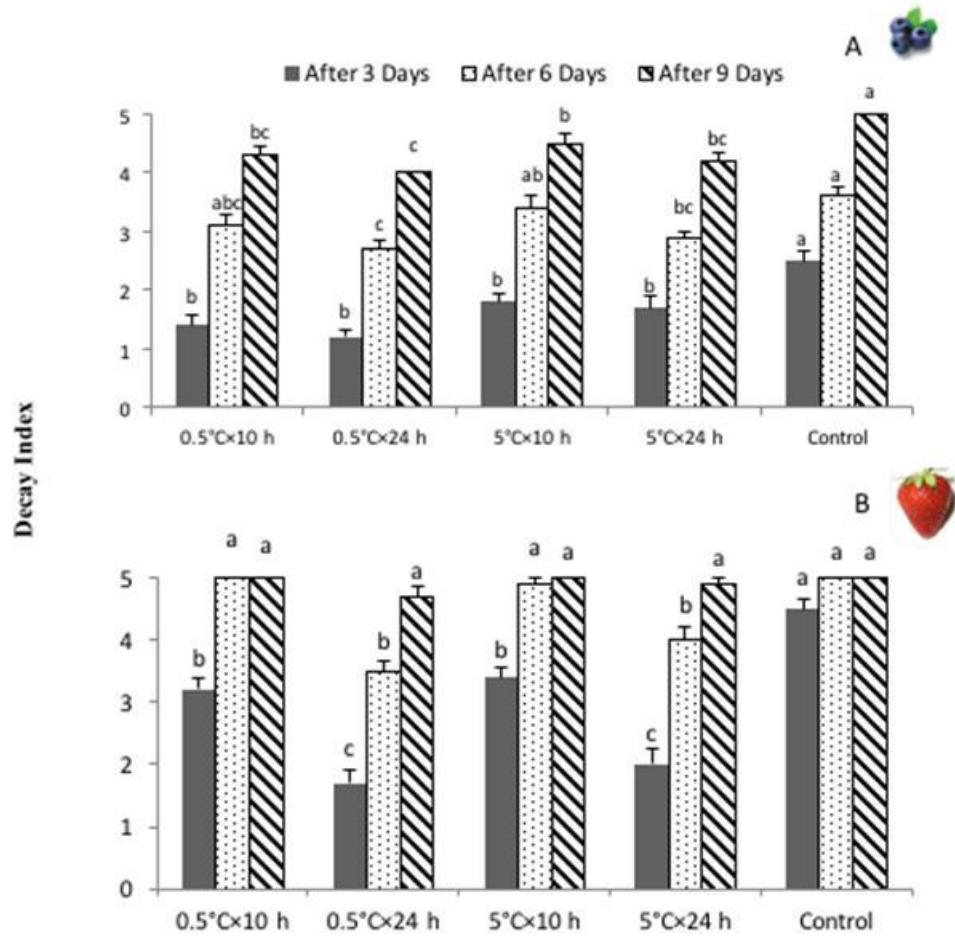


Figure 2. Effect of treatment on decay level on (A) Blueberry and (B) Strawberry after 3, 6 and 9 days from treatment. Bars show standard error and different letters show significant differences among treatments within day at Tukey's HSD test; $p=0.05$

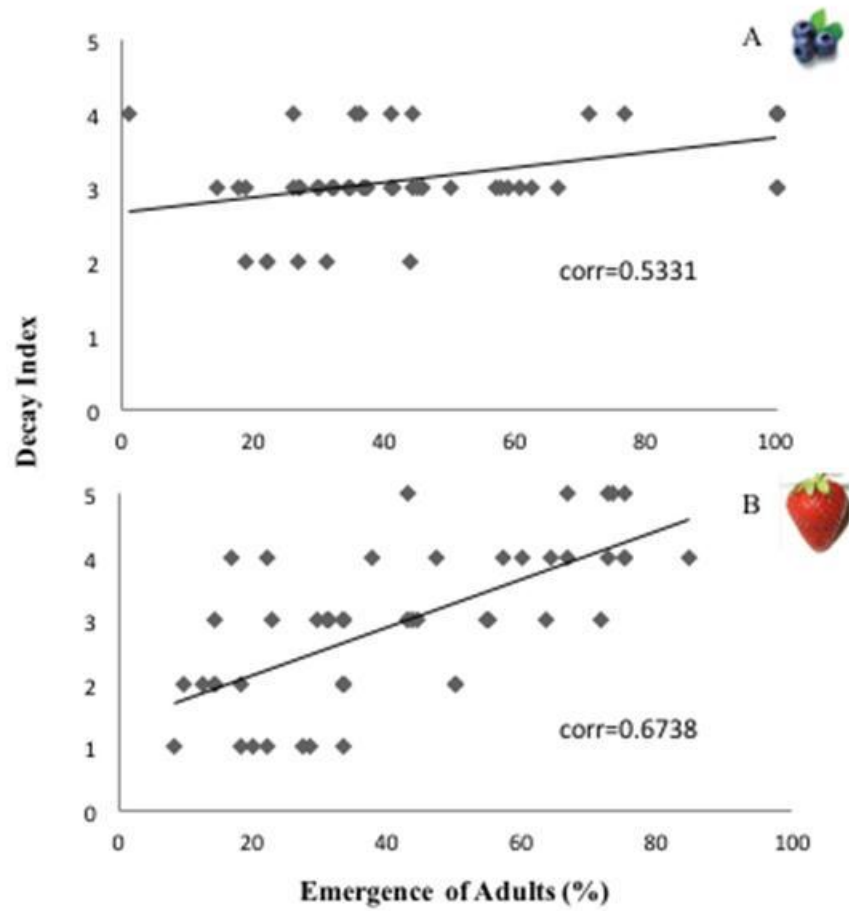


Figure 3. Correlation between decay level and adult emergence (as percentage of laid eggs) in (A) blueberry 6 days after treatments and (B) strawberry 3 days after treatments

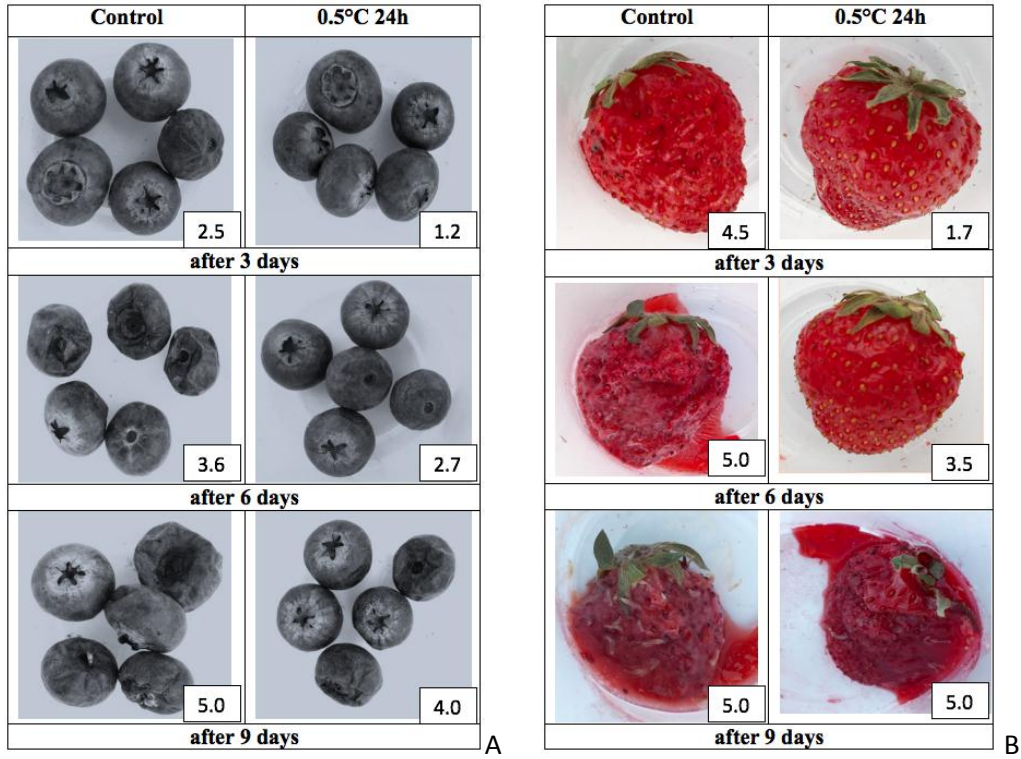


Figure 4. Quality decay level on infested (A) blueberry and (B) strawberry after cold stress at 0.5°C×24h. Numbers indicate the average decay index obtained in each treatment.

Chapter 5

Conclusions

Conclusions

Since 2008, the appearance of *Drosophila suzukii* in Europe and its large spread has alarmed vital regions of fruit production (Cini *et al.*, 2012; Deprá *et al.*, 2014; Hauser, 2011). Being a pest, its performance has adversely influenced by creating loss of produce and higher price in manufacturing of small and stone fruits (Bolda *et al.*, 2010). Growers mainly depend on the use of insecticides in order to handle populations of *D. suzukii*, although this is difficult because of the concealed nature of the insect, the short development time, and the limitation of registered molecules. Management measures can benefit from information regarding thermal response of *D. suzukii* to factors such as *Wolbachia*, pesticides, and host fruits. Similar investigations on *D. melanogaster* have revealed how temperature affects the response to insecticide applications on this fly in from three regions (Fournier-Level *et al.*, 2016), and how *Wolbachia* infection interacts with temperature (Kusmintarsih 2012; Strunov *et al.*, 2013). The present study has specifically addressed the relationships between temperature and performance of *D. suzukii* under variable conditions of *Wolbachia* infection, pesticide applications, and host fruits.

The first objective was to explore the effects of nutritional stress and *Wolbachia* on survival, fecundity, cytoplasmic incompatibility and viability of *D. suzukii* at fluctuating temperatures. Cultures of *D. suzukii* were maintained at different temperature regimes in order to check the number of eggs, hatch rate, adults and survival on two types of diet media (poor and normal). Development time from egg to adult and lifespan of adults were used as dependent variables. Elevated temperature significantly impacted the survival, fecundity, CI and viability. *Wolbachia* did not show any impact on nutritional and starvation stress whereas it responded to temperature. At 29°C, treated flies displayed longer survival rather than untreated. Fecundity was not variable in treated and untreated flies while egg-hatching rate was. This study suggests that the lower performance of *D. suzukii* at high temperature can be mediated by the effects on its symbiont.

The second objective was to explore how temperature modifies the efficacy of insecticides by considering exposure through topical and residual methods. Field and laboratory experiments were designed in order to test the efficacy of major types of insecticides available in Italy against this pest. Pyrethroids and spynosins proved to be the most effective under all

temperature conditions. Organophosphates, tested only in the laboratory and neonicotinoids showed lower efficacy at low temperatures, indicating that they are not suitable to protect crops under those conditions. The results indicate that the management of the pest in cold habitats, that are very suitable for the cultivation of high quality berries such as in mountain farming systems, should take into account such limitations and organize IPM strategies accordingly.

The third objective was to elucidate the effect of short exposures to low temperature on fruits containing eggs/larvae of *D. suzukii* and the consequent decay level of fruits. Strawberries and blueberries were artificially infested and then exposed to different combinations of low temperature and time interval. Short cold stress highly suppressed the pest and reduced decay index of fruits. This experiment may open a way to the exploration of thermal treatments right after harvest, for reducing the development of *D. suzukii* and maintain a quality of fruits suitable for food chain process.

Upcoming efforts should be targeted at discovering how bacterial symbionts could be manipulated with temperature to achieve better pest control, especially under conditions of highly fluctuating temperatures. Additional studies involving more populations of *D. suzukii*, different host plants and a wider range of temperatures are needed. A similar approach should be used to better understand the efficacy of insecticides under different environmental conditions in the field, where the need for a safer management of the pest are highly needed. Cold storage could be an interesting addition to the pool of management measures available against the pest, and it need to be tested on more diverse host plants and storage conditions.

Chapter 6

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