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Doctoral Thesis

"Exploitation of population dynamics and chemical communication for integrated management of *Drosophila suzukii*"

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Preface

This Ph.D. thesis is the result of three years of work carried out at the Edmund Mach Foundation (FEM) under the supervision of Professor Gianfranco Anfora and at the Oregon State University under the supervision of Professor Vaughn M. Walton. Financial support for this study was provided by the University of Udine, Italy and Edmund Mach Foundation.

The Ph.D. project proposal was conceived to elucidate multiple aspects of the biology of the invasive species *Drosophila suzukii,* and the main goal was to find management alternatives to chemical treatments.

The thesis is thus focused on different life traits of *D. suzukii*, presenting an innovative and effective free-insecticide attractant matrix for controlling damages and spreads of this invasive species. The short general discussion at the end aims at putting the obtained results into a broader perspective.

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Publications during candidature and my contribution

I have participated in all stages of the preparation of these papers, which included the experimental plan design, performing the experiments, data analysis and writing the manuscript.

Tait G*., Vezzulli S., Sassù F., Antonini G., Biondi A., Baser N., Sollai G., Cini A., Tonina L., Ometto L., Anfora G. (2017) **Genetic variability in Italian populations of *Drosophila suzukii***. *BMC Genetics* **18**:87.

Tait G*., Grassi A., Pfab F., Crava M. C., Dalton D. T., Magarey R., Ometto L., Rossi-Stacconi M. V., Gottardello A., Pugliese A., Firrao G., Walton M. V., Anfora G. (2018) **Estimates of *Drosophila suzukii* population dispersal and dynamics in Trentino, Italy**. *Journal of Pest Science.* 10.1007/s10340-018-0985-x.

Tait G., Kaiser C., Dalton D.T., Rossi-Stacconi M. V., Anfora G., Walton M. V. (2018) **A food-grade gum as a management tool for *Drosophila suzukii*.** Bulletin of Insectology LXXI(2):295-307

Tait G., Cabianca A., Grassi A., Pfarb Ferdiand., Oppedisano T., Puppato S., Mazzoni V., Anfora G., Walton M. V. (2018). **Mark-capture based monitoring to evaluate *Drosophila suzukii* daily small-scale movement.**

Tait G., Park K., Nieri R., Clappa E., Boyer G., Crava M. C., Rewerts J., Mermer S., Dalton T. D., Carlin S., Brewer L., Field J., Walton M. V., Anfora G., Rossi Stacconi M. V. (2018). **Egg–laying behavior of *Drosophila suzukii* with evidence of an oviposition marking pheromone.**

I have contributed to perform the experiments, data analysis and writing of the manuscript.

Grassi A., Gottardello A., Dalton D. T., **Tait G**., Rendon D., Ioriatti C., Gibeaut D., Rossi Stacconi M. V., Walton M.V. (2017) Seasonal Reproductive Biology of *Drosophila suzukii* (Diptera: Drosophilidae) in Temperate Climates. *Journal of Environmental Entomol*ogy**.** 10.1093/ee/nvx195

Rendon D., Lee J., **Tait G**., Buser J., Walton M. V. (2018). **Survival and fecundity parameters of two *Drosophila suzukii* morphs on variable diet under suboptimal temperatures.** Journal of Insect Science DOI:10.1093/jisesa/iey113

Rendon D., Walton M. V., **Tait G.**, Buser J., Lemos Souza I., Wallingford A., Loeb G., Lee J., (2018). **Interactions among morphotype, nutrition, and temperature impact fitness of an invasive fly**. *Ecology and Evolution.* DOI: 10.1002/ece3.4928

Crava M. C., Sassu F., **Tait G**., Becher P., Anfora G. (2018). **The antennal transcriptome of *Drosophila suzukii*, and transcriptional changes associated with female mating status.** https://doi.org/10.1016/j.ibmb.2018.12.012

Sierras N., Marin C., Botta A., **Tait G**., Merli D., Carlin S., Malacrida A. R., Gasperi G., Anfora G., Scolari F. (2018). **Olfactory responses of the Mediterranean fruit fly, *Ceratitis capitata*, to the Cera trap® lure: exploring the bases of attraction.** Submitted to Frontiers Cellular Neuroscience

Aim of the work

Drosophila suzukii (Matsumura), also known as Spotted Wing Drosophila (SWD), is a vinegar fly belonging to the Diptera order and *Drosophilidae* family. It is native to South East Asia and it has recently invaded western countries, affecting fruit production. This pest species is considered alien owing to its great tolerance of a wide range of climatic conditions, extreme fecundity, wide host range and high dispersal potential. Because of its rapid spread, the economic impact has been huge in most of the invaded countries. *D. suzukii* infestations generate direct and indirect economic impacts, through yield losses, shorter shelf life of infested fruits, extra labor and costs for monitoring, field sanitation and post-harvest handling (especially in organic production) as well as the closure of international markets for fruits produced in infested areas. For all these reasons, deeper information about the biology, ecology, chemical-ecology, genetics and behavior are necessary to better manage the species. This thesis was developed to contribute towards a better understanding of these topics, in order to find a scientifically supported and effective solution. In particular, this work focused on four aspects: 1) analyze the genetic structure of different Italian populations, 2) understand the spread and therefore the capacity of movement of *D. suzukii* in large and small scale, 3) study the oviposition behavior with an in depth characterization of the chemical volatiles involved in the substrate choice and 4) manipulate the egg-laying behavior thanks to an innovative lure, which can work as an alternative oviposition substrate.

Taken together, the results presented in this thesis highly contribute to enhancing our understanding of *D. suzukii* biology, ecology and chemical and to expand integrated pest management options.

Abstract

Drosophila suzukii, an insect pest from South-East Asia, invaded America (USA) and Europe (EU) continents in 2008 and since then has caused extensive economic damage to the soft fruits' industry all over the world. *D. suzukii* population control mostly relies on the use of chemical pesticides, practice with serious drawbacks because of their use close to harvest and the following risk of high residues left on fruits. Pest management programs are, therefore, in permanent search for alternative strategies including those based on biocontrol.

In order to identify new solutions, in depth studies concerning the spread capacity, ecology, behavior and chemical ecology of the species are requested. For this reason, part of my thesis focused on some basic aspects of *D. suzukii* biology and ecology, in particular the genetic structure characterization of Italian *D. suzukii* populations (chapter 2), the dispersal ability of the species (either large and small scale, and in close proximity of host fruits) (chapters 3 and 4), the oviposition behavior (Chapter 5) and its oviposition manipulation (chapter 6). In the first chapter, we characterized the genetic structure of the invasive species *D. suzukii* in Italy. Our analysis showed extensive genetic homogeneity among *D. suzukii* collected in Italy. The relatively isolated Sicilian population suggests a largely human-mediated migration pattern while the warm climate in this region allows the production of soft fruit, and the associated *D. suzukii* reproductive season occurring much earlier than on the rest of the peninsula.

Understanding seasonal and daily migrations *D. suzukii* populations is fundamental, since it could help farmers and industries decide where to apply specific management techniques. This was the aim of the third and fourth chapter. Furthermore, the finding of a consistent seasonal and daily movement from the crop to the surrounding vegetation and towards areas with different altitude, may lead to use treatments, especially chemical, in precise periods of the year and in defined zones on the border of the crop. In both papers we found that abiotic factors, such as temperature and humidity, play a fundamental role in the environmental distribution of the species. In particular, in the third chapter data seem to indicate that as soon as seasons change, and as a consequence the temperature and the availability of food, *D. suzukii* adults are able to move towards different altitude in order to exploit better conditions for their survival. In the fourth chapter, focused

on the movement of the species between a crop and the surrounding forests, results provide evidence that suboptimal cropping conditions with respect with fly development coupled with more optimal surrounding vegetation environments can respectively act as ‘push and pull’ forces for *D. suzukii* populations.

The fifth chapter was focused on the oviposition behavior of *D. suzukii*. The goal of this work was to elucidate behavioral and chemical aspects of short-range site selection of this species. Video recording evidenced several discrete and repeated steps during oviposition. In particular the final step was the release of an anal secretion over the fruit surface near the oviposition site. Choice and non-choice tests indicated oviposition preference towards egg-infested fruits, suggesting a role of the released liquid as a marking pheromone attracting multiple oviposition. The knowledge gained with this study may accelerate establishment of control strategies based on the interference and disruption of the *D. suzukii* communication during the oviposition processes.

Finally, the main target of my sixth chapter had more practical purposes focusing on the identification of new attractants for *D. suzukii* able to interfere with its movement and, consequently, with the potential to be used as baits for pest monitoring and control. This project, carried out at the Oregon State University, led to the development of a new gum matrix as a potential sink for *D. suzukii* eggs in a commercial-standard cropping system. Field trials were thereafter conducted over periods of 72 ± 2 to 96 ± 2 hours on commercial-standard blueberry bushes. Fruit on bushes exposed to predetermined numbers of *D. suzukii* displayed 50 to 76% reductions of fruit infestation and total eggs laid, respectively, in gum treatments.

In conclusion we can say that all together the data collected during three years of PhD and the performed analyses have definitely increased the knowledge about the species and more importantly, support a valid new control alternative against the invasive species *D. suzukii*.

Riassunto

Drosophila suzukii, una specie invasiva proveniente dal sud-est asiatico, dal 2008 ha invaso sia il continente americano che l'Europa, e da allora ha causato danni ingenti all'industria dei piccoli frutti. Il controllo di questa specie dannosa si basa principalmente sull'uso di insetticidi, pratica che a lungo andare causa seri effetti negativi agli ambienti naturali e agli agroecosistemi e, per via dei residui che si possono accumulare sui frutti trattati prossimi alla raccolta, alla salute umana. I nuovi programmi di controllo sono pertanto rivolti alla ricerca di strategie alternative incluse quelle basate sul biocontrollo.

Per accellerare la messa a punto di nuovi strumenti, sono necessari studi approfonditi riguardo la capacitá di diffusione, l'ecologia, la biologia e il comportamento. Per tale ragione, parte della mia tesi si basa su aspetti quali la struttura genetica di popolazioni campionate in diverse regioni della nostra Penisola (capitolo 2), sulla capacitá di dispersione della specie (sia ad ampia che a piccola scala, e in prossimitá dei campi coltivati) (capitoli 3 e 4), il suo comportamento di oviposizione (capitolo 5) e come si puó manipolare il suo comportamento di oviposizione attraverso la creazione di un alternativo substrato di oviposizione (capitolo 6). Nel secondo capitolo i dati raccolti hanno permesso di definire come la popolazione Siciliana sia quella geneticamente piú isolata e che il trasporto di merci sia la principale fonte di dispersione tra diverse localitá della specie invasiva. Allo stesso tempo risulta fondamentale conoscere come la specie percorre lunghe o brevi distanze stagionali e/o giornaliere in modo da poter aiutare gli operatori agricoli a definire quando e dove applicare i trattamenti siano essi chimici o di altra natura. Questo é stato lo scopo del terzo e del quarto capitolo. I risultati ottenuti evidenziano uno spostamento giornaliero tra il campo coltivato e il bosco adiacente in relazione al cambio di temperatura e umiditá, cosí come migrazioni tra altitudini differenti sempre ricoducibili alle variazioni di paramentri abiotici e di risorse alimentari. In particolare nel terzo capitolo i dati sembrano indicare che quando la temperatura, e di conseguenza la disponibilitá di cibo iniziano a variare, *D. suzukii* é in grado di migrare ad altitudini diverse per riuscire a sfruttare al meglio queste risosre e trovare un ambiente favorevole alla sopravvivenza. Nel quarto capitolo, focalizzato sul volo giornaliero tra ambiente coltivato e bosco circostante, i risultati sembrano evidenziare che condizioni subottimali del campo

coltivato accoppiato con migliori condizioni abiotiche nelle zone circostanti possono rispettivamente fungere da ‘push and pull’ nella distribuzione di *D. suzukii*.

Il quinto manoscritto di questa tesi si focalizza sul comportamento di oviposizione di *D. suzukii*. Il goal finale di questo progetto é quello di elucidare l’aspetto comportamentale e chimico della selezione di siti di oviposizione della specie. Le registrazioni video hanno permesso di evidenziare diversi comportamenti ripetuti durante la fase di oviposizione. In particolare, dopo l’oviposizione dell’uovo all’interno del frutto, la femmina rilascia sulla superficie dello stesso una secrezione anale. I test di comportamento indicano che le femmine sono attratte maggiormente verso frutti giá infestati, suggerendo pertanto la presenza di feromoni di attrazione in tale secrezione. Le conoscenze che traiamo da tale lavoro potrebbero acellerare metodi di controllo innovativo basati sull’interferenza della communicazione olfattiva durante il comportamento di oviposizione.

Il mio ultimo progetto presentato in questa tesi, sesto capitolo, ha uno scopo piú strettamente pratico basato sull’identificazione di attrattivi per *D. suzukii* capaci di interferire con il comportamento di oviposizione della specie in pieno campo, e pertanto con l’obiettivo di essere usati in trappole innovative per il monitoraggio e controllo della specie. Questo lavoro, sviluppato all’Oregon State University, ha portato allo sviluppo di substrato di oviposizione artificiale altamente attrattivo. I risultati ottenuti in campo, dimostrano una riduzione dell’infestazione dei frutti pari al 50%, e una riduzione del numero di uova deposte su di essi pari al 76%.

In conclusione possiamo affermare che le ricerche svolte durante i tre anni di dottorato incrementano significativamente le conoscenze sulla specie e cosa piú importante supportano lo sviluppo di nuovi validi mezzi di controllo di *D. suzukii.*

CHAPTER 1.
Introduction

Introduction

1.1 *Drosophila suzukii*, a new global pest

The spotted wing drosophila (SWD), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), is a pest species which has spread from its original range in Asia to a number of western countries in the past decade, including the Mediterranean basin (Asplen *et al.*, 2015; Atallah *et al.*, 2014), Europe, and USA (Hauser *et al.,* 2011; Cini *et al.,* 2012). The history of the geographical spread and infestation of *D. suzukii* is still under investigation: it is known that this species was first recorded in Japan in 1939. Initially it was described by Dr. Shounen Matsumara in 1931 as *Leucophenga suzukii* then amended by Kanzawa as *Drosophila suzukii* cherry fruit fly (Kanzawa, 1934). Since it first recognized it was then recorded in other Asian countries included Thailand, Korea, China, India and Russia (Hauser *et al.,* 2011; Calabria *et al*., 2012; Walsh *et al.,* 2011). In Hawai islands the first recognition happened in 1983 (Kaneshiro, 2983). Europe and the Americas were colonized much later, possibly during the last 9 years (Lee *et al*., 2011; Hauser *et al.*, 2011; Cini *et al.*, 2012; Deprà *et al.*, 2014). First adults of *D. suzukii* were caught contemporaneously in the region of Catalonia, Spain (Calabria *et al.*, 2012) and in Tuscany, Central Italy, in 2008 (Cini *et al.,* 2012). In 2009 *D. suzukii* individuals were found on both wild hosts (Vaccinium, Fragaria and Rubus spp.) and several species of cultivated berries in Trento Province, North Eastern Italy, where also the first economically important damage by this species in Europe was reported (Grassi *et al.*, 2011). In Italy the species was registered in multiple regions: Piemonte, Valle d'Aosta, Lombardia, Veneto, Emilia Romagna, Liguria, Marche and Campania. Always during years 2010 and 2011, *D. suzukii* was recorded also in France, Switzerland, Slovenia, Croatia, Austria, Germany and Belgium (Cini *et al*., 2012). During the following years, *D. suzukii* has been spreading rapidly across Europe, with documented infestations ranging from Mediterranean regions (i.e. Greece, Turkey) to northern latitudes (i.e. Sweden, Poland, UK) (EPPO Global Database, *Drosophila suzukii* – DROSSU, 2017) (Figure 1).

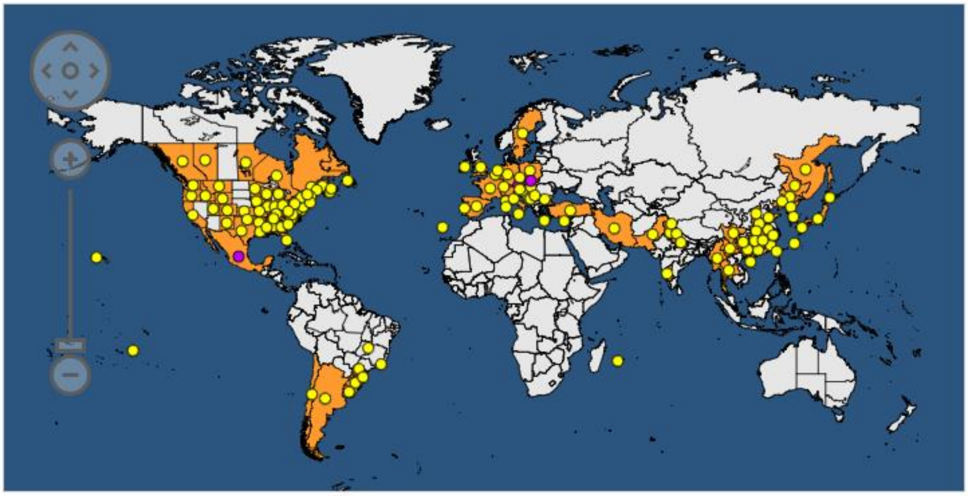

Figure 1: *D. suzukii* worldwide distribution (https://gd.eppo.int/taxon/DROSSU/distribution).

In contrast to other *Drosophila* species, *D. suzukii* threats ripe and un-damaged fruits by laying eggs under their skin by means of a serrated ovipositor of females (Walsh *et al.*, 2011; Cuthbertson *et al.*, 2014; Emiljanowicz *et al.*, 2014). Evolution of the ovipositor in *D. suzukii* acted to make females able not only to puncture skin of intact fruits and laying eggs inside, but also to change the insect diet from protein-rich (as other *Drosophila* flies that feed on rotten fruits) to carbohydrate rich (Rendon *et al*., 2018). Although it is still not known how can *D. suzukii* larvae develop with low dietary protein, this adaptation is important for larvae to avoid interspecific competition by utilizing unused food resource, whereas adults still feed on rotten fruits and fermenting material (Jaramillo *et al.*, 2014; Rendon *et al*., unpublished data).

D. suzukii is a highly polyphagous species, attacking several economically important crops (Kanzawa 1939; Bolda *et al.,* 2010; Grassi *et al.,* 2011; Lee *et al.,* 2011; Walsh *et al.,* 2011; Bellamy *et al.,* 2013). In addition to cultivated crops, this alien pest has been reported to attack more than 50 wild plants in Europe and the USA (Baroffio *et al.,* 2015; Lee *et al.,* 2015; Poyet *et al.,* 2013; Briem *et al.,* 2016; Kenis *et al.,* 2016). For example, jujube (*Ziziphus jujube* Mill) and rough bindweed (*Smilax aspera L.*) are wild hosts that serve as reservoirs of the pest and insure the re-infestation of the crops throughout the year (Baser *et al.,* 2015). This frugivorous pest is known for being a multivoltine species due to its high fecundity and short developmental time per generation thus resulting in fast outbreaks of attacks (Kanzawa 1939). *D. suzukii* is not only highly polyphagous (Cini *et al*., 2014; Kenis *et al*., 2016), but is well adapted to temperate climatic conditions characterized by alternations of warm summers and cold winters (Mitsui *et al*., 2010; Anfora *et al.,* 2012; Ometto *et al.,* 2013; Shearer *et al.,* 2016; Rossi Stacconi *et al.,* 2016). All these factors allow the species to survive even in an alpine climate, where the temperature has ample fluctuations during the year. Another important characteristic is that *D. suzukii* developed the ability to overwinter in reproductive diapause in a distinct relatively cold-resistant morphotype (Ometto *et al*., 2013; Stacconi *et al*., 2016; Shearer *et al*., 2016). Actually, it has developed a tolerance to climatic variations by morphological changes of the colour and thickness of the body (Anfora *et al.,* 2012; Ometto *et al.,* 2013; Shearer *et al.,* 2016; Stacconi *et al.,* 2016).

Such escalating records highlight the role of *D. suzukii* as a major global pest of fruit industry, calling for an immediate and a serious pest management program. Various researchers, local growers, producers from different European countries met in 2012 to exchange information and research ideas to understand this pest' biology and ecology (Cini *et al*., 2012). It has been since then that almost 400 research articles have been published in the last five years describing the bio-ecological features, invasive history and management practices of this pest.

1.2 Morphology and identification

The species is also known as Spotted Wing Drosophila (SWD). The name comes from the male individuals carrying a dark spot on the top edge of each wing. Females do not carry any wing-spot, instead, possess large serrated ovipositor (Kanzawa 1934; Hauser *et al*., 2011; Calabria *et al*., 2012; Walsh *et al.,* 2011) (Figure 2): this, together with the typical *Drosophila* male tarsal sex combs, make it a certain sexual dimorphic species. Based on phylogenetic analysis, *D. suzukii* belongs to the *suzukii* subgroup within the *melanogaster* species group, along with its close relatives *D. pulchrella* (Van der Linde *et al.,* 2008) and *D. biarmipes* (Chiu *et al*., 2013; Ometto *et al*., 2013). *D. takahashii* of the *takahashii* subgroup is sister to the *suzukii* subgroup (Van der Linde *et al*., 2008; Ometto *et al*., 2013; Chiu *et al*., 2013). The drosophila flies ancestors were characterized by short, weakly sclerotized ovipositors, which specifically evolved in long, strongly saw-like ovipositors in *D. suzukii* and some closely related species such as *D. pulcherella* and *D. subpulcherella* (Sasaki *et al.,* 1996). Since these species share similar serrated ovipositor morphology but vary in their shapes, Atallah *et al*. (2014) suggested that ovipositor shape evolved after the evolution of enlarged bristles around it. The female serrated ovipositor and the male wing spot are the key characteristic used for identifying *D. suzukii* in the field, although their presence in other species can make identification more challenging. For example, the serrated ovipositor is shared with *D. subpulchrella* and *D. pulchrella* (Takamori *et al.,* 2006), while similar wing-spots characterize *D. biarmipes* (Hauser *et al*., 2013) and *D. immacularis* among others (Okada 1954; Kimura 2004). Other characters, such as the number and position of sex combs on the fore tarsi may definitively guide to their correct identification (Cini *et al.,* 2012). *D. suzukii* males have two sets of sex combs on their front legs: one on the first tarsal segment and another on the second, placed in the direction of the leg (Cini *et al*., 2012; Hauser *et al*., 2013). Sex combs of *D. biarmipes*, however, are only present on the first tarsomere and placed in two separated rows (Hauser *et al.,* 2013), and *D. subpulchrella* has sex combs arranged in two separated rows (Takamori *et al.*, 2006).

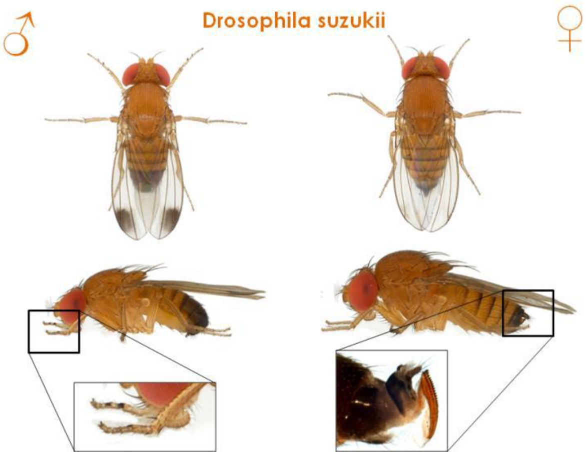

Figure 2: Male (left) and female (right) of *D. suzukii* (source: Gompel, 2012).

1.3 Biology and ecology

D. suzukii is not a single strict fruit-specialist but can easily attack a large variety of soft-bodied fruits such as blueberries, strawberries, cherries, raspberries and blackberries although it can occasionally infect hard fruits likes apples and pears as well (Walsh *et al*., 2011, Dreves *et al.,* 2009; Poyet *et al*., 2013). The wide range of hosts (more than 30 fruit species) and the fast dispersal potential have allowed *D. suzukii* to fully exploit wild land hosts that ripe throughout the year (Bolda *et al*., 2010; Lee *et al*., 2011; Asplen *et al*., 2015). An adult of *D. suzukii* reaches the sexual maturity one-two days after the emergence, in relation to the weather condition. The coupling is possible since the first day of life and females are able to lay eggs since the second day. During the life a mature female can lay around 400 eggs. Eggs hatch after one-two days after having been laid, and larvae need 3-15 days before becoming pupae. The pupae can persist inside the berry until the hatching or directly in the soil for 3-15 days always in relation with the weather conditions. This short life cycle allows the species to have 7-25 generations per year (Cini *et al*., 2012).

The species is able to survive during winter condition thanks to the capacity to go into reproductive diapause (Bouletreau and Fouillet 2002; Kimura 2004; Dalton *et al*., 2011). Such ability has left a genetic signature in *D. suzukii* genome (Ometto *et al*., 2013). Because overwintering implies fewer generations per year, the *D. suzukii* genome is accordingly slower evolving than other *Drosophila* genomes likely because fewer generations have resulted in less changes of passing mutations to the offspring (Ometto *et al.,* 2013). *D suzukii* can both overwinter and survive dry summers: it can easily tolerate temperature as low -1.6°C for females and -1.0°C for males, and as high as 32.6°C for females and 32.2°C for males (Kimura 2004). Such a broad range of temperature tolerance in *D. suzukii* is also one of the reasons for its worldwide invasive spread. However, too little is known about how this species manages to survive in winter and maintains their colonization. Two scenarios have been proposed. The first assumes that *D. suzukii* is cold-susceptible and it would try to escape cold by migrating to warm areas (Mitsui *et al.,* 2010; Kimura 2004). Alternatively, if they are unable to escape the cold, they would die in a given location which will be re-colonized from infested fruits transported from other warmer places in the following spring (Dalton *et al*., 2011). It is also possible that the species might manage to survive by hiding under debris or trees-barks (Kanzawa 1939; Park *et al*., in preparation) or exploit a natural adult plasticity and/or develop into winter-morphs by undergoing population bottleneck and regaining their effective population size in forthcoming spring (Asplen *et al*., 2015; Ometto *et al*., 2013; Jakobs *et al*., 2017; Shearer *et al*., 2016). Although scientists propend for the second hypothesis, one paper of mine (Publication II, Tait *et al*., 2018) describes how collected data suggest a natural migration of *D. suzukii*, at least in the studied area (Valsugana Valley, Trentino-Alto Adige, Italy).

1.4 Weather resistance

D. suzukii survival, number of eggs laid, and generation time all depend on temperature. The optimal temperature for the pest (i.e. the temperature at which more eggs are produced, and less time is required to complete a full egg-adult cycle) is ≈ 25°C. *D. suzukii* is considered to be highly adapted to temperate climate and has a considerable thermal tolerance (Kanzawa *et al.*, 1939; Lee *et al.*, 2011; Enriquez & Colinet, 2017). Enriquez & Colinet (2017) tested the fly survival at extreme temperatures, taking also into account humidity, sex and life stage (either pupa or adult). Males resulted colder tolerant than females while females were more tolerant to extreme heat than males. At temperature above 5 °C, adult mortality dropped under 20% after one month of exposure. Pupae were less cold tolerant than adults but more heat tolerant. Finally, high humidity values enhanced the fly survival at high temperature but had no effect on low temperature survival. Another key factor of *D. suzukii* efficiency as a pest is its ability to overwinter. Only adult *D. suzukii* flies can successfully overwinter (Kanzawa, 1939; Lee *et al.*, 2011; Shearer *et al.*, 2016). The winter morph is characterized by a darker pigmentation and a longer wing length, and has a higher cold tolerance compared to the summer morph (Hamby *et al.*, 2016; Shearer *et al*., 2016) (Figure 3). In fact, winter morph flies have a lower lethal temperature and can survive for longer periods at 1 °C compared to summer morph flies (Hamby *et al.*, 2016; Shearer *et al.*, 2016; Grassi *et al.*, 2017). Shearer *et al.* (2016) sequenced the RNA of winter morph *D. suzukii* adults in order to find DEGs. Genes involved in known mechanisms of cold-hardening (for example carbohydrate metabolism) were found to be up-regulated. Moreover, genes related to oogenesis and DNA replication were found to be down-regulated: this is consistent with a reproductive diapause during winter months and confirms several other studies that suggested the presence of this diapause (Enriquez & Colinet, 2017; Grassi *et al.*, 2017). Grassi *et al.* (2017) confirmed phenotypically the presence of a reproductive diapause during winter months, showing that *D. suzukii* females have a decreased level of mature eggs during the dormant period and that this level is positively correlated to Degree Days (i.e. cumulative heat).

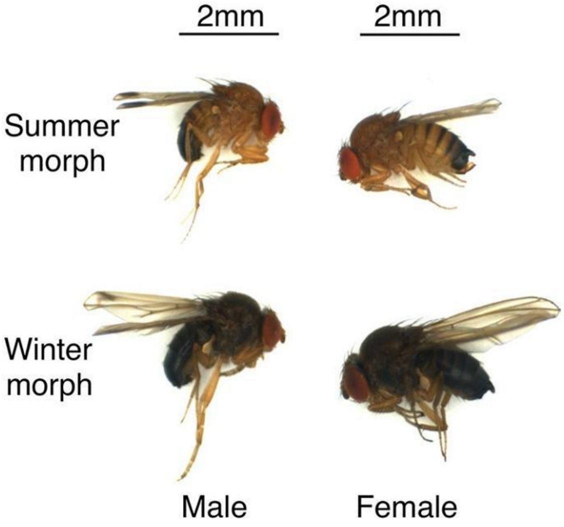

Figure 3: summer morph and winter morph of *D. suzukii* of both sexes, with clear phenotypic differences (Shearer *et al.*, 2016).

1.5 Dispersal ability and genetic spread

Anthropogenic activities play a major role in the worldwide spread of invasive alien species (Taylor 1974; Taylor *et al*., 1984; Bigsby *et al*., 2011). Animal dispersal is typically studied by following the movement patterns of marked individuals (Nathan *et al*., 2003). Therefore, while human movements and global trade are likely the cause of the rapid passive spread of *D. suzukii* on a continental scale (Cini *et al*., 2012), little information is available on its active shorter-range dispersal capacity, apart from limited small-scale field studies (Klick *et al*., 2015). According to the number of trap catches throughout the season and across elevational gradients, Mitsui et al. (2010) hypothesized that *D. suzukii* migrates progressively towards higher elevations during summer and returns to progressively lower elevations as temperatures decrease. It was speculated that this behavior would enable *D. suzukii* to minimize exposure to suboptimal high temperatures and resource-limited conditions at low elevations during summer. Consequently, this behavior would also allow such populations, generation after generation, to exploit suitable food resources, i.e. ripening fruits, as they progressively become susceptible for colonization at higher elevations as the season progresses (Tonina *et al*., 2016).

There are multiple approaches to study the dispersal capacity of insects (Hagler and Jackson 2001). Insect marking techniques include use of stable radio isotopes (Hagler and Jackson 2001; Boina *et al*., 2009; Zhang *et al*., 2016), fluorescent dusts (Schneider 1989; Skovga 2002; Coviella *et al*., 2006), and immunomarking (Hagler and Jackson 2001; Hagler and Jones 2010; Klick *et al*., 2014; Lessio *et al*., 2014). An ideal marker should have no effect on insect behavior, resist severe weather conditions, be inexpensive, be easily applicable to the study species over large areas, and be readily detectable (Hagler and Jackson 2001). Immunomarking techniques, which make use of proteins from animal or plant sources, fulfill most of these requirements and have been applied successfully in mark–release–recapture studies, whereby wild or reared insects were marked by contact with contaminated plants in the field or directly marked with the proteins under controlled conditions (Jones *et al*., 2006; Klick *et al*., 2014; Lessio *et al*., 2014; Blaauw *et al*., 2016).

Invasion dynamics can be studied using molecular markers that can discriminate and characterise the genetic relationships between source and derived populations, migration flows and population expansion patterns (Sunnucks *et al*., 2015; Schlötterer *et al*., 2004). In particular, Single Nucleotide Polymorphism (SNP) and Simple Sequence Repeat (SSR) markers have played an increasingly significant role in the study of genetic differentiation across species populations (Morin et al., 2007). Thanks to their great discrimination power and high reproducibility and variability, SSRs represent one of the most robust and informative molecular markers available for genotyping individuals (Murphy *et al.,* 2016). For instance, their use in *Drosophila* species was pivotal in studying intra-population genetic variation and evolution (Hutter *et al*., 1998; Amos *et al*., 2003).

In relation to *D. suzukii*, SSRs have been exploited to study genetic aspects of the colonisation process in the USA and Europe. Jeffrey and colleagues based their research on the use of six X-linked genes and suggested that the invasions of the USA and Europe are two independent events (Adrion et al., 2014). Bahder et al., (2015) in particular analysed samples of *D. suzukii* populations collected in California and Washington and determined that while *D. suzukii* in the former region had high levels of genetic variation, the latter was highly monomorphic (Bahder *et al*., 2015). Furthermore, Fraimout's group investigated Hawaiian and Spanish populations by exploiting microsatellite markers, finding a significant level of genetic differentiation (Fraimout *et al.,* 2017). Although both studies exploited two different sets of microsatellites and tested different populations, the authors were led to similar conclusions: they demonstrated the presence of a specific differentiation process among ancestral and derived populations and suggested that for *D. suzukii* a genetic analysis approach is valuable not only to better understanding of the evolutionary history of the species, but also to manage its great potential for invasiveness. Different studies on the invasiveness of species, including *D. suzukii*, have demonstrated the relationship between their spread and human trade (Hulme 2009; Cini *et al*., 2014; Manni *et al*., 2017).

1.6 Control strategies

In order to stop *D. suzukii* from spreading into new countries and to avoid an increase in its population in already infested areas, integrated pest management techniques have to be developed and consistently applied. Several different approaches are being studied and some are already being used in the field. Removing any possible oviposition target of *D. suzukii*, either inside or outside the crop, is fundamental to prevent new infestations or an increase in the fly population (Walsh *et al.*, 2011; Cini *et al.*, 2012). This means removing overripe fruits or non-collected fruits as well as non-crop hosts and ornamental host plants. After removal, fruits have to be destroyed. Composting and similar techniques are definitely to be avoided, since larvae and eggs are not destroyed and can quickly complete their development thanks to the warm environment produced by the decomposition of the fruits (Walsh *et al.*, 2011; Cini *et al.*, 2012). Several options for disposal of potentially contaminated fruits have been proposed and, on the EPPO (European Plant Protection Organization) website, guidelines on how to decrease the risk of persistence of sources of re-infestation can be found (EPPO Alert list, "*Drosophila suzukii*" entry). Among the possible IPM approaches, the scientific community has chosen to concentrate on environmentally safe strategies. Here are examples of major environmentally safe *D. suzukii* control strategies: RNA interference, parasitoids and microorganisms.

RNA interference is the process by which the presence of dsRNA triggers a sequence-specific gene silencing cascade. In insects, it has been shown how the ingestion of dsRNA can cause RNAi that can lead to larval stunting and mortality (Baum *et al.*, 2007; Taning *et al.*, 2016). RNA interference to control pest population can be supplied to insects either by spraying of the dsRNA on infested plants or by genetically engineering crops to produce the dsRNA (Baum *et al.*, 2007; Taning *et al.*, 2016). Both mechanisms have advantages and disadvantages. dsRNA spraying fits well with current pesticides delivery methods but is not cost effective because of the use of in vitro synthesized RNA (Taning *et al.*, 2016). Engineering plants, on the opposite, can be cost effective because of the production of transgenic strains that do not need continuous RNA synthesis (Baum *et al.*, 2007; Taning *et al.*, 2016). However, this approach is hindered by public hesitancy in accepting transgenic food (Taning *et al.*, 2016).

Biological control aims at exploiting natural enemies of the pest, like predators, parasitoids, microbes and viruses, introducing them into the environment to decrease pest population (Zabalou *et al.*, 2004; Cini *et al.*, 2012; Pfab *et al*., 2018). Parasitoids are the organisms most used as biological controls; they are arthropods (mainly Hymenoptera and Diptera) which develop inside or on the surface of other arthropods and consume the host's tissues during their own development (Chabert *et al.*, 2012; Cini *et al.*, 2012, Stacconi *et al*., 2016). The possible practical usage of parasitoids to control *D. suzukii* population has been debated, because of the pest high-reproduction rate (Cini *et al.*, 2012, Pfab *et al*., 2018). Nonetheless, parasitoids are being studied intensively because of the long-term negative effect that they have on phytophagous populations if permanently introduced in infested environments (Walsh *et al.*, 2011; Chabert *et al.*, 2012; Cini *et al.*, 2012; Stacconi *et al.,* 2018). The absence of invasive species natural enemies is often regarded as one of the key factors for a successful biological invasion; parasitoid introduction aims at removing this key factor, slowing the invasion of the pest (Chabert *et al.*, 2012; Ioriatti *et al.*, 2018). However, this approach has some practical difficulties, among which there is the usually low efficiency of local parasitoids in attacking new pests. Insects that invade new environments can often survive local parasitoids thanks to strong immune reactions or because they are not targeted at all by parasitoids, and this can happen even if the exotic insect is similar or closely related to local parasitoids hosts (Zabalou *et al.*, 2004; Chabert *et al.*, 2012; Cini *et al.*, 2012; Mazzetto *et al.*, 2016). For this reason, local European parasitoids have been tested for *D. suzukii* parasitization rate (Chabert *et al.*, 2012; Stacconi *et al*., 2018). Among the major European parasitoids, the pupal parasitoid *Trichopria drosophilae* showed high parasitization rate against *D. suzukii* (Chabert *et al.*, 2012; Mazzetto *et al.*, 2016; Zhu *et al.*, 2017; Stacconi *et al*., 2018) and several authors have argued that it could be used to efficiently control *D. suzukii* population.

Among the microorganisms used as biological control, endosymbionts are the most common. In arthropods, microorganisms can strongly interfere with their host biology (Cini *et al.*, 2012). Control strategies targeting these microorganisms could thus be directed to reduce the pest population. One of the most known examples of microorganisms interacting with host insect biology is *Wolbachia* (Werren *et al.*, 2008; Kaur *et al*., 2017). *Wolbachia* are gram negative bacteria that infect more than half of the living insect species as well as several nematodes (Werren *et al*., 2008; Cini *et al.*, 2012). *Wolbachia* are vertically and horizontally transmitted and can manipulate their hosts in several different ways (Zabalou *et al.*, 2004; Werren *et al.*, 2008; Cattel *et al*., 2016). These bacteria can induce different phenotypes depending on the host species and on the bacterium strain. Known phenotypes caused by *Wolbachia* infections are Cytoplasmic Incompatibility (CI), parthenogenesis induction, feminization of genetically male individuals and male killing (Werren *et al.*, 2008; Zabalou *et al.*, 2008). CI, the most common effect caused by *Wolbachia* infections, could be exploited in pest control strategies (Zabalou *et al.*, 2008; Cini *et al.*, 2012). Cytoplasmic Incompatibility prevents infected males from successfully mating with females with different *Wolbachia* infection status, i.e. non-infected or infected with incompatible strains (Werren *et al*., 2008, Cini *et al.*, 2012; Rota-Stabelli *et al*., 2016).

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Chapter 2.

Genetic variability of Italian population of *Drosophila suzukii*

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Genetic variability in Italian populations of *Drosophila suzukii*

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Abstract

Background: *Drosophila suzukii* is a highly destructive pest species, causing substantial economic losses in soft fruit production. To better understand migration patterns, gene flow and adaptation in invaded regions, we studied the genetic structure of *D. suzukii* collected across Italy, where it was first observed in 2008. In particular, we analysed 15 previously characterized Simple Sequence Repeat (SSR) markers to estimate genetic differentiation across the genome of 278 flies collected from nine populations.

Results: The nine populations showed high allelic diversity, mainly due to very high heterozygosity. The high Polymorphism Information Content (PIC) index values (ranging from 0.68 to 0.84) indicated good discrimination power for the markers. Negative fixation index (F_{IS}) values in seven of the populations indicated a low level of inbreeding, as suggested by the high number of alleles. STRUCTURE, Principal Coordinate and Neighbour Joining analysis also revealed that the Sicilian population was fairly divergent compared to other Italian populations. Moreover, migration was present across all populations, with the exception of the Sicilian one, confirming its isolation relative to the mainland.

Conclusions: This is the first study characterizing the genetic structure of the invasive species *D. suzukii* in Italy. Our analysis showed extensive genetic homogeneity among *D. suzukii* collected in Italy. The relatively isolated Sicilian population suggests a largely human-mediated migration pattern, while the warm climate in this region allows the production of soft fruit, and the associated *D. suzukii* reproductive season occurring much earlier than on the rest of the peninsula.

Keywords: Spotted wing drosophila, SSR markers, Population structure, Gene flow, Bottleneck, Human trade

Background

The spotted wing drosophila (SWD), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), is a pest species which has spread from its original range in Asia to a number of western countries in the past decade, including the Mediterranean basin [1], Europe, and USA [1–3]. The history of the geographical spread and infestation of *D. suzukii* is still under investigation: it is known that in 1939 this species was first recorded in Japan (Kanzawa 1939), while in the 1980s it was collected on the island of Hawaii [4]. Europe and the Americas were colonized much later, possibly during the last 9 years [2, 3, 5, 6]. First adults of *D. suzukii* were caught contemporaneously in the region of Catalonia, Spain [7] and in Tuscany, Central Italy, in 2008 [3]. In 2009 *D. suzukii* individuals were found on both wild hosts (*Vaccinium*, *Fragaria* and *Rubus* spp.) and several species of cultivated berries in Trento Province, North Eastern Italy, where also the first economically im- portant damage by this species in Europe was reported [8]. During the following years, *D. suzukii* has been spreading rapidly across Europe, with documented infesta- tions ranging from Mediterranean regions (i.e. Greece, Turkey) to northern latitudes (i.e. Sweden, Poland, UK) (EPPO Global Database, *Drosophila suzukii* – DROSSU, 2017). In Italy, after the first detection, infestations were reported from the regions of Bolzano, Piedmont, Liguria, Campania and Veneto in 2010, from Lombardy, Emilia Romagna, Marche, Aosta Valley, Marche, Calabria and Sicily in 2011 [3], Sardinia in 2012 [9], Apulia in 2013 [10], Umbria in 2014 [11], and Latium in 2015 (Antonini G., present paper). Invasion dynamics can be studied using molecular markers that can discriminate and characterize the genetic relationships between source and derived pop- ulations, migration flows and population expansion pat- terns [12–14].

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In particular, Single Nucleotide Polymorphism (SNP) and Simple Sequence Repeat (SSR) markers have played an increasingly significant role in the study of genetic differentiation across species populations [15]. Thanks to their great discrimination power and high reproducibility and variability, SSRs represent one of the most robust and informative molecular markers available for genotyping individuals [16]. For instance, their use in *Drosophila* species was pivotal in studying intra- population genetic variation and evolution [14, 17–20].

In relation to *D. suzukii*, SSRs have been exploited to study genetic aspects of the colonization process in the USA and Europe. Jeffrey and colleagues based their research on the use of six X-linked genes and suggested that the invasions of the USA and Europe are two independent events [21]. Bahder et al. in particular analysed samples of *D. suzukii* populations collected in California and Washington and determined that while *D. suzukii* in the former region had high levels of genetic variation, the latter was highly monomorphic [22]. Furthermore, Fraimout's group investigated Hawaiian and Spanish populations by exploiting microsatellite markers, finding a significant level of genetic differentiation [23]. Although both studies exploited two different sets of microsatellites and tested different populations, the authors were led to similar conclusions: they demonstrated the presence of a specific differentiation process among ancestral and derived populations and suggested that for *D. suzukii* a genetic analysis approach is valuable not only to better understanding of the evolutionary history of the species, but also to manage its great potential for invasiveness. Different studies on the invasiveness of species, including *D. suzukii*, have demonstrated the relationship between their spread and human trade [24–26]. For this reason, it is very important to consider the correlation between gene flow analysis and the sale of soft fruit all over the country. Taking into account this aspect, we used a population genetic approach to characterize genetic diversity among *D. suzukii* individuals collected in different regions of Italy. In order to perform this work a set of 15 microsatellites validated by Fraimout and colleagues [23] were employed. The current research is the first study that provides new insights on the trend of genetic diversity in Italian populations of *D. suzukii*.

Methods

D. Suzukii collection, identification and DNA extraction

A total of 278 individuals of *D. suzukii* collected from nine populations in Italy were analysed (Fig. 1). Adult *D. suzukii* were collected between October 2015 and April 2016 using Droskidrink®-baited traps [27] left exposed for 3 days. In order to limit the likelihood of sampling individuals related to each other, three traps per location were used, at a distance of at least 500 m from each other. In the laboratory, *D. suzukii* individuals were identified using a 7×-45× stereomicroscope, according to Hauser's (2011) morphological characteristics, such as the structure of the ovipositor for females and spots on the wings and tarsal combs for males. Samples were preserved in 96% ethanol and kept at 4 °C until DNA extraction. For each location, we selected 15 females and 15 males for DNA extraction, with genomic DNA being extracted from each individual separately using the Macherey Nagel Kit (NucleoSpin Tissue, Macherey Nagel, Düren, Germany).

Microsatellite analysis

The SSRs used for this work were selected from a set of microsatellites previously designed and validated [23]. Of the 28 published SSRs, 22 continuous di-nucleotide loci were tested on a pool of 20 *D. suzukii* individuals. Seven of these loci were discarded because of amplification problems, leaving 15 SSR markers distributed across chromosomes 2 and 3 (Fig. 2) [28].

Each pair of primers was used for PCR amplification in 25 μL final volume, containing 1X GoTaq G2 Master Mix, 0.5 μL of each primer, 10.5 μL of distilled deionized water and 1 μL of genomic DNA. The PCR program was set with an initial period of denaturation at 94 °C (30 s) followed by 32 cycles of additional denaturation at 94 °C (30 s), an annealing phase at 57 °C (1 min 30 s), an elongation phase at 72 °C (1 min) and ending with another extension phase at 72 °C (30 min). PCR products were checked using electrophoresis on 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. Each amplicon was then diluted 1:10 in distilled water and 1 μL of this dilution was added to 12.5 μL of a mixture of deionized Formamide (Sigma-Aldrich) and GeneScan-500 ROX size standard (Life Tech, Waltham, MA USA). Prior to denaturation for 4 min at 94 °C, capillary electrophoresis was carried out in an ABI PRISM 310 Genetic Analyzer (Life Tech) and the fragments were sized with GeneMapper v.4.0 software in binning mode. If no sample amplification was obtained after two PCR attempts, the locus was classified as missing data.

Statistical analysis

Microsatellite allele data were processed with Tandem program v.1.08 [29]. GenAIEx software v.6.41 [30] was run to study the genetic variability between populations using the following statistics: mean number of alleles (N_a), effective number of alleles (N_e), expected heterozygosity (H_E), observed heterozygosity (H_O), number of

Number on the map	Sample site	Nb samples	Collection Time	Elevation	UTM Coordinate
1	Trentino1	98	2015	1455 m a.s.l	46.138100 11.320927
2	Trentino2	174	2015	473 m a.s.l	46.074247 11.250031
3	Latium	64	2015	240 m a.s.l	42.140862 12.487181
4	Sicily	55	2016	60 m a.s.l	37.635348 14.879720
5	Veneto	100	2015	1437 m a.s.l	45.374117 11.755821
6	Tuscany	73	2016	80 m a.s.l	43.503177 10.449749
7	Liguria	114	2015	32 m a.s.l	44.376027 9.125623
8	Sardinia	53	2015	8 m a.s.l	39.774671 8.572070
9	Apulia	66	2015	15 m a.s.l	41.112437 16.903723

Fig. 1 Field collected samples of the *D. suzukii* analysed in this study. This image has been adapted from the original (https://it.wikipedia.org/wiki/File:Italy_topographic map-blank.svg) whose author is Eric Gaba, and it is licensed through Creative Commons Attribution 3.0

private alleles (N_p), frequency of private alleles (A_p) and inbreeding coefficient (F_{IS}). Allelic richness was calculated using FSTAT v.2.9.3 software [31]. Deviation from Hardy-Weinberg equilibrium after the Bonferroni multiple correction test and allelic Polymorphic Information Content (PIC) were tested using CERVUS software v.3.0 [32]. N_e and H_E were chosen as the basic genetic variability and estimated for each population. N_e was ana- lysed with ANOVA using origin as a factor. N_e was taken from the formula $1/(1\text{-}H_E)$ and then tested with the non-parametric Tukey test [33]. N_e was used instead of (N_a), considering that it is less sensitive to rare alleles and sample size. H_E was taken from the formula $H_E = 1\text{-}(\Sigma q^2)$, where q represents the frequency of the i^{th} allele in the population. H_E was converted into $1/H_E$ and then tested with the non-parametric Kruskal Wallis test. All statistical analyses were performed using R software v.3.3.2. The significance level was set below 0.001 ($P < 0.001$) to minimize sources of uncertainty.

To evaluate the genetic structure of populations, we relied on multiple approaches: Principal Coordinate Analysis (PCoA), Neighbour Joining Tree, AMOVA ana- lysis, measurement of the index of differentiation (F_{ST}) and use of a non-spatial Bayesian algorithm. These ap- proaches were chosen in order to obtain a broad view of the genetic structure of this invasive species in Italy. PCoA, obtained with GenAIEx software, was used to display genetic divergence across *D. suzukii* in a multidi- mensional space, considering frequency data. Unrooted Neighbour Joining Tree based on Nei's genetic distance constructed using DARwin software was complementary to PCoA analysis [34]. AMOVA analysis obtained using

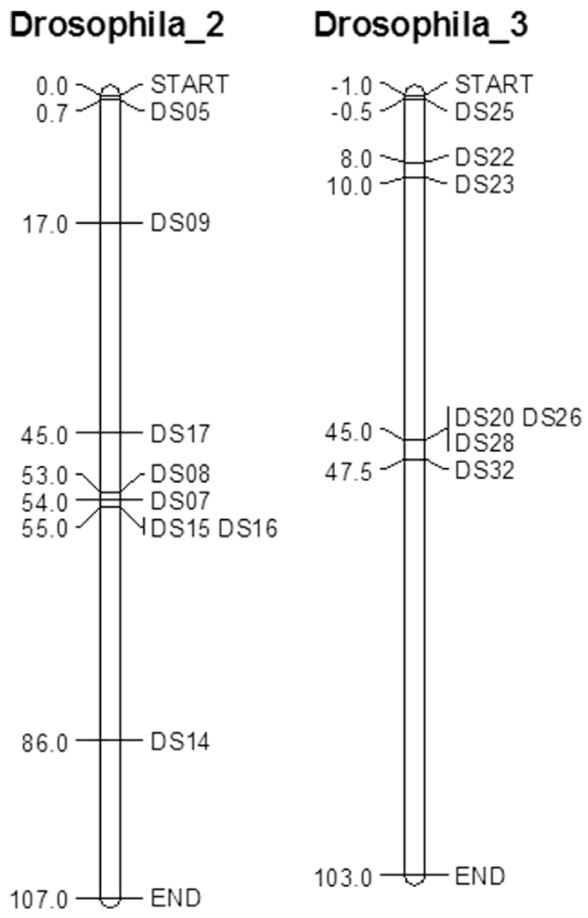

Fig. 2 Location of the 15 microsatellite markers distributed across the chromosomes 2 and 3

the Arlequin v.3.5 [35] program was performed to estimate variability distribution within and between the tested groups. The level of genetic differentiation in populations was detected using the F_{ST} values obtained with Microsatellite Analyzer (MSA) v.4.05 software [19]. The program allows comparison of each observed F_{ST} value with that obtained in 10,000 matrix permutations in order to define the statistical significance of each F_{ST}.

The Bayesian method was implemented with STRUCTURE software v.2.3.3 [36, 37]. This program was employed in order to obtain clusters of individual genotypes. The analysis was run using the admixture hypothesis, which is based on correlated allele frequencies, in which each sample contains a portion of the genome of each ancestral population. This, correlated to the allele frequency model, allows calculation of the log likelihood for the data, L(K). Not knowing the origin and the degree of isolation of the studied populations a priori, this model is considered to be the most appropriate in these situations [36]. Prior probability, i.e. the probability that an individual belongs to any K reference populations, is defined as l/K. The K value was fixed from 1 to 10 with 20 replicates of each K to test the convergence of the Markov chain. A total of 1000,000 simulations per run and 500,000 Markov Chain Monte Carlo MCMC repetitions were fixed. Once the results were obtained, they were scored with STRUCTURE HARVESTER software to detect the number of K groups that best fit the dataset according to the Evanno test [38, 39]. GENECLASS v.2.0 [40] was run to estimate the probability of each individual in a population belonging only to that population, the probability of it being an immigrant from each of the other populations, and the probability of it being a migrant to the other populations. BOTTLENECK v.1.2.02 [41] was run in order to evaluate whether demographic events such as population contraction or expansion took place in each population.

Heterozygosity excess, which is associated with a population expansion, was tested with the two-phase mutation model (TPM) using Wilcoxon signed-rank test, which according to Piry et al. is the most appropriate and powerful test when dealing with less than twenty loci [41]. Parameters were set as 20% multiple-step mutations and 80% single-step mutations with 1000 iterations. In order to verify the effect of isolation by distance, and therefore to find possible correlation between genetic and geographical distances, the ISOLDE option in GENEPOP software was run.

Results

Genetic diversity

The variability indices of the 15 SSR loci are shown in Table 1. The number of alleles per locus across populations ranged from 8 (DS17) to 20 (DS07), with an average (± standard deviation) of 13.6 ± 3.37. The PIC estimate ranged from 0.68 (DS14) to 0.84 (DS07), suggesting that this set of loci is informative for population analysis. Only five alleles were in Hardy-Weinberg Equilibrium (DS07, DS09, DS22, DS23, DS26), while the other 10 showed significant HWE deviations, with nine loci having an excess of H_O (Table 1). The reason for the HWE disequilibrium could be the presence of null allele that may affect estimation of population differentiation [42, 43]. The mean H_O across loci ranged from 0.68 (DS32) to 0.91 (DS16), while H_E ranged from 0.71 (DS14) to 0.86 (DS07). Mean H_O across populations ranged from 0.66 ± 0.16 (Trentino2), to 0.89 ± 0.09 (Tuscany) (Table 2). Allelic richness ranged from 6.23 in Trentino1 to 8.58 in Apulia. For most of the loci the F_{IS} was negative. F_{IS} values ranged from −0.28 in Sicily to 0.07 in Trentino2. In the Additional file 1 are reported all the data concerning the observed and expected heterozygosity, the number of alleles, the effective number of alleles, the number of private alleles, the F-statistic (*F*is, *F*it and *F*st) and the fixation index. The Tukey test revealed a significant effect of population origin on the heterogeneity of N_e on comparing the following sampled populations: Trentino1 and Apulia, Trentino2 and Apulia, Sicily and Apulia, and Sicily and Tuscany ($P < 0.001$). On analysing all the populations together with ANOVA, using the collection site as a factor, N_e showed a significant difference between populations ($F = 3.86$, $P < 10^{-10}$). The effect of the collection site was also evident in mean H_E (F 4.19, P < 0.001).

Table 1 Summary of the genetic variability at 15 microsatellite loci

Locus	Repeat	PIC	Allele range	*N*	H_O	H_E	H-W
DS05	(TG)10	0.74	250–284 bp	11	0.79	0.77	**
DS07	(CA)13	0.84	180–220 bp	20	0.89	0.86	NS
DS08	(AG)10	0.81	118–158 bp	17	0.84	0.83	***
DS09	(AC)15	0.72	200–230 bp	13	0.71	0.75	NS
DS14	(TG)10	0.68	136–239 bp	11	0.75	0.71	**
DS15	(GT)11	0.76	238–278 bp	13	0.88	0.79	*
DS16	(AC)13	0.78	85–119 bp	15	0.91	0.81	***
DS17	(GT)10	0.70	93–113 bp	8	0.90	0.74	***
DS20	(AG)12	0.74	207–235 bp	13	0.84	0.77	*
DS22	(GT)11	0.71	304–334 bp	13	0.83	0.75	NS
DS23	(AC)10	0.75	236–266 bp	13	0.76	0.77	NS
DS25	(CA)10	0.71	222–280 bp	18	0.74	0.74	***
DS26	(CA)10	0.73	79–109 bp	10	0.76	0.77	NS
DS28	(TG)11	0.78	141–161 bp	11	0.86	0.81	***
DS32	(TG)15	0.83	310–376 bp	18	0.68	0.85	***

Repeat, motive of the microsatellite; *PIC* polymorphic information content; Allele range, *N* number of alleles, *Ho*,observed heterozygosity, *He* expected heterozygosity

Table 2 Level of genetic diversity across 9 populations of *D. suzukii*

	N_a	N_e	A_r	N_p	A_p	H_O	H_E	A_n	F_{IS}
Trentino1	6.40 ± 1.29	3.65 ± 0.93	6.23	1	0.06	0.68 ± 0.18	0.70 ± 0.07	0.01	0.02
Trentino2	7.60 ± 1.50	3.83 ± 1.01	7.36	8	0.53	0.66 ± 0.16	0.72 ± 0.08	0.03	0.07
Sardinia	7.87 ± 1.40	4.37 ± 0.80	7.69	6	0.40	0.82 ± 0.10	0.76 ± 0.05	0.03	−0.08
Latium	7.80 ± 1.37	4.18 ± 0.87	7.44	6	0.40	0.83 ± 0.13	0.74 ± 0.06	0.04	−0.11
Sicily	5.60 ± 0.98	3.25 ± 0.58	5.44	1	0.06	0.87 ± 0.11	0.68 ± 0.05	0.11	−0.28
Apulia	8.87 ± 3.44	5.22 ± 2.53	8.58	25	1.66	0.81 ± 0.13	0.77 ± 0.07	0.01	−0.04
Tuscany	7.47 ± 1.12	4.68 ± 0.98	7.23	2	0.13	0.89 ± 0.09	0.77 ± 0.04	0.06	−0.14
Liguria	7.40 ± 1.50	4.41 ± 0.77	7.11	3	0.20	0.87 ± 0.10	0.76 ± 0.03	0.05	−0.13
Veneto	7.53 ± 1.30	4.14 ± 0.50	7.26	5	0.33	0.83 ± 0.16	0.75 ± 0.02	0.04	−0.10

N_a mean number of alleles, N_e mean effective number of alleles, A_r mean of allele richness, N_p number of private alleles, A_p mean frequency of private alleles, H_O mean observed heterozygosity, H_E mean expected heterozygosity, A_n mean frequency of null alleles, F_{IS} mean inbreeding coefficient

Genetic population structure and gene flow

An estimate of variability distribution (AMOVA) within the tested populations indicated that 96% of the variation occurred within individuals, while only 4% of total variation was detected between populations. Table 3 gives a summary of analysis of variance for the nine *D. suzukii* populations. The results of PCoA are shown in Fig. 3. The first axis explains 57.9% of genetic variation, while the second axis explains 18.9%. The first axis separates the Sicilian population from the remaining populations. The second axis mainly divides Apulia, Tuscany, Liguria and Veneto from the others. The unweighted Neighbour-Joining dendrogram represented in Fig. 4 supports data obtained using PCoA: the Sicilian group has the same origin as the other populations, but individuals belong to a separated cluster.

The F_{ST} values confirmed the genetic differentiation between the Sicilian group and the others. Considering all the populations, 30 of the 36 pairwise comparisons tested were significantly different from zero (Table 4). The least significant differentiation was between Liguria and Veneto ($F_{ST} = 0.003$), while the greatest divergence was between Sicily and Trentino1 ($F_{ST} = 0.135$). Population structure analysis led to the identification of two clusters (K = 2), based on the Evanno method (Fig. 5) and revealed genetic homogeneity between most populations, with the exception of flies collected in Sicily. The data of gene flow are reported in Table 5. The findings show that the Sicilian population did not migrate significantly to any other populations (m < 0.100). Although there was no gene flow from Sicily to the other regions, migrant flow occurred from Trentino2 (m = 0.100), Sardinia (m = 0.176), Lazio (m = 0.281), Tuscany (m = 0.195), and Liguria (m = 0.111). On the other hand, migration occurred from Apulia to other regions, but not to Apulia (m < 0.100) from other regions. Excluding Sicily and Apulia, the remaining seven populations both received and provided significant genetic information in relation to other populations (m > 100). Comparisons of Trentino2 and Veneto (m = 0.430 and m = 0.485 respectively), Sardinia and Tuscany (m = 0.316 and m = 0.327 respectively) and Lazio and Liguria (m = 0.376 and m = 0.315 respectively) revealed a similar migration rate in both directions. Considering the likelihood of the presence of migrants across populations, nine migrants (seven females and two males) were detected with a probability of less than 0.01.

Table 3 Analysis of molecular variance test (AMOVA)

Source of variation	DF	SS	VC	%PV
Among Populations	8	165.16	0.24	4%
Within Individuals	278	167.05	6.00	96%

DF degree of freedom, *SS* sum of squares, *VC* variance components, *%PV* percentage of total variation

Results from the individual population analysis using the TPM model did not support an expansion scenario. On the contrary, population bottleneck, defined by significant heterozygote deficiency was present in the Trentino2 population ($P = 0.004$). The nine populations showed no significant correlation when comparing genetic and geographical distances [$R^2 = 0.014$, $P = 0.568$, $F_{ST}/(1- F_{ST}) = 0.049 + \text{Ln (geographical distance)} = 0.001$].

Discussion

Genetic diversity

The introduction of invasive species to new environments poses threats to biodiversity, agriculture, public health and ecosystem integrity [44–47]. For this reason, considerable attention is paid to the rapid spread of alien species [46, 48]. Genetic characteristics deeply affect the capacity for expansion [49]. Therefore, in order to mitigate their impact and define management strategies it is imperative to study these fundamental characteristics. Currently techniques such as genomics [50–52], transcriptomics [53, 54], and metagenomics [55, 56] allow us to investigate these basic traits.

This research investigated the genetic structure of *D. suzukii* collected in different areas of Italy. In particular,

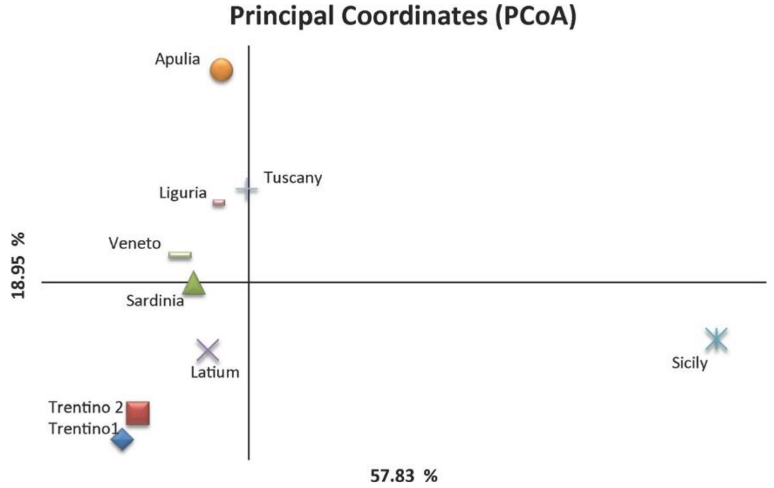

Fig. 3 Principal coordinate analysis (PCoA) of nine populations generated from genetic distance calculation in GenAlEx program

the aim of the analyses was to understand the gene flow of this species in a newly colonized environment. Our findings help to better understand the dynamics and complexity of this invasive species in Italy. The nine populations studied show a high level of genetic variation. The high number of alleles per locus detected clearly demonstrated the discriminatory power of these markers. Taking into consideration N_e, H_E and H_O, it is evident that the level of genetic differentiation is similar in *D. suzukii* collected across Italy, even in the locations at the greatest distance from the likely spreading centre of the species in Italy [25]. The high level of heterozygosity could be explained by good adaptation to new ranges due to a favorable environment, their reproductive power, and the absence or limited presence of natural competitors and predators [57, 58]. Bahder et al. found that populations from Washington were much less polymorphic than those in California, suggesting a recent strong population bottleneck associated to the recent invasion of the former [22]. Washington has a much cooler climate than California, similar to the contrast between Trentino and the rest of Italy. However, we did not observe such a contrast in heterozygosity, probably due to the highly favorable habitat found in Trentino coupled with a high migration rate with the rest of the Italian populations. Heterozygosity deficiency was

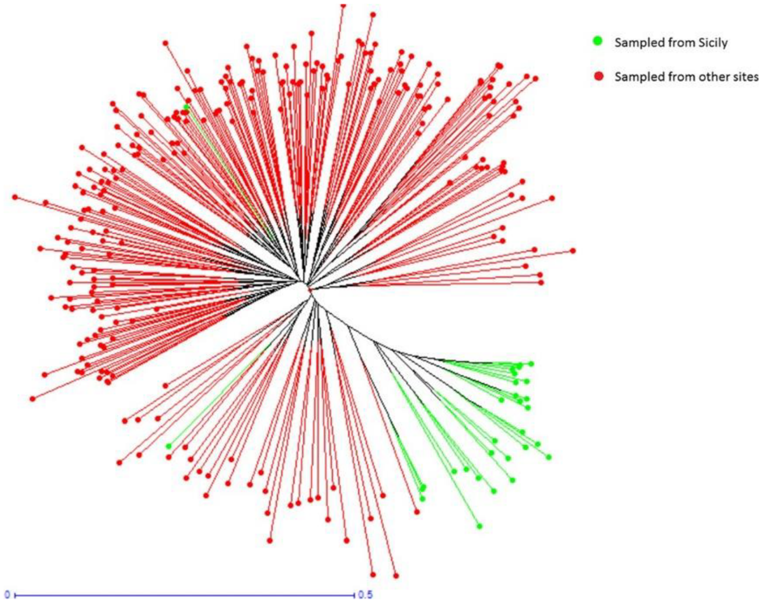

Fig. 4 Unrooted neighbour joining (UNJ) tree obtained from DARwin software. Each brunch represents single individual

Table 4 Pairwise *F*st among the nine populations of *D. suzukii*

	Trentino1	Trentino2	Sardinia	Latium	Sicily	Apulia	Tuscany	Liguria	Veneto
Trentino1	0.000								
Trentino2	0.006	0.000							
Sardinia	**0.022**	**0.011**	0.000						
Latium	0.009	0.007	**0.017**	0.000					
Sicily	**0.135**	**0.121**	**0.109**	**0.097**	0.000				
Apulia	**0.051**	**0.040**	**0.038**	**0.036**	**0.116**	0.000			
Tuscany	**0.039**	**0.031**	**0.015**	**0.022**	**0.097**	**0.038**	0.000		
Liguria	**0.026**	**0.017**	**0.010**	**0.018**	**0.096**	**0.033**	**0.015**	0.000	
Veneto	**0.022**	0.006	0.008	**0.012**	**0.113**	**0.036**	**0.017**	0.003	0.000

Boldface indicates values significantly different from zero ($P = 0.05$)

detected for one out of the nine investigated populations. In contrast, the remaining eight groups showed heterozygosity excess. Sicily in particular had the lowest heterozygosity value (−0.28). Negative results indicate random mating, therefore a lack of inbreeding among the collected individuals. In contrast, Trentino1 and Trentino2 had a positive F_{IS} value, indicating inbreeding. The Apulian population showed the greatest number of private alleles (25). This could be the consequence of a steady introduction of new alleles due to migration, possibly associated with human-mediated transport [25, 59].

Genetic structure analysis

Moderate genetic differentiation between most of the groups was in evidence for the nine populations, while the Sicilian population was the most differentiated from the others. This is supported by the NJ tree, PCoA data and structural analysis. At the same time, low differentiation between the other populations may be due to gene flow, which can homogenize gene frequency across populations. Data concerning the reduction or expansion of the studied populations indicated that Trentino2 was the only group having indices of genetic bottleneck.

Migration pattern

Human transportation is the most probable explan- ation for the extensive spread of *D. suzukii*. [25, 59]. When an alien species is introduced into an environment outside its native range, expansion can be identified not only by analysing genetic diversity indices, but also by analysing the genetic flow between populations, which is a direct proof of rapid distribution. [60, 61]. In particular, in the last 40 years, the risk of biotic invaders has increased significantly because of levels of international trade not seen before [62]. This situation facilitates genetic flow between groups located in different locations and may well apply to the results of our study. For instance, the observation that the level of heterozygosity (F_{st}) does not clearly decline (increase) from the hypothetical source population (Livorno, Tuscany) and that there is a high migration rate among localities, suggests that *D. suzukii* moves extensively across most of the Italian peninsula. Most of the Sicilian production of vegetables and fruit, including high *D. suzukii* susceptible hosts, is frequently exported to central and northern Italy. While this could suggest a high probability of flies being transported between Sicily and the rest of the peninsula, our results indicate that there was no gene flow from Sicily to other regions. This is probably due to the fact that ripe fruits are exported from Sicily mostly during the cold season, when moderate temperatures allow the production of berry fruit in Sicily, but not in the rest of Italy. Therefore, any *D. suzukii* accidentally moving from Sicily to the rest of

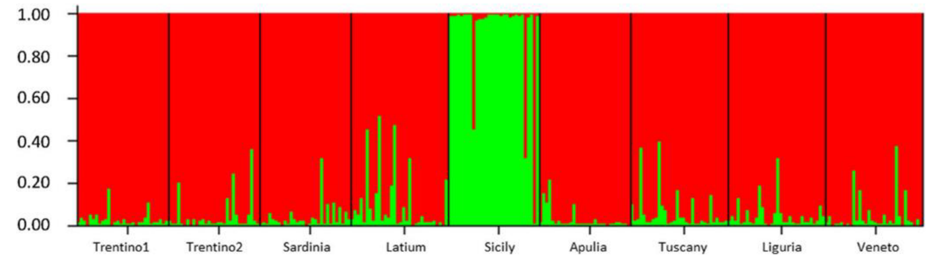

Fig. 5 Genetic structure of nine Italian *D. suzukii* populations estimated by structure analysis

Table 5 Individual assignment analysis obtained using GENECLASS

	Trentino1	Trentino2	Sardinia	Latium	Sicily	Apulia	Tuscany	Liguria	Veneto
Trentino1	**0.391**	**0.512**	**0.518**	**0.604**	0.004	**0.272**	**0.344**	**0.482**	**0.485**
Trentino2	**0.288**	**0.477**	**0.492**	**0.524**	0.008	**0.307**	**0.394**	**0.506**	**0.485**
Sardinia	0.087	**0.254**	**0.444**	**0.270**	0.005	**0.138**	**0.327**	**0.305**	**0.301**
Latium	**0.149**	**0.386**	**0.349**	**0.472**	0.008	**0.194**	**0.324**	**0.315**	**0.270**
Sicily	0.015	**0.100**	**0.176**	**0.281**	**0.611**	0.064	**0.195**	**0.111**	0.089
Apulia	0.008	0.084	0.051	0.056	0.001	**0.377**	0.049	0.040	0.053
Tuscany	0.052	**0.162**	**0.316**	**0.209**	0.006	**0.115**	**0.567**	**0.266**	**0.176**
Liguria	**0.109**	**0.362**	**0.423**	**0.376**	0.004	**0.204**	**0.388**	**0.466**	**0.344**
Veneto	**0.183**	**0.430**	**0.454**	**0.456**	0.006	**0.283**	**0.401**	**0.473**	**0.564**

Boldface indicates significant migration rate values (m ≥ 100)

Italy would arrive at a time when the local population is made up of a few individuals in winter diapause [27].

A second interesting piece of information revealed by our results is related to the scenario in Sardinia. To satisfy local demand for berry fruit, this region imports fruit from Italy and northern Europe, Spain, the USA and South America. The flies used in this study were collected in Arborea, a town 13 km away from the port of Oristano, one of the most important commercial ports in Italy. Thus, it is likely that the Sardinian population is made up of immigrants from other regions, as suggested by the low differentiation between this population and those on the mainland.

Conclusion

This research represents the first study investigating the pattern of genetic variability for *D. suzukii* following its introduction to Italy. Defining the population structure of a species, in particular of an invasive species, it is necessary not only to improve our knowledge of the genetic architecture, but also to apply knowledge. Indeed, understanding the current genetic structure of *D. suzukii* has significant implications in relation to geographical and economic impact. The evaluation of the genetic status of the *D. suzukii* populations in newly invaded areas and their expansion or reduction phases during defined periods of the year, may thus provide valuable information for predicting population spread, outbreaks, and improve integrated pest management programmes. Proper genetic management practices for *D. suzukii* and constant monitoring are therefore critical for maintaining populations under control.

The information obtained can be applied in particular to the management of coastal areas; one important action could be to increase monitoring control with the use of traps and other early warning tools in order to limit either multiple reintroductions of the same species or new introductions of exotic organisms.

Additional file

Additional file 1: Data explained allele frequencies for each locus at each location. Are in more reported the observed and expected heterozygosity, the number of alleles, the effective number of alleles, the number of private alleles, the F-statistic (*F*is, *F*it and *F*st) and the fixation index. (XLSX 246 kb)

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Availability of data and materials

The data supporting the results of this article are included in the article and the supplementary information.

Authors' contributions

GT contributed to all the steps, planning the experimental design, sampling populations, conducting laboratory tests and data analysis, as well as writing the manuscript. SV participated in genetic data analysis and drafting the manuscript. SV and LO contributed to data interpretation. FS contributed to laboratory experiments. GfA and GlA contributed to sampling populations, suggesting professional contacts and conceiving the main idea. AB, NB, GS, AC and LT contributed to sampling populations. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

No specific permits were required for this project. *Drosophila suzukii* is an agricultural pest, not a protected species. All the insects analysed were collected in the open field and not from national parks or protected areas.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interest.

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CHAPTER 3.

Large-scale spatial dynamics of *Drosophila suzukii* in Trentino, Italy

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ORIGINAL PAPER

Large-scale spatial dynamics of *Drosophila suzukii* in Trentino, Italy

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Abstract

Drosophila suzukii (Diptera: Drosophilidae) is an invasive alien species devastating soft fruit crops in newly invaded territories. Little is known about the importance and potential of long-distance dispersal at a regional scale. The goal of this work is to investigate *D. suzukii* dispersal ability during different times of the season, and along an elevational gradient in a mountain valley in Trentino Province, Italy. We employed a mark–release–recapture strategy using protein markers. Flies were recaptured using fruit- baited traps. The protein-marked flies were positively identified using ELISA procedure. Additional microsatellite analyses were performed on *D. suzukii* collected during autumn at different elevations to characterize the population structure. Results suggest that a portion of the local *D. suzukii* population moves from low to high elevations during spring and summer and travels back to low elevations in autumn. Genetic analysis further revealed that samples collected during autumn at different elevations belong to the same population. These results show that *D. suzukii* are able to fly up to about 9000 m away from the marking point and that seasonal breezes likely facilitate long-distance movement. We suggest that these migrations have multiple functions for *D. suzukii*, including conferring the ability to exploit gradual changes of temperature, food, and ovipositional resources in spring and autumn, as well as to assist in the search for suitable overwintering sites in late autumn. Our findings help to unveil the complex ecology of *D. suzukii* in Italian mountainous regions and provide important clues for improving the efficacy of integrated pest management control techniques to combat this pest.

Keywords Spotted wing drosophila · Mark–release–recapture · Seasonal movement · ELISA · Microsatellite markers · Abiotic factors

Key message

- This study was conducted to investigate *D. suzukii* dispersal ability during different times of the season, and along an elevational gradient in a mountain valley in Trentino Province, Italy.
- The findings of the current study add to scientific understanding of *D. suzukii* seasonal migration patterns, suggesting that this species is able to move and select the most suitable sites in accordance with the change of sea-sons.
- Our data are the first to document *D. suzukii* dispersal over distances of up to 9 km.

Introduction

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae) is an invasive insect species belonging to the *Drosophila melanogaster* species group (Yang et al. 2004) . It is pre-sumed to be native to Japan, where it was first described in 1931 (Kanzawa 1939). The species has been collected in other Asiatic regions (Cini et al. 2012) and the first individuals of *D. suzukii* were recorded outside of its indigenous range in the early 1980s in Hawaii (Lee et al. 2011; Asplen et al. 2015). In 2008, *D. suzukii* was found in California, Spain and Italy, and the species rapidly expanded throughout

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Europe and the Americas (Hauser 2011; Calabria et al. 2012; Cini et al. 2012; Mazzi et al. 2017; Tait et al. 2017). The economic losses due to *D. suzukii* are considerable in Trentino Province, Italy, where in 2011 damage was estimated at €3.3 million. However, the economic impact of the pest in Tren-tino declined from an estimated 13% of potential revenue in the absence of management to 7% under an integrated pest management strategy (De Ros et al. 2015). In North America, Farnsworth et al. (2017) estimated an annual total of $US 511 million in potential damage.

Drosophila suzukii is highly polyphagous (Cini et al. 2014) and well adapted to temperate climatic conditions characterized by alternations of warm summers and cold winters (Mitsui et al. 2010; Ometto et al. 2013). These key factors allow the species to survive even in an alpine climate, where the temperature has significant fluctuation during the year. Another important characteristic is that *D. suzukii* has developed the ability to overwinter in reproductive diapause in a distinct, relatively cold-resistant morphotype (Dalton et al. 2011; Ometto et al. 2013; Rossi-Stacconi et al. 2016; Shearer et al. 2016). Furthermore, according to the number of trap catches throughout the season and across elevational gradients, Mitsui et al. (2010) hypothesized that *D. suzukii* migrates progressively towards higher elevations during summer and returns to progressively lower elevations as temperatures decrease. It was speculated that this behaviour would enable *D. suzukii* to minimize exposure to suboptimal high temperatures and resource-limited conditions at low elevations during summer. Consequently, this behaviour would also allow such populations, generation after genera-tion, to exploit suitable food resources, i.e. ripening fruits, as they progressively become susceptible for colonization at higher elevations as the season progresses (Tonina et al. 2016).

Anthropogenic activities play a major role in the world-wide spread of invasive alien species (Taylor 1974; Taylor et al. 1984; Bigsby et al. 2011). Animal dispersal is typically studied by following the movement patterns of marked individuals (Nathan et al. 2003). Therefore, while human movements and global trade are likely the cause of the rapid passive spread of *D. suzukii* on a continental scale (Cini et al. 2012), little information is available on its active shorter-range dispersal capacity, apart from limited small-scale field studies (Klick et al. 2015). There are multiple approaches to study the dispersal capacity of insects (Hagler and Jackson 2001). Insect marking techniques include use of stable radio isotopes (Hagler and Jackson 2001; Boina et al. 2009; Zhang et al. 2016), fluorescent dusts (Schneider 1989; Skovga 2002; Coviella et al. 2006), and immunomarking (Hagler and Jackson 2001; Hagler and Jones 2010; Klick et al. 2014; Lessio et al. 2014). An ideal marker should have no effect on insect behaviour, resist severe weather conditions, be inexpensive, be easily applicable to the study species over large areas and be readily detectable (Hagler and Jackson 2001). Immunomarking techniques, which make use of proteins from animal or plant sources, fulfill most of these requirements and have been applied success-fully in mark–release–recapture studies, whereby wild or reared insects were marked by contact with contaminated plants in the field or directly marked with the proteins under controlled conditions (Jones et al. 2006; Klick et al. 2014; Lessio et al. 2014; Blaauw et al. 2016).

In the present study, we wanted to assess scale dispersal ability and the seasonal elevational movement capacity of *D. suzukii*. To attain this aim, we used a procedure that combined marking wild *D. suzukii* individuals by contact with plants contaminated with chicken egg albumin with a capture–release–recapture protocol. The experimental site was a steep alpine valley located in Trentino Province (Italy) with a known high *D. suzukii* population density (Rossi Stacconi et al. 2016) . Genetic analyses were performed on *D. suzukii* collected during early autumn at different elevations to permit characterization of the local population structure. Our hypothesis was driven by observed trap catches along an elevation gradient (Rossi Stacconi et al. 2016) similar to those found by Mitsui et al. (2010). We hypothesized that *D. suzukii* individuals would move from low to high elevations in late spring or early summer. As temperatures become unfavourable at lower elevations individuals would move to more favourable temperatures at higher elevations as host plants become available during the progression of the season. Conversely, during late summer this trend would be reversed in order to exploit available food resources, suit-able climatic conditions, and adequate overwintering sites. Results are discussed in light of the current knowledge about the biology and ecology of *D. suzukii* and in consideration of implications to pest management.

Materials and methods

Mark–release–recapture trials

Three trials were conducted in a forested habitat with elevations ranging from 509 to 1520 m above sea level (a.s.l), along the Mocheni Valley, a lateral valley of the Valsugana Valley (Trento Province, Italy). This experimental site was selected because it hosts a previously studied *D. suzukii* pop-ulation that is causing important losses to soft fruit produc-tion (Fig. 1) (Rossi Stacconi et al. 2016). Each trial included a single marking point (Fig. 2), respectively, at 664 m (Serso, hereafter trial A), 871 m (Mala, trial B) and 1471 m elevation (Passo Redebus, trial C). The mark–release–recapture procedure made use of chicken egg albumin as the protein marker (Klick et al. 2014). The marker was applied twice weekly as solution (C6534, Sigma–Aldrich) using a

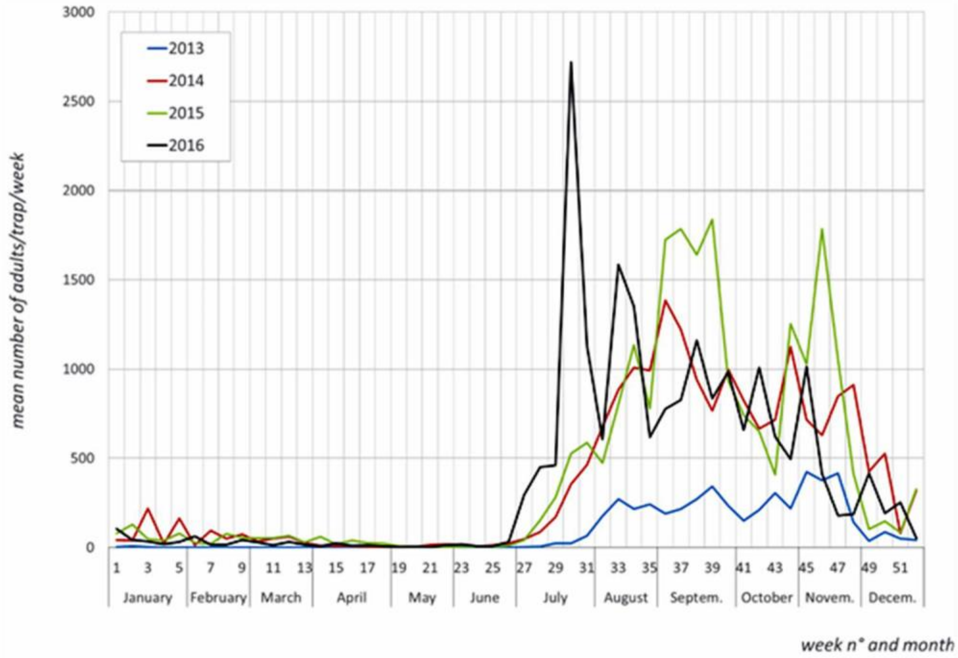

Fig. 1 Captures of *D. suzukii* during 2013–2016 in Valsugana and Mocheni valleys. Traps were Droso-Traps (Biobest, Westerlo, Belgium) baited with Droskidrink® liquid

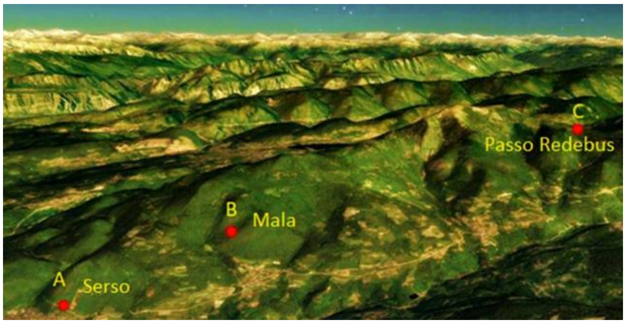

Fig. 2 Study area along the Valsugana and Mocheni valleys. Red points indicate the position of the three immunomarking points (low, middle and high elevations): Site A (Serso) corresponds to the low elevation spring marking point (46°04′54.91″N, 11°15′58.00″E); Site B (Mala) to the middle elevation summer marking point (46°05′42.01″N, 11°16′29.60″E); and Site C (Passo Rede-bus) to the high elevation autumn marking point (46°07′59.62″N, 11°18′41.24″E). This image has been adapted from https://earth.google.com/web/@0,0,24018.82718741a,36750128.22569847d,35y,0h,0t,0r/data=CgAoAQ

backpack sprayer with an electric pump (ITALDRIFA, OSS company, Trento, 16 L) to soak 300 m^2 (mostly consisting out of coniferous and deciduous species) and thereby marking wild *D. suzukii* adults within the experimental plot. Wild *D. suzukii* individuals were attracted to the plot by installing two food-baited Droso-Traps (Biobest, Westerlo, Belgium) in each of the three marking sites. These bait stations attracted flies and increased the probability of insect contact with albumin-contaminated vegetation as well as to catch additional *D. suzukii* for the controlled mark-release.

These traps contained small cups comprising a cotton wick soaked with Droskidrink-® liquid to avoid *D. suzukii* drowning in the liquid bait. The -Droskidrink® (Azienda Agricola Prantil, Priò, Trento, Italy) liquid consisted of 75% apple cider vinegar and 25% red wine + 20 g/l of unrefined brown sugar. Individuals found in these traps were collected three times per week and were confined in a plastic cage soaked with albumin by means of a manual sprayer (Growland, ELHO-002, 0.7 L) after which they were released into the environment after 5 m in exposure.

The first and second trials (A, B) were conducted from March 18 to May 19, 2016 and from June 26 to August 8, 2016, respectively, in order to study *D. suzukii* dispersal ability from low to high elevations. Trial C was conducted from September 29 to October 31 to determine dispersal from high to low elevations. For each trial, food-baited traps as described above were deployed around the three marking sites at different distances along an elevational gradient (Fig. 3). At Serso (46°04′54.91″N, 11°15′ 58.00″E), traps were positioned at a linear distance of 40, 100 and 500 m from the marking point in each cardinal direction with a range of elevation from 509 to 790 m a.s.l. Six traps were located at higher elevations from the marking point, while another six traps were located at lower elevations. At Mala (46°05′42.01″N, 11°16′29.60″E), traps were placed at a linear distance of 390, 750, 1100, 2043 and 3000 m from the marking point. Two of these traps were located at a lower elevation than the marking point. At Passo Redebus (46°07′59.62″N, 11°18′41.24″E), traps were placed at a linear distance of 1080, 1935, 3092, 7716 and 9050 m from the marking point, one located at a higher elevation from

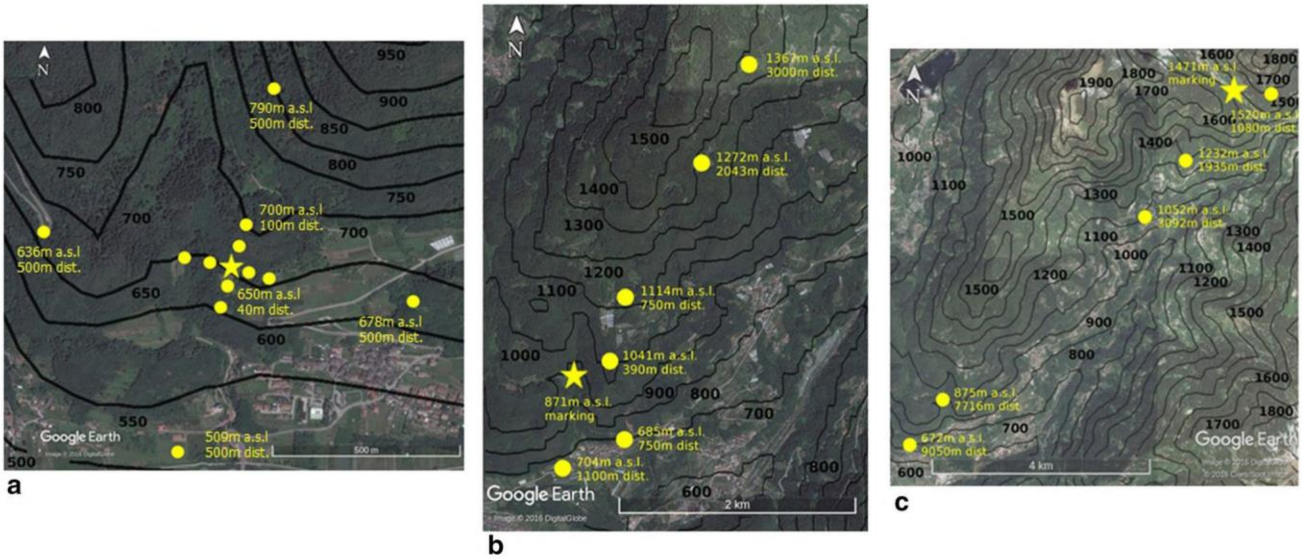

Fig. 3 Topographic map of experiment 1 (**a**) (March 29 to May 13, 2016, Serso marking site, at 664 m a.s.l.), experiment 2 (**b**) (June 29 to August 8, Mala marking site, 871 m a.s.l) and experiment 3 (**c**) (September 21 to October 31, Passo Redebus marking site, 1471 m a.s.l). The star indicates the position of the marking point and the dots indicate the positions of the traps. For Experiment 1, the nearest set of 4 traps was placed at a distance of 40 m from the marking point, with the other two sets of traps placed at a distance of 100 and 400 m, respectively

the marking point (1520 m a.s.l). Flies in each trap were collected three times per week and placed in Falcon tubes (Fisher Scientific Company, Hampton, NH, USA) separately from the other traps in the study; traps were serviced by first rinsing them with 70% EtOH and then with deionized water to provide a sterile surface area within each of the traps.

Protein-specific analysis

Insects collected in the mark–release–recapture experiment were placed individually into vials within an insulated cold box (~ 0–5 °C) and brought to the laboratory. Flies were identified to species under a binocular stereoscope. *D. suzukii* individuals were separated from other drosophilids and placed individually into 1.5 mL microcentrifuge tubes. All samples were subsequently stored at − 80 °C until ELISA was performed. For each sample, the ELISA procedure for the identification of chicken egg albumin was carried out by analysing pools of two same-sex individuals into a single well of a 96-well ELISA microplate (Nunc polypro; Nalge Nunc, Naperville, IL, USA). Two insects per well maximized the number of screened individuals and allowed us to better estimate *D. suzukii* seasonal movements at a population level. Positive results were counted as a single marking event, using a modified protocol from Jones et al. (2006). Paired insects were placed into a 1.5 mL microcentrifuge tube containing 1 mL tris-buffered saline (TBS, pH 8.0; T- 664, Sigma-Aldrich, Milan, Italy) to which 0.3 g/l EDTA (Sodium (tetra) ethylenediamine tetra acetate, S657; Sigma-Aldrich, Milan, Italy) was added. Each tube contain-ing the submerged insects was vortexed for 5 s and left to stand for no more than 3 min at room temperature. Two ali-quots (two replicates) of 80 µL from each tube were pipetted into individual wells of the 96-well ELISA microplate. Each plate was subsequently covered with aluminium foil and incubated for 2 h at 37 °C. After incubation, all wells were emptied and washed five times with 300 µL phosphate-buff-ered saline (PBS, pH 7.4; P3813, Sigma-Aldrich) + 0.09% Triton-X100 (PBST) (Triton-X100; 9002931, MP Biomedi-cals, Irvine, CA, USA) followed by the addition of 300 µL of Blocker Solution to each well (37426, MP Biomedicals). One hour later, the Blocker Solution was discarded and 300 µL of PBST was used to wash the wells, and then 80 µL of primary antibodies was added. The primary anti-body was diluted in Blocker Solution + 1300 ppm Silwet-77 (2 µL in 8.0 mL), while the secondary antibody was diluted only in Starting Block PBS Blocking Buffer solution (1.4 µL in 8.0 mL) (37538, ThermoFisher, Waltham, MA, USA). After incubation for 30 min, the primary antibody was discarded and wells were washed five times with 500 µL of PBS-SDS and with three times with 300 µL of PBS. After this step, 80 µL of TMB (ImmunoPure Ultra TMB substrate kit, 34028, Pierce Biotechnology Inc., Rockford, IL, USA) was added to each well. The plate was placed on a rotary shaker at 37 °C in the dark for 10 min. In order to stop the reaction, 80 uL of 2 -NH_2SO_4 was then added to each well.

Flies were scored for the presence of the albumin marker protein using a dual wavelength plate reader (Universal

Microplate Reader EL 800; Bio Tek Instruments Inc, Winooski, VT) at 450 and 490 nm. Samples were scored as albumin- positive if the ELISA optical density (OD) was greater than the threshold suggested by Sivakoff et al. (2011). Calculation of the threshold is as follows: $\mu_j + 4s_j$ where μ_j and are the mean and the standard deviation of the 10 negative controls on the plate.

Weather data

Daily weather data were obtained from the Climate Forecast System Reanalysis (CFSR) software (Saha et al. 2010). CFSR is an historical grid weather data set that is derived from station and remote sensing data sources. These weather data were plotted for 2016 at the 11 sites where *D. suzukii* were marked, released and recaptured. For each day, the maximum and minimum temperatures were recorded. The daily temperature values were extracted from the hourly 0.25 °C CFSR model grid. A lapse rate correction (− 0.65 °C per 100 m) was applied between the elevation of each point and the mean elevation of the grid (Stone and Carlson 1979; Magarey et al. 2015).

DNA extraction and microsatellite amplification

Genomic DNA was extracted from 90 *D. suzukii* individuals collected during early autumn of 2015 at the three different elevations in order to characterize the genetic structure of *D. suzukii* present in the Mocheni Valley. Samples were collected at the bottom of the valley near Serso, at the middle of the valley near Mala, and at Passo Redebus. Fifteen highly polymorphic microsatellite simple sequence repeats (SSRs) were selected from a set of microsatellites designed and validated in previous works and amplified to characterize the genetic structure of the sampled insects (Fraimout et al. 2015; Tait et al. 2017). All SSRs are continuous di-nucleotide combinations. Our choice was based on the known allele number of *D. suzukii*. For each locality, 30 samples were genotyped (15 females and 15 males). The DNA was isolated from each individual separately using the Macherey–Nagel Kit (NucleoSpin Tissue, Macherey–Nagel, Düren, Germany). Our choice was based on the known allele number of *D. suzukii* (Fraimout et al. 2015; Tait et al. 2017). Each pair of primers was used for PCR amplification in 25 µL final volume containing 1X GoTaq G2 Master Mix, 0.5 µL of each primer, 10.5 µL of ddWater and 1 µL of genomic DNA. The PCR program was set with an initial 30 s period of denaturation at 94 °C followed by 32 cycles of 30 s of additional denaturation at 94 °C, an annealing phase of 90 s at 57 °C, an elongation phase of 60 s at 72 °C and a final extension phase of 30 min at 72 °C. The PCR products were checked by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under UV light. Each amplicon was then diluted 1:10 in distilled water; 1 µL of the dilution was added to 12.5 µL of a mixture of deionized Formamide (Sigma–Aldrich) and GeneScan-500 ROX size standard (Life Tech, Waltham, MA). Prior denaturation for 4 min at 94 °C, capillary electrophoresis was carried out in an ABI PRISM 310 Genetic Analyzer (Life Tech) and the fragments were sized with the GeneMapper v.4.0 software in binning mode. Following two PCR attempts with no sample amplification, the locus was scored as a negative result.

Statistical analysis

All the statistical analyses were conducted with the software *Mathematica 11* (Wolfram 1991) and the computing environment R (R Core Team 2014).

For each mark–release–recapture experiment, the relation between trap elevation and number of marked sam-ples was analysed by Poisson regression. This regression is based on the assumption that for a trap with elevation (h) and distance from the marking point (d) the num-ber of marked catches is Poisson -distributed with the mean = c × $\exp^{(\alpha \times h + \beta \times d)}$, where c is a scaling parameter proportional to the total number of recaptured marked insects and α and β are parameters describe how the expectation for marked catches depends on the elevation of the trap and its distance to the marking point, respectively.

The maximum likelihood estimators were obtained for parameters α and β. The sign of the parameter α deter-mines whether elevation and marked catches are correlated positively or negatively. Parameter β is constrained to be less than or equal to zero because we assume that, in a flat landscape, the number of marked insects should decrease or at least not change with the distance from the marking point. Significance of those trends was estimated by testing the null hypothesis that number of catches is independent of trap elevation and trap distance. Dispersal parameter β was additionally expressed as the median dispersal dis-tance in a flat landscape $r_{0.5} = \log(2)/\beta$ (Northfield et al. 2009), together with a confidence interval for this estima-tion. This model is known to provide an adequate phe-nomenological description of insect dispersal (Lessio et al. 2014; Northfield et al. 2009).

Finally, data from the three mark–release–recapture experiments were used to investigate possible trends in the insect sex ratio. Trends were investigated by testing, with appropriate χ^2 tests, the null hypothesis that the sex ratio is independent of the time of the year, and that it is the same for total catches and marked catches. The ratio between *D. suzukii* males and females was calculated for total catches and marked catches in Trials A, B, and C, computing 95% confidence intervals of the binomial proportion.

Genetic data analysis

For microsatellite analysis, data obtained upon the fragment analysis were processed with Tandem program (Matschiner and Salzburger 2009) in order to avoid genotyping errors. GENALEX software v.6.41 (Peakall and Smouse 2006) was run to determine the genetic variability among populations and included the following parameters: mean number of alleles (N a), effective number of alleles (N_e), expected and observed heterozygosity (H_e and H_o, respectively), number of private alleles (N_p) and inbreeding coefficient (F_{is}). Deviation from Hardy–Weinberg equilibrium and the allelic polymorphic information content were tested using CER-VUS software v.3.0, with Bonferroni correction for multiple testing (Kalinowski et al. 2007). The genetic structure of populations was evaluated using neighbour-joining tree, AMOVA analysis and measuring the index of differentiation F_{st}. The unrooted neighbour-joining tree, based on Nei's genetic distance, was constructed using DARWIN v.5.0 soft-ware (Peakall and Smouse 2006). AMOVA analysis obtained from the Arlequin v.3.5 program was performed to estimate the variability of the distribution within and between the tested groups (Excofer et al. 2005).

Results

Mark–release–recapture and weather effect

Insects collected in modified food-baited traps during the three seasonal experiments were screened for the presence of the albumin protein on their bodies. The number of marked and non-marked insects caught during the three trials is presented in Tables 1, 2, and 3. The statistical analysis on the regression of the number of marked insects in the trap elevation and their distance from the marking point is shown in Table 4. For trials A and B (spring and summer experi-ments), catches of marked insects significantly increased with an increase in trap elevation, while the number of flies decreased numerically with an increase in distance from the marking point. The data points and regression curves for trial A are shown in Fig. 4. In trial C (autumn), catches of marked insects increased with the distance from the marking point in a flat landscape. Since this seems unrealistic and the trend was not statistically significant, we set the corresponding parameter to zero and tested the influence of the trap elevation alone. However, the data displayed no clear correlation between trap elevation and the number of catches of marked *D. suzukii*. The null hypothesis that the number of marked insects is independent of trap elevation likewise cannot be rejected. The median dispersal distance in Trial A and B was, respectively, $r_{0.5}$ = 176 m and $r_{0.5}$ = 2525 m with lower limits for the 95% confidence interval of 81 and 1244 m. No upper limits could be found for either trial possibly because of the low number of catches. In summer and autumn, marked *D. suzukii* were recorded as far as 3000

Table 1 Catches of marked *D. suzukii* during the first experiment A (marking at 664 m a.s.l.)

Elevation a.s.l. (m)	Distance from marking point (m)	Catches (marked/total)	OD value
672	40	2/26	1.21 ± 0.74
683	40	2/6	1.92 ± 0.02
650	40	2/14	1.27 ± 0.65
653	40	0/14	0
667	100	2/30	1.39 ± 0.57
700	100	2/5	1.94 ± 0.00
620	100	2/29	1.79 ± 0.08
626	100	0/20	0
635	500	0/26	0
790	500	3/15	1.24 ± 0.77
509	500	0/21	0
678	500	0/16	0

In the table, the elevation of the traps, their distance from the marking point, the number of positive wells and the optical density at 450 nm (OD) are reported

Table 2 Catches of marked *D. suzukii* during the second experiment B (marking at 871 m a.s.l.)

Elevation a.s.l. (m)	Distance from marking point (m)	Catches (marked/total)	OD value
685	750	20/352	0.63 ± 0.52
704	1100	4/142	0.47 ± 0.19
1041	390	14/251	0.46 ± 0.28
1114	750	19/488	0.41 ± 0.27
1272	2043	39/777	0.26 ± 0.28
1367	3000	5/479	0.69 ± 0.41

In the table, the elevation of the traps, their distance from the marking point, the number of positive wells and the optical density at 450 nm (OD) are reported

Table 3 Catches of marked *D. suzukii* during the third experiment (marking at 1471 m a.s.l.)

Elevation a.s.l. (m)	Distance from marking point (m)	Catches (marked/total)	OD value
672	9050	2/887	0.10 ± 0.04
875	7716	7/332	0.09 ± 0.03
1052	3092	5/101	0.12 ± 0.01
1232	1935	0/78	0
1520	1080	5/51	0.17 ± 0.09

In the table, the elevation of the traps, their distance from the marking point, the number of positive wells and the optical density at 450 nm (OD) are reported

Table 4 Statistical analysis of dependence of dispersal on elevation in the three marking experiments

Period	α ±standard error	p value	β ±standard error	p value
Trial A: 29/3–13/5/16	$1.8 \times 10^{-2} \pm 8.^{3} \times 10^{-3}$	0.031	$-3.9 \times 10^{-3} \pm 2.4 \times 10^{-3}$	0.096
Trial B: 26/6–8/8/16	$1.4 \times 10^{-3} \pm 5.7 \times 10^{-4}$	0.013	$-2.7 \times 10^{-4} \pm 1.4 \times 10^{-4}$	0.057
Trial C: 21/9–31/10/16	$-3.0 \times 10^{-6} \pm 7.9 \times 10^{-4}$	0.997	0	–

The parameters α and β express the relation between the number of marked catches and the height of the trap and its horizontal distance to the marking point

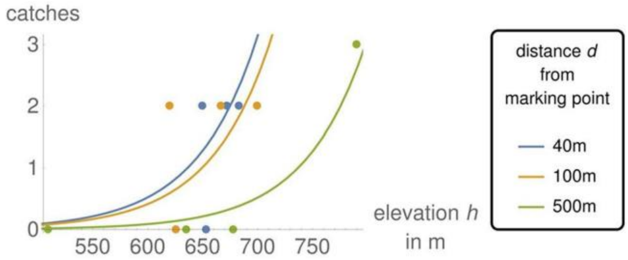

Fig. 4 Number of marked catches in the traps in Trial A, together with the regression curves. This regression is based on the assump-tion that for a trap with elevation (h) and distance from the marking point (d) the number of marked catches is Poisson distributed with the mean $= c \times \exp^{(\alpha \times h + \beta \times d)}$, where c is a scaling parameter propor-tional to the total number of recaptured marked insects and α and β are parameters describing how the expectation for marked catches depends on the elevation of the trap and its distance to the marking point, respectively

and 9050 m away from the marking point (Tables 2, 3), suggesting that these distances are highly likely for seasonal migration.

There is a clear trend towards skewed sex ratios of *D. suzukii* that change throughout the growing season, as was confirmed by a test on the independence between sex ratio and season. Significantly higher numbers of female flies were captured in spring and significantly higher numbers of male flies were captured in summer and autumn ($P = 0.005$ for marked insects and $P < 0.001$ for total captures, Fig. 5). The sex ratio, however, did not differ significantly when comparing marked and total caught insects in any of the three experiments ($P = 0.9$, $P = 0.8$ and $P = 0.6$ for spring, summer and autumn, respectively), suggesting similar migration patterns for males and females.

Concerning the daily weather conditions, temperature values were always notably higher at the lowest-elevation experimental site (Serso, 515 m) compared to the highest elevation trap locality (Passo Redebus, 1520 m) (Fig. 6), supporting the possibility of migration due to the suit-ability of local temperatures at different elevations during different times of the year.

Genetic diversity study

In total, 92 *D. suzukii* adults were sampled and subjected to genotyping for 15 previously identified microsatellite markers. The allele distribution of the tested insects is summarized in Table 5. A limited expected genetic variability across *D. suzukii* collected in the three sample sites was measured. DARWIN software was run to estimate pairwise genetic distances and subsequently for the construction of a dissimilarity matrix. Once the dissimilarity matrix was obtained, data were subjected to cluster analysis using the unweighted neighbour-joining method (UNJ). Using the parameters of this analysis, no individu-als belonged to a separate cluster (Fig. 7), indicating that *D. suzukii* collected at the three different locations share the same genetic pattern. F_{st} values (Table 6) provide no evidence of genetic differentiation among the three ana-lysed groups.

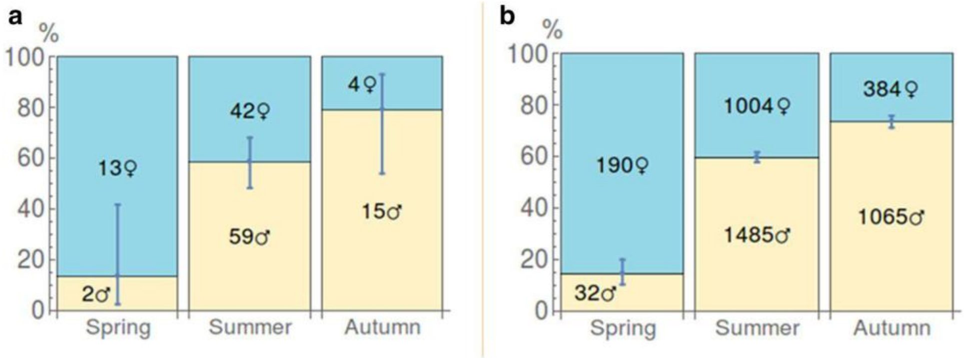

Fig. 5 Marked catches in the three experiments by gender (**a**) and total catches in the three experiments by gender (**b**). The error bars correspond to 95% confidence intervals for the proportions and values outside the intervals can be rejected on a P = 5% significance level

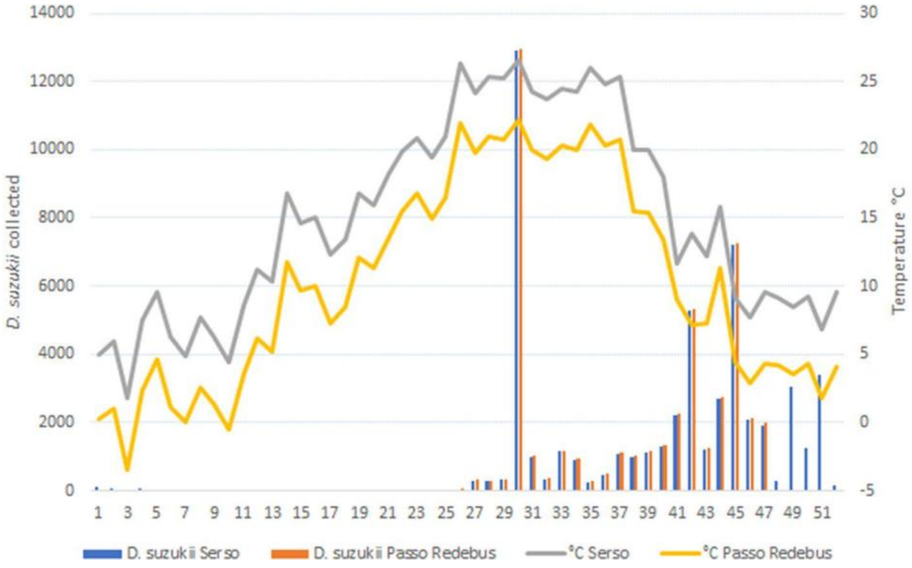

Fig. 6 Weekly temperature trends in Serso (545 m a.s.l.) and Passo Redebus (1455 m a.s.l) during year 2016. Columns represent the mean of the *D. suzukii* adults collected weekly from the first week of the year to the last

Table 5 Level of genetic diversity across 3 groups of *D. suzukii* collected at the 3 different elevations

	N_a	N_e	A_r	H_o	H_e	F_{is}
Passo Redebus	6.06	3.56	5.96	0.708	0.702	0.00
Mala	6.53	3.87	6.39	0.791	0.726	-0.08
Serso	7.13	3.71	7.00	0.679	0.709	0.04

N_a mean number of alleles; N_e mean effective number of alleles; A_r mean of allele richness; H_o mean observed heterozygosity; H_e mean expected heterozygosity; F_{is} mean inbreeding coefficient

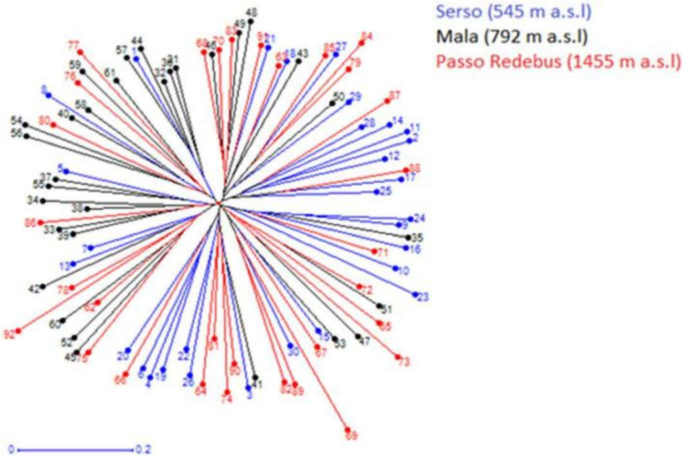

Fig. 7 Neighbour-joining tree Unrooted neighbour join-ing (UNJ) tree obtained from DARWIN software based on pairwise genetic distances. Each branch represents an individual fly

Table 6 Pairwise F_{st} among three groups of *D. suzukii*

	Mala	Passo Redebus	Serso
Mala	0		
Passo Redebus	0.011936	0	
Serso	0.006613	0.007712	0

Collected data provide no evidence of genetic differentiation between the three analysed groups

Discussion

It is known that diverse arthropod species move season-ally from one area to another (Coyne et al. 1982; Riley et al. 1995; Ahmed et al. 2009; Stefanescu et al. 2013). In *Drosophila*, some temperate species migrate from low to high elevations in early summer and probably return to low elevations in autumn (Kimura et al. 1977; Kimura and Beppu 1993; Kimura 2004; Mitsui et al. 2010). Mitsui et al. (2010) described *D. suzukii* as a fruit specialist spe-cies that occurs at specific times of the year in mountain-ous regions of Japan. For this reason, it may be reason-ably assumed that this species is able to perform seasonal migration between low and high elevations. The findings of the current study add to the scientific understanding of *D. suzukii* seasonal migration patterns, suggesting that this species moves and selects environmentally suitable sites. Klick et al. (2015) observed that *D. suzukii* population within the cultivated crop is associated with high activity levels in the field margin containing host plants. This behaviour could be driven both by host crop availability and suitable environments within the surrounding vegeta-tion and the crop alike. Overall, the results of the current work provide additional information on the magnitude of seasonal long-distance movement of *D. suzukii*. Our data are the first to document *D. suzukii* dispersal over dis-tances of up to 9 km and further suggest that during spring and summer, flies move from low to higher elevations. The difference in temperature between these elevations may play a role in the migration of *D. suzukii* populations toward more suitable temperature regimes (Tochen et al. 2014; Ryan et al. 2016; Wiman et al. 2014; Grassi et al. 2017). Migration towards higher elevations in spring might be exploratory movement to search for additional oviposition sites and food resources in favourable envi-ronments (Mitsui et al. 2010; Tochen et al. 2014, 2016; Wiman et al. 2014; Evans et al. 2017). Summer migration may additionally be a strategy to avoid suboptimal high temperatures at lower elevations. It is possible that migra-tion in autumn towards lower elevations might be driven by suitable climatic conditions during the latter portion of the season here. (Evans et al. 2017; Stephens et al. 2015). In spring and summer, the number of catches of marked individuals significantly increased with trap elevation, while it decreased with distance from the marking point. This suggests temporal dispersion towards outlying regions and towards different elevations. With regard to the autumn experiment, data showed no clear relationship between trap elevation and the number of marked catches but, despite this, positive marked flies were recorded at different distances and elevations, suggesting a season-ally differentiated spread of *D. suzukii*. In our experi-ment, *D. suzukii* movement was most likely facilitated by updrafts (Beerwinkle et al. 1994; Brattström et al. 2008). We believe that the dispersal of *D. suzukii* from a low to a high elevation during spring and summer could be facilitated by diurnal mass movement of air due to fluctuations in temperature from the day to the night. The breeze regime in Trentino valleys has been previously described by Giovannini et al. (2013). During spring and summer, daily wind speed is greater than in autumn and winter (Goldreich et al. 1986). With the arrival of cooler autumn temperatures, air movement from high to low elevations likely promotes the movement of flies towards lower elevations (Giovannini et al. 2013). Genetic analysis provided data to support our hypothesis. Results from genetic analysis revealed that tested insects belonged to the same population. The low genetic differentiation among the three collected groups in our trials indicates recent colonization by *D. suzukii* in Italy (Tait et al. 2017) and validates the hypothesis of genetic homogeneity between *D. suzukii* insects collected at different elevations. This gene homogeneity is not only due to transport by human movement towards the valley but may also be attributable to a natural migration towards different elevations during different times of the year (Mitsui et al. 2010). It is clear that the study area provides ideal conditions for the persistence and reproduction of *D. suzukii* populations. Strategies to control this destructive species must take into account its high dispersal potential. Finally, our results suggest that *D. suzukii* populations are present at lower altitudes during the early portion of the season. No *D. suzukii* are found at high altitudes during the early season. Factors contributing to this finding include suboptimal winter conditions and the lack of wild fruits (Tonina et al. 2016). Considering these observations, a valid approach could be to be plant early producing berry varieties in high -elevation sites. Such crops might benefit from temporal escape from the onset of seasonal migration.

Furthermore, knowledge concerning the capacity of *D. suzukii* dispersal is crucial to better-forecast development of pest populations, to validate and optimize population models, and to more effectively design management strategies.

Author contributions

GT contributed to all the steps, planning the experimental design, sampling populations, conducting laboratory tests and data analysis, as well as writing the manuscript. AG contributed to the research concept and design as well to insect sampling. FP contributed to data analysis and to the manuscript draft. CMC contributed to data interpretation and the manuscript. DTD contributed to data analysis, data interpretation and the manuscript draft. RM provided weather data. LO and SV participated in genetic data interpretation and drafting the manuscript. MVRS participated in drafting the manuscript. AG contributed to insect samplings. AP contributed to statistical data interpretation. GF contributed to genetic data interpretation. VMW contributed to data interpretation and drafting the manuscript. GA conceived the central idea and contributed in drafting the manuscript. All the authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All applicable international, national, and/or institu-tional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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CHAPTER 4.

Drosophila suzukii daily dispersal between crop and surrounding vegetation

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Drosophila suzukii daily dispersal between commercial blackberry tunnels and and surrounding vegetation

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Abstract

Drosophila suzukii, Matsumara, (Diptera: Drosophilidae) is an insect pest species originating in South-East Asia that has invaded both Europe (EU) the Americas. This insect has subsequently caused extensive economic damage to the soft fruit industry. *D. suzukii* population management relies primarily on chemical pesticides, a technique with serious drawbacks related to the risk of residues on fruit. *D. suzukii* migration from surrounding vegetation is believed to significantly increase the risk of crop damage. Targeted solutions towards *D. suzukii* management are therefore dependent on more detailed knowledge concerning its daily migration patterns between the crop and surrounding vegetation. This work had the goal to study the daily dispersal patterns between the crop and surrounding vegetation. During the summer of 2017, we employed a mark-capture strategy using protein markers. Flies were captured using fruit-baited Droso-Traps and later analyzed for the presence of the marker through an ELISA procedure. Results clearly indicate daily movement from the crop to the surrounding woody vegetation. Our data suggest that this migration is related to optimal temperature, humidity, and suitable alternative hosts found in the surrounding woody vegetation. Up to 78% of *D. suzukii* marked within the crop were collected in surrounding woody vegetation within 24 hours of the marking event. These observations will help direct more optimal pest management for this pest and could help farmers and industries decide when, where and how to manage *D. suzukii* pest populations. Furthermore, the finding of consistent daily movement from the crop to the surrounding vegetation under these conditions may lead to improved *D. suzukii* targeting and improved management.

Keywords Spotted Wing Drosophila, Immunosorbent technique, migration, abiotic factors

Introduction

Among the 1500 and more species belonging to the *Drosophila* genus, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), a member of the “oriental lineage” of the melanogaster subgroup (Markow and O’Grady, 2006), can be considered the only real threat to cultivations and agriculture and is now acknowledged as an important pest of various fruit plants of different families (Rota Stabelli *et al.*, 2013). *D. suzukii* is also known as cherry drosophila in Japan, because of its ability to penetrate and damage healthy cherry fruits, and spotted-wing drosophila in the USA, since males have a black spot on each of their wings. This fly combines high dispersal ability (Calabria *et al.,* 2012; Cini *et al.*, 2012, Tait *et al*., 2018) with a wide thermal tolerance that enhances its ability to overwinter (Walsh *et al*., 2011; Enriquez & Colinet, 2017; Shearer *et al.*, 2016; Rossi Stacconi *et al.*, 2016; Rendon *et al*., 2018), and a short generation time, especially in spring and summer (Grassi *et al*., 2017). These characteristics, together with a serrated and more streamlined ovipositor, considerably increase the species’ capacity to feed on and oviposit in healthy, ripe fruit. There are at least 15 different susceptible fruit, ranging from soft-skinned berry fruits, like raspberries, blackberries, blueberries and strawberries, to more resistant stone fruits, like peaches, almonds and plums, and grapes (EPPO, 2013; Walsh *et al.*, 2011; Ioriatti *et al.,* 2015; Ioriatti *et al*., 2018; Tonina *et al*., 2016; Tait *et al.*, 2017). Because of these characteristics, *D. suzukii* has been able to cause substantial economic damage to production regions that are heavily reliant on the cultivation and marketing of these products. It is estimated that in 2009 this insect caused up to $500 million of losses in the three major Pacific soft fruits production states (Bolda *et al*., 2010). Two years later, in 2011, the Trentino region (Italy) suffered losses of around € 3 million, (c.a.~10% of the average expected revenues of the soft fruit industry, which is one of the main local agricultural activities) (De Ros *et al*., 2013; De Ros *et al.,* 2015). *D. suzukii* damage can be categorized as direct and indirect. Direct damage due to oviposition begins when the female lays eggs into the fruit’s pulp: eggs hatch inside the fruit and develop through three larval instars, causing the fruit to rapidly soften and become unmarketable (Walsh *et* *al.,* 2011; EPPO, 2013; Cini *et al*., 2014). Indirect damage is due to oviposition wounds that can become sites of secondary infections by pathogens such as fungi, yeasts and bacteria, leading to additional revenue loss (Chabert *et al.,* 2012; Atallah *et al*., 2014; Wiman *et al.*, 2014; Ioriatti *et al.*, 2018). To try limit the negative impact of this pest on economically important production systems, many management techniques are being studied, from agronomic and biological approaches, although most control still relies heavily on pesticides. A critical aspect to optimizing *D. suzukii* control depends on multiple strategies aimed at reducing the potential damage and thus the economic losses. For this reason, it is important to increase our knowledge of the daily movement of *D. suzukii* between the cultivated crop and nearby uncultivated areas. This knowledge will improve improved targeting of management programs against this pest. *D. suzukii* has a high dispersal ability and is able to travel up to 9 km in 4 to 5 days and to move along extreme elevation gradients during the year (Calabria *et. al*, 2012; Tait *et al*., 2018). Little is known about *D. suzukii's* small-scale movements or the relation between near-crop vegetation and the quantity of flies infesting the field. Klick et al. (2016) explained how the presence of “Himalayan Blackberry”, a non-crop *D. suzukii* host, near the border of a crop, increased the number of flies that were captured in the crop itself, providing evidence that presence of these plants in the crop’s surroundings may increase crop damage. His study, however, did not take into account the relative difference and suitability of the crop and border microclimates in relation to each other, nor did it provide details of non-crop flora composition, or *D. suzukii* movement from the crop to the surrounding vegetation within daily cycles. Kirkpatrick et al. (2018) demonstrated that the maximum dispersal distance from the crop edge to the crop interior is 90 m per day, while Leach et al. (2018) in her work underlined deeper the importance of wild alternative host for the survival of *D. suzukii*. The aim of this study was to investigate 1) how *D. suzukii*’s movement is affected by the time of the day, 2) how temperature and humidity can affect the daily migration between different environments, and 3) how the presence of non-crop alternative hosts in the surrounding wild area can influence the presence of *D. suzukii.*

Material and Methods

To directly document the movement of flies, we marked a sector of a cultivated field with chicken egg albumin, which would stick to any flies that came in contact with it. This approach is known as “mark-capture,” and differs from “mark-release-recapture.” The collected flies were analyzed for the marker protein following a slightly modified version of the protocol in Jones et al. (2006).

Field sites, trap positions and insect marking

Trials were conducted in a commercial (*Rubus fruticosus*) field located at San Rocco di Villazzano, Trento, Italy (46° 1'58.19"N, 11° 8'43.25"E, 360 m a.s.l) (Figure 1). Spotted-wing drosophila was managed during the experimental conditions, using deltamethrin sprays (Decis Evo, Bayer - 50 ml/hl) at 7-day intervals.

The blackberries were grown inside nylon-covered tunnels (5 m width, 4 m height, covered with Multisolar 3 nylon - Eiffel S.P.A, shielded with shading white ReduSol - ReduSystems®, Mardenkro B.V. on the roof) of variable length for a total surface area of 1800 m^2. The west side of the field was bordered by a wood that consisted of a mix of weeds, shrubs and trees (Table 1). The surrounding vegetation was identified to species level in a representative 10x13m area where traps were placed within the woods. A relative abundance level was assigned to each of the main plants, which were further classified as weeds, shrubs or trees based on their mean height. The number of individuals in the selected area was visually estimated and a +++ level was assigned when a plant was found in ≥ 10 individuals, a ++ level was assigned when a plant was found in ≥ 5 but <10 individuals, a + level was assigned when a plant was found in <5 individuals. The known host status for each of the respective non-crop plants is provided (Table 1).

We assumed that the blackberry field contained *D. suzukii* based on continued observed fruit infestation within this field during the preceding two weeks of monitoring and fruit inspection (data not shown). *D. suzukii* present in the field likely came into direct contact with the marking protein during the marking events (7:00 am on July 11 and July 31 2017). The mark-capture procedure made use of food-grade chicken egg albumin (Egg white, EUROVO srl, S. Maria in Fabriago, RA, Italy) as a protein marker (Klick *et al.*, 2014). The albumin was applied in a 10% solution with water using an electric backpack sprayer (ITALDRIFA, OSS company, Trento, 16 L) on a 900 m^2 area at the center of the crop (Figure 1). A total of eight food-baited Droso-Traps (CRI, Biobest, Westerlo, Belgium) within the crop were spaced approximately 10 meters apart. Traps were numbered from one to eight along s north-south axis, with trap eight situated at the southernmost location (Figure 1). Similarly, numbered and spaced traps were placed at the crop border (CRB) and within the woods (W). CRB traps were located parallel to the west and were 20 meters away from the traps within the crop, and W traps were 30 meters west of the CRB traps. Woods traps were 20 meters higher in altitude compared to traps within or on the border of the crop. Traps contained small cups with a cotton wick soaked with Droskidrink® liquid (Azienda Agricola Prantil, Priò, Trento, Italy –75% apple cider vinegar and 25% red wine + 20 g/liter of unrefined brown sugar) to attract flies and avoid insect drowning. Samples were grouped by their collection date, time and trap location. Collection dates were in two distinct periods: July 11 to July 14 and July 31 to August 4. Traps were checked twice daily at 7 am and 5 pm. Flies collected from traps were individually placed into 1.5 ml tubes, sited into cooled containers and taken to the laboratory within 2 hours. Flies were subsequently stored at -80 °C after being brought to the laboratory, and the egg albumin enzyme-linked immunosorbent assay (ELISA) was performed.

ELISA bioassay– OD reading

Presence of egg albumin on individual flies was assessed using an indirect enzyme-linked immunosorbent assay (ELISA). A slightly modified version of the protocol from Jones et al. (2006) was followed. For each sample, the ELISA procedure was carried out by analyzing single flies or pools of two individuals of the same sex. One or two flies were placed into wells of a 96-well ELISA microplate (Nunc Polypro; Nalge Nulc, Naperville, IL, USA). In cases when 15 or more flies were collected, we placed two insects per well in order to maximize the number of screened individuals and to estimate *D. suzukii* seasonal movements at the population level. Positive results were counted as a single marking event. Single and paired flies were placed into a 1.5 mL microcentrifuge tube containing 0.5 mL tris-buffered saline (TBS, pH 8.0; T-664, Sigma-Aldrich, Milan, Italy), to which 0.3 g/L EDTA was added (Sodium (tetra) ethylenediamine tetra acetate, S657; Sigma-Aldrich, Milan, Italy). Each 1.5 mL tube was retained for a minimum of 4 min. Two aliquots (replicates) of 80 µL were transferred from each tube into two wells of the 96-well microplate and replicates from the same 1.5 mL tube were put into contiguous wells of the same microplate. Each microplate (8 x 12 wells) was laid out as follows: on the first column, three wells (A1, B1 and C1) contained 80 µL of TBS plus 4 µL of marker protein, while the last two columns (11-12) only contained 80 µL of TBS. This layout left 81 wells available for use, but because two aliquots from each tube were used, one well (H10) remained empty. The samples in the plates were then treated following the ELISA protocol used in Tait et al. (2018). The optical density (OD) of the samples was measured using a dual wavelength plate reader (Universal Microplate Reader ELx800; Bio Tek Instruments Inc., Winooski, VT) at 450 nm, with 490 nm reference standard. Samples were scored as albumin-positive if the mean OD of the two aliquots (μ_i) was greater than the mean μ_j of the OD of the two negative control columns plus five times their standard deviation (i.e if $\mu_i > \mu_j + 5 \cdot \sigma_j$).

Weather data

Daily weather data (temperature, °C and humidity RH, %) were recorded (TinyTag data logger, Omni Instruments, Dundee, Scotland). These weather data were plotted for 2017 at 12 locations within the experimental area where *D. suzukii* were marked and recaptured (in proximity to traps 1, 3, 5 and 7, Figure 1). For each day, temperature and humidity values were acquired every five minutes from 9 am to 9 pm, providing 288 measurements per day, allowing more detailed description of environmental changes during the days of the experimental period. Relative humidity, daylight temperatures and humidity (~12-13 hours per day) values were normalized within the range of 0 to 1 for each day. Numbers between 0 and 1 represent values at the lower and upper-recorded temperature or humidity respectively. The values were calculated using the formula: (value - min)/(max - min). Values close to 0 and 1 represent temperatures close to the minimum and maximum-recorded temperatures for each day. We also calculated the maximum and minimum daily temperature and humidity value. Finally, we created an index to characterize the normalized range (0 = low variation, 1 = high variation) of respective temperature and humidity observations. Values closer to 1 illustrated relatively high differences. Values closer to 0 illustrated lower levels of differences between treatments.

Statistical analysis

Statistical analyses were performed using a one-way Anova as well as the Chi-square test to describe levels of movement of *D. suzukii* as indicated by marked flies caught within the crop inside (CRI), crop border (CRB), and woods (W) using R software (R Core team, 2013). Factors included date of collection, location of collection, trap position on the north – south axis and gender.

Data were fitted to a Poisson distribution as necessary to account for over-dispersion in the data. A chi-squared goodness-of-fit test (Yates' corrected) was conducted using R v.3.03 (R core Team 2013) to determine significant differences among the numbers of marked flies in the three selected areas. The aggregation of catches in the different traps was expressed as an index of dispersion D, which, assuming Poisson distributed catch numbers, equals the ratio of variance and the mean of the data (Madden and Hughes, 1995). $D = 1$ corresponds to random data, $D < 1$ to under-dispersed data and $D > 1$ to over-dispersed data. Significance of the dispersion trend was tested using the corresponding chi square test.

A correspondence analysis was additionally performed using Statistica™ (StatSoft, 2012), in order to provide statistical parameters to correlate temperature encountered during each period of the day (morning or afternoon), location, and trap counts. We categorized capture events i.e.: none (0 captures), low (1-6), medium (7-14), high (15-55) and very high (56+). Student's t-tests were performed on the number of captures to attest the influence of various variables on the number of captured flies.

Results

During the 11-14 July collection period, a total of 402 insects were collected within traps. Of these, 313 (77.86%) were collected in W traps, 59 (14.68%) in CRB traps and 30 (7.46%) in CRI traps. The sum of captured flies was statistically different between the woods and the other two areas (1-Way ANOVA: W vs CRB: F= 40.27, df = 3.67, p = 0.0044; W vs CRI: F = 53.67, df = 3.183 p = 0.004). The number of marked flies collected at CRB and CRI were however not statistically different (F= 3.88, df = 4.51, p = 0.112). The index of dispersion was D=22, suggesting an aggregation of the catches (Chi square: 505; df = 23; $p < 0.001$). The number of flies captured in the morning was statistically higher than in the evening in the three locations, (W: F= 38.17, df = 3.369, p = 0.006; CRB: F= 13.29, df = 3.444, p = 0.028; CRI: F= 12.1, df = 3.118, p = 0.0237).

During the 1-4 August collection period, a total of 737 flies were collected within traps. Of these, 452 (61,3%) were collected in W traps, 256 (34,7%) in CRB traps and 29 (3,9%) in CRI traps. W traps collected a non-significantly higher number of flies compared to the CRB traps (F= 1.75, df = 4.40, p = 0.239) but a significantly higher number compared to the CRI traps (F= 10.01, df = 3.10, p = 0.048). The index of dispersion was D=28, suggesting an aggregation of the catches (Chi square: 638; df = 23; $p < 0.001$). The number of flies captured in the morning was statistically higher than in the evening in the CRB traps but not in the other two areas (W: F= 5.09, df = 3.44, p = 0.097; CRB: F= 13.11, df = 3.01, p = 0.036; CRI: F= 2.85, df = 3.02, p = 0.189).

During 11-14 July, a total of 23 flies scored positive using ELISA. The largest number (16/23, 69.6%; F= 10.57, df = 3.88, p = 0.032) were collected from W traps. Only 4 and 3 (17.4% and 13%) were respectively collected from the CRB and CRI traps (Table 2). As for the W traps, the number of captures of marked flies was significantly higher in the morning than in the evening (W: F = 8.727, df = 3.924, p = 0.042; CRB: F = 2, df = 3.35, p = 0.241; CRI: F = 0.09, df = 3.87, p = 0.771).

During 1-4 August, a total of 31 flies scored positive using ELISA. Positive flies were respectively distributed in W (23, 74.2%), CRB (6, 19.4%) and CRI (2, 6.5%) (Table 2). Again, a significant difference was found between the number of marked flies captured by W traps and the others (Chi square: 21.9; df = 2; $p < 0.001$). The first marked insects were collected within 24 hours after the marking procedure, during both trial periods.

Overall, daily trends of both temperature and humidity in both trials are shown in relation with the daily *D. suzukii* fly captures (Figure 2). Overall, temperatures recorded in W traps were lowest followed by CRB; those recorded in the CRI location were warmest as indicated by the normalized values (Table 3). Humidity recorded in W was highest during the first trial period followed by CRB, and those recorded in the CRI location were lowest. This trend however, was, reversed during the second trial period.

The smallest and largest daily temperature difference between the hottest and coolest locations (CRI and W in all cases) were 0.93 and 3.12 °C respectively. Larger temperature differences were recorded during the first trial period compared to the second trial period as indicated by the high range index for this period. Larger humidity differences were recorded during the first trial period compared to the second trial period as indicated by the high range index for this period.

The influence of trap position and period of the day on the number of captures are represented in the correspondence analysis (Figure 3). P-values obtained from Student's t tests further confirmed the significance of trap position and period of the day, with morning collections having more captures than afternoon collections (p-value 0.005) and woods traps collecting more than crop inside traps (p-value 0.0022). To visualize the difference in captures between morning and afternoon as well as the influence of temperature we created two box plots: one compares morning and afternoon captures and the other one compares the average temperature value of the seven hours before collection (figure 4). Figures 5a, 5b, 6a and, 6b graphically represent the spatial distribution of the species for both periods.

Discussion

This is the first study to describe the daily movement of *D. suzukii* from the crop into surrounding vegetation, taking into consideration the relation with temperature and humidity. The trials were conducted during two climatically distinct periods within one growing season. The first had relatively higher temperature and humidity compared to the second. Relatively higher *D. suzukii* fly counts were made during the second period. Despite these differences and the fact that this work was carried out in one season, 2017, the collected data showed a consistent trend. These observed data are strongly supported by several other studies conducted at lower data resolution levels between crop and surrounding vegetation (Klick *et al*., 2016), indicating the importance of microclimate (Rogers *et al*., 2016, Diepenbruck and Burrack 2017, Tochen *et al*., 2016). These data strongly evidenced the daily *D. suzukii* migration between crop and surrounding area. We believe that these data describe in more detail the underlying reasons for such daily migration (Schneider 1989; Carriere *et al*., 2006; Swezey *et al*., 2013; Swezey *et al*., 2014; Blaauw *et al*., 2015). Our data, together with earlier studies under tunnels (Rogers *et al*., 2016), suggest that temperature and humidity, among others, are factors that influenced the migration between these distinct habitats. The first marked insects were collected within 24 hours after the marking procedure, during both trial periods. The relative high resolution of data collection over this short time span allowed the description of diurnal *D. suzukii* movements between the distinctly different ecosystems. For both trials in July and August, a statistically higher number of marked flies were found in the wood (69.6% and 74.2% respectively), suggesting a migration from the marking point into the crop towards the forest. In relation to this, the index of dispersion evidenced an aggregation status of the species into the wood (D = 22 and D = 28 in July and August respectively). It is possible that the presence of DroskiDrink baited traps may have influenced the shorter-range movement of *D. suzukii* flies. The distance between traps in the three distinct locations were however more than 3 m, the estimated area of attractive action of these baited traps. The temperature and humidity recorded fluctuated significantly, but general trends within each location were different. During the two experimental trials (11 to 14 July; 31 July to 4 August), the wood had consistently lower temperatures. These values were closer to 20 °C, the optimal *D. suzukii* developmental temperature compared to the other two areas. The mean humidity values were normal or higher in five out of eight days during the experimental periods. These data suggest that the more suitable micro-environment for *D. suzukii* were in the woods (Ometto *et al.*, 2013; Tochen *et al.*, 2014; Tochen *et al.*, 2016; Enriquez & Colinet, 2017). The microclimate was likely moderated by the architecture within the wood resulting in comparatively higher and more optimal levels of humidity than the other two environments. Similarly, more optimal and moderated lower temperatures were also found in the woods. In relation to the important influence of temperature, there is a strong inverse correlation between the daily temperature's change and the number of collected flies: during the early morning during when the mean temperature is 20 °C we collected the highest number of *D. suzukii* (both total and marked), while during the evening the temperature reached an average of 30 °C, the number of catches was lower. This underlying the fact that *D. suzukii* activity is lower when temperature are suboptimal (Wiman *et al*., 2014; Tochen *et al*., 2014).

An additional contributing factor to environmental suitability is the presence of alternative host plants that provide valuable food and reproductive resources. It was reported that such non-crop hosts may provide suitable ovipositional media, and also provide energy in the form of nectar (Lee *et al.*, 2016, Tochen *et al*., 2016). We believe that the significantly higher number of marked *D. suzukii* found in the woods confirms this assertion and that some of this movement was toward nutritional resources located in the wood. It is known that multiple species are able to migrate from the crop to a nearby crop habitat for more suitable micro and macro environmental conditions including refuge, food and protection (Boina *et al*., 2009, Basoalto *et al.,* 2010; Lessio *et al*., 2014; Grassi *et al*., in preparation). The woods as described in this study likely provided such an optimal nutritional environment for *D. suzukii*. The biotic elements that likely enhanced the suitability are three known *D. suzukii* non-crop hosts: *Cornus sanguinea*, one of the most frequently encountered shrubs and *Taxus baccata*, a tree also found in the surveyed area (Kenis *et al*., 2016; Lee *et al*., 2015). *Hedera helix* is additionally known to serve as an alternative oviposition host during the late dormant period (Grassi *et al.,* 2018). In particular, Kenis et al. (2016), reports that these two species were frequently found infested by *D. suzukii*, showing a high level of attraction to *D. suzukii*. (Poyet *et al*., 2015). *T. baccata* was also commonly found in the woods and is also a known host of *D. suzukii*'s. Lee et al. (2015) states that many members of the genus *Cornus* contained populations of *D. suzukii*. Two other plants found in the area that have been reported as hosts of *D. suzukii*, have not been considered in this analysis, because their presence was infrequent and because the literature on their relations with the pest is not clear i.e: *Rosa canina* and *Viburnum lantana*. Finally, *Rubus idaeus*, red raspberry, was also found in the surveyed area: this plant is one of the main hosts of *D. suzukii* (Cini *et al*., 2012) and it may have increased the levels of resources in the woods area for the insect, even if it was found only sparsely. Elsensohn & Loeb (2018) investigated the infestation of non-crop plants by *D. suzukii*, underlining the important role of the alternative vegetation as a key survival and population buildup factor. For these reasons, we believe that in this case the surveyed woods margin played a critical ecological role for *D. suzukii* populations. An additional factor possibly affecting daily movement, could be related not only because the above mentioned favorable abiotic and biotic conditions in the surrounding vegetation, but also because pesticide applications within the crop during the experimental period. Deltamethrin was applied against *D. suzukii* (Smirle *et al*., 2017; Shawer *et al*., 2018) every seven days (at least). This, together with the suboptimal abiotic factors could lead flies to move away from the field. An important point that underlies the movement of flies towards the wood instead of remaining into the crop, is the fact that most of the marked flies was found in the forest and not in the nearest traps to the marking point. Applying attractants in surrounding vegetation together with toxicants/repellents in the crop (push-pull may also result in lower rates of *D. suzukii* damage within the cultivated crop.

The data collected from this study support previous assertions that surrounding vegetation can provide a benefit to migrating *D. suzukii* populations in two ways. First, the woods provided *D. suzukii* with a refuge from adverse climatic conditions. Second, by containing alternative host plants the wood likely provided food and reproductive resources. There is increasing evidence that the presence of suitable surrounding vegetation may contribute to *D. suzukii* risk to crop fields. Our data support these studies, but also suggest that *D. suzukii* can migrate to and from surrounding vegetation on a daily basis (Kenis *et al*., 2016; Diepenbrock *et al.*, 2016). Leach et al., (2018) demonstrated that in the landscape surrounding fruit farms may strongly influence the dispersal of *D. suzukii*. Our study supports the assertion that the surrounding vegetation can play an important role in *D. suzukii* management. If the elements described in this and other supporting studies exist in areas surrounding the commercial crop, these areas can provide both nutritional resources and suitable habitat during environmentally and other adverse conditions within the crop. These areas can conversely provide refuge for parasitoids and at the same time aid in the management *D. suzukii* infestation (Santoiemma *et al*., 2018).

In the case of the current study, we demonstrated movement away from the crop to the surrounding vegetation. Results of the present study support the hypothesis that *D. suzukii* migrate from the crop to the surrounding vegetation during the day, which has implications for the pest management programs. These results provide valuable details to more effectively manage *D. suzukii*, providing further insights into daily activities between distinctly different habitats. This information could help farmers and industries decide when and where to apply insecticides or other *D. suzukii* controls, as well as with which intensity, where to add more number of attractant traps and in which way to use behavioral manipulation techniques (Landolt *et al*., 2012; Kirkpatrick *et al*., 2016; Kirkpatrick *et al*., 2017; Wallingford *et al.,* 2018, Tait *et al*., 2018), highlighting the possibility to apply such management actions directly to the surrounding vegetation. This strategy would be safer to humans compared to applying pesticides directly on fruit, providing that there is no contamination of soils and water with toxic pesticides.

In conclusion, these data provide valuable and detailed information on the daily movement of *D. suzukii* between different ecosystems. A trend of aggregation in more suitable abiotic and biotic conditions suggest adaptability of *D. suzukii* in order to result in increased overall success of these damaging populations.

Author contribution

GT and AC contributed in conducting laboratory test, data analysis as well as writing the manuscript. AG and VMW conceived the central idea, contributed in planning the experiment design and sampling population as well as writing the manuscript. FP contributed in data analysis and drafting the manuscript. TO contributed to laboratory test. SP contributed in sampling insects. VM contributed in data analysis as well as drafting the manuscript. GA contributed in drafting the manuscript. All the authors have read and approved the final manuscript.

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Figure 1. *Drosophila suzukii* daily movement study area in a commercial blackberry (*Rubus ulmifolius*) tunnel production unit at San Rocco di Villazzano, Trento, Italy during 2017. The west side of the field was bordered by a wood.

Figure 2. Number of total collected *Drosophila suzukii* in relation to temperature and humidity, in a commercial blackberry (*Rubus ulmifolius*) tunnel production unit at San Rocco di Villazzano, Trento, Italy during 2017.

Figure 3. Correspondence analysis. in a commercial blackberry (*Rubus ulmifolius*) tunnel production unit at San Rocco di Villazzano, Trento, Italy during 2017. Each rhombus represents a capture category while circles, squares and triangles represent wood, crop border and crop inside capture events, respectively. The specific rhombi illustrate variable values of increase or decrease in the quantity of captured flies. Arrows connect morning captures with the respective afternoon captures and shows the differences by period of day. The longer the arrow, the higher the difference between morning and afternoon captures.

Figure 4 a, b. Comparison of average temperature values recorded during seven hours before protein-marked *Drosophila suzukii* collections in a commercial blackberry (*Rubus ulmifolius*) tunnel production unit during two trial periods at San Rocco di Villazzano, Trento, Italy during 2017.

Figure 5 a, b. Trap catches for both trials (July and August 2017). Each number corresponds to the total number of 2017 of protein-marked *Drosophila suzukii* collected in the three studied areas (wood, border and crop) in a commercial blackberry (*Rubus ulmifolius*) tunnel production unit at San Rocco di Villazzano, Trento, Italy during 2017 at the corresponding trap location.

Figure 6a, b: Spatial representation of two trial periods, a) July and b) August 2017 of protein-marked *Drosophila suzukii* collected in the three studied areas (wood, border and crop) in a commercial blackberry (*Rubus ulmifolius*) tunnel production unit at San Rocco di Villazzano, Trento, Italy during 2017 using the inverse distance weighting method in Surfer. Crosses indicate the position of the traps.

Table 1. Species composition and relative abundance of flora directly adjacent to a *Drosophila suzukii* daily movement study in a commercial blackberry (*Rubus ulmifolius*) tunnel production unit at San Rocco di Villazzano, Trento, Italy during 2017. There were eight 10x13 m surface areas directly surrounding each of the traps in the surrounding vegetation (known *D. suzukii* hosts are indicated by an asterisk *).

Layer	Type	Common name	Family	Species	Abundance
Weed (0-200 mm stratified	herbaceous	Nettle	Urticaceae	*Urtica dioica*	++

layer)					
	herbaceous	Dandelion	Asteraceae	*Taraxacum officinale*	+
	herbaceous	Ground ivy	Lamiaceae	*Glechoma hederacea*	+
	herbaceous	Wild raspberry	Rosaceae	*Rubus idaeus*	+
	herbaceous	Field bindweed	Convolvulaceae	*Convolvulus spp.*	++
	herbaceous	Common wormwood	Asteraceae	*Artemisia vulgaris*	+++
	herbaceous	Absinthe	Asteraceae	*Artemisia absinthium*	++
	herbaceous	Red clover	Fabaceae	*Trifolium pratense*	+
	herbaceous	Common wetch	Fabaceae	*Vicia sativa*	++
	herbaceous	Common comfrey	Boraginaceae	*Symphytum officinale*	+
	herbaceous	Lamb's quarters	Amaranthaceae	*Chenopodium spp.*	++
	herbaceous	Meadow buttercup	Ranunculaceae	*Ranunculus acris*	+
	herbaceous	Blue grass	Poaceae	*Poa pratensis*	++
	herbaceous	White bedstraw	Rubiaceae	*Galium album*	+
	herbaceous	Barrenwort	Berberidaceae	*Epimedium spp.*	++
	herbaceous	Bastard balm	Labiateae	*Melittis melissophyllum*	+
Shrub (0.2m-1m stratified layer)	shrub	Common hazel	Betulaceae	*Corylus avellana*	+
	shrub	Butterfly bush	Buddlejaceae	*Buddleja spp.*	+
	shrub	Common dogwood	Cornaceae	*Cornus sanguinea **	+++
	shrub	Common hornbeam	Betulaceae	*Carpinus betulus*	++
	shrub	Manna ash	Oleaceae	*Fraxinus ornus*	+++
	shrub	Common barberry	Berberidaceae	*Berberis vulgaris*	++
	shrub	Common ivy	Araliaceae	*Hedera helix**	++
	shrub	Wayfarer	Caprifoliaceae	*Viburnum lantana*	+
	shrub	Dog rose	Rosaceae	*Rosa canina*	+

Tree **(1-10 m stratified layer)**	tree	European yew	Taxaceae	*Taxus baccata **	++
	tree	Field maple	Sapindaceae	*Acer campestre*	++
	tree	Silver fir	Pinaceae	*Abies alba*	+
	tree	Common hornbeam	Betulaceae	*Carpinus betulus*	+++
	tree	Manna ash	Oleaceae	*Fraxinus ornus*	+++
	tree	Field maple	Aceraceae	*Acer campestre*	++
	tree	Norway spruce	Pinaceae	*Picea abies*	+

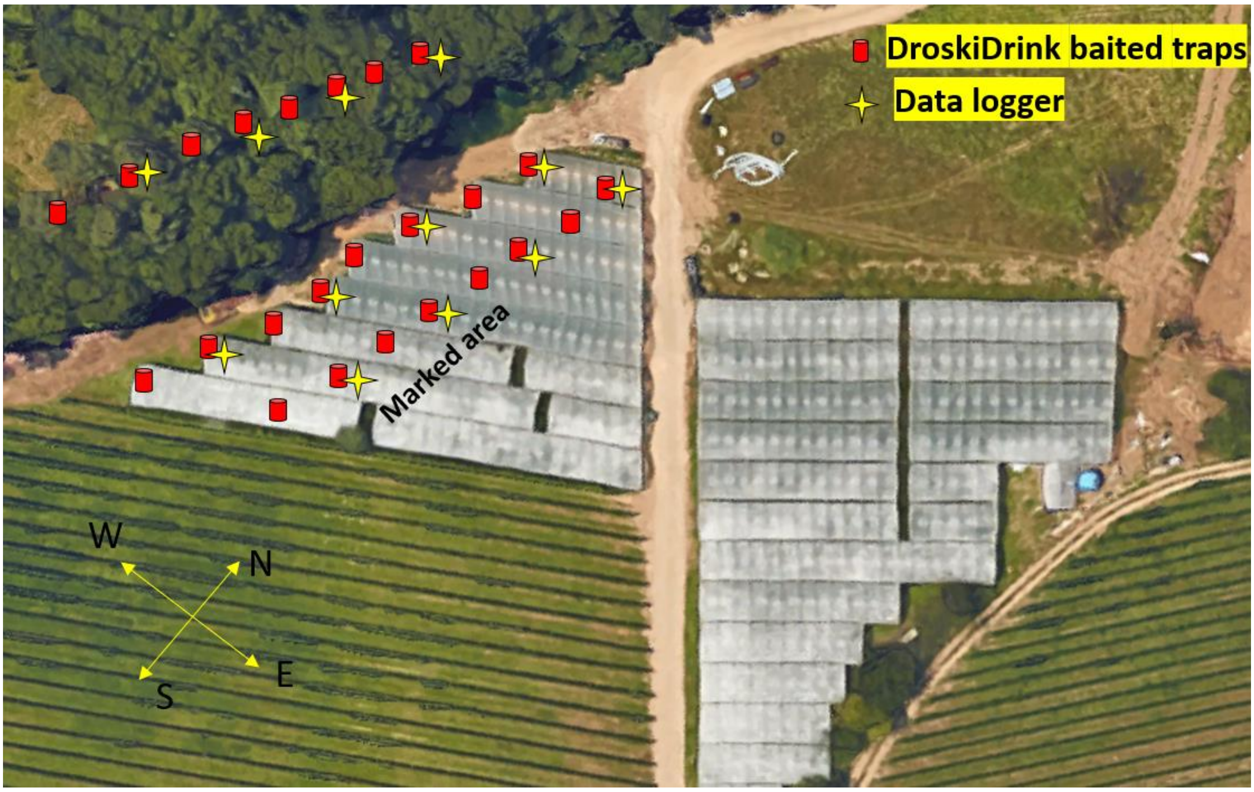
Table 2. Number of marked catches (Nm) on the total collected *Drosophila suzukii* (Nt), % of marked samples and OD value in each of two mark recapture trial periods in San Rocco di Villazzano, Trento, Italy during 2017.

	I trial – July 2017			**II trial – August 2017**		
N/S axis	**Nm/Nt**	**%**	**µ OD**	**Nm/Nt**	**%**	**µ OD**
1	0/38	0	0	2/110	1.82	0.303
2	0/26	0	0	3/68	4.41	0.277
3	0/51	0	0	1/63	1.59	0.075
4	0/29	0	0	4/67	5.97	0.316
5	1/49	2.04	0.092	2/50	4	0.126
6	10/68	14.71	0.314	2/78	2.56	0.144
7	4/60	6.67	0.145	7/144	4.86	0.145
8	8/81	9.88	0.194	8/157	6.37	0.237

Table 3. Mean comparative normalized, daylight temperature and humidity, extreme daily temperature and humidity, normalized difference between extremes of daily temperature and humidity between three locations in San Rocco di Villazzano, Trento, Italy during 2017.

Date	**11-July**	**12-July**	**13-July**	**14-July**	**31-July**	**1-August**	**2-August**	**3-August**
Location								
Normalized temperature								
Wood	0	0	0	0	0	0	0	0
CRB	0.488	0.389	0.352	0.419	0.427	0.585	0.165	0.264
CRI	1	1	1	1	1	1	1	1

Normalized humidity								
Wood	1	1	1	1	0.112	0.379	0	0
CRB	0.538	0.740	0.937	0.592	0	0	0.565	0.450
CRI	0	0	0	0	28.49	30.74	31.27	31.25
Daily temperature and humidity extremes								
Temperature ^{max} (°C)	26.99	28.48	30.39	25.25	26.46	29.45	30.33	30.32
Temperature ^{min} (°C)	24.25	25.87	27.45	22.13	68.21	62.22	64.08	61.92
Relative humidity ^{max} (%)	73.55	67.69	60.17	79.41	67.23	58.38	56.69	55.14
Relative humidity ^{min} (%)	65.21	62.04	55.48	70.83	0.202	0.129	0.094	0.092
_Normalized difference between daily temperature and humidity extremes								
Temperature	0.273	0.261	0.293	0.312	0.202	0.129	0.094	0.092
Humidity	0.834	0.565	0.468	0.858	0.097	0.383	0.738	0.678



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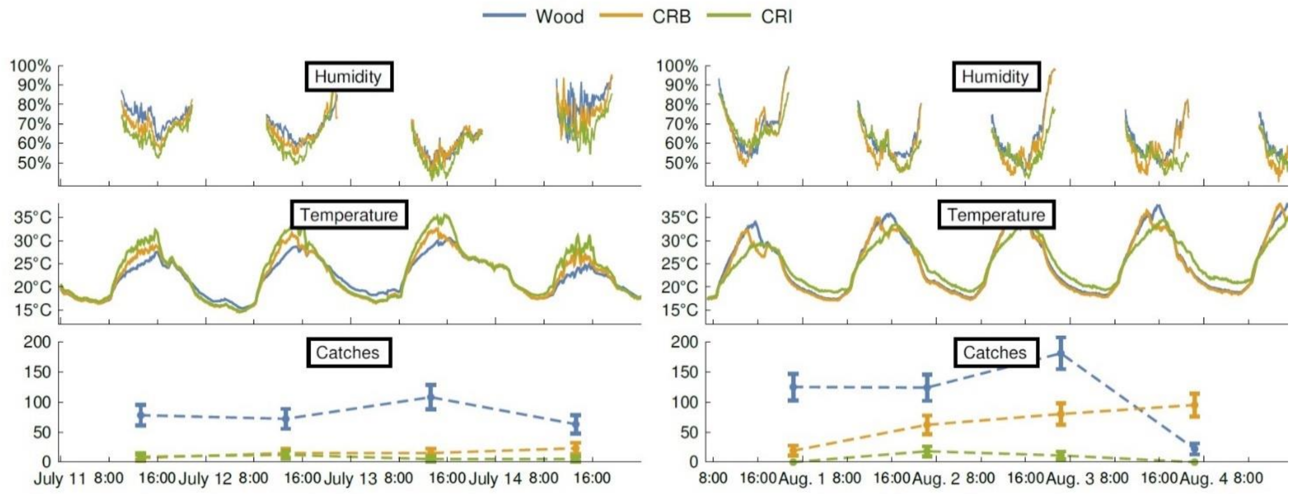

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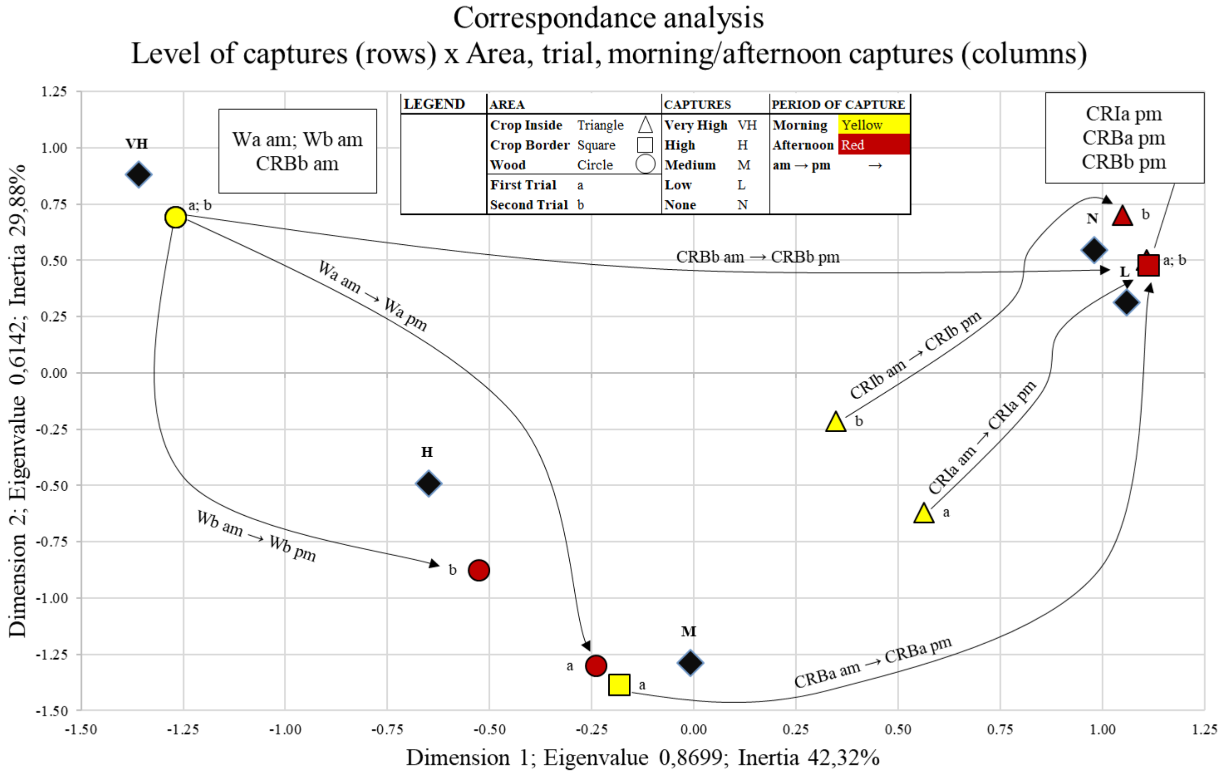

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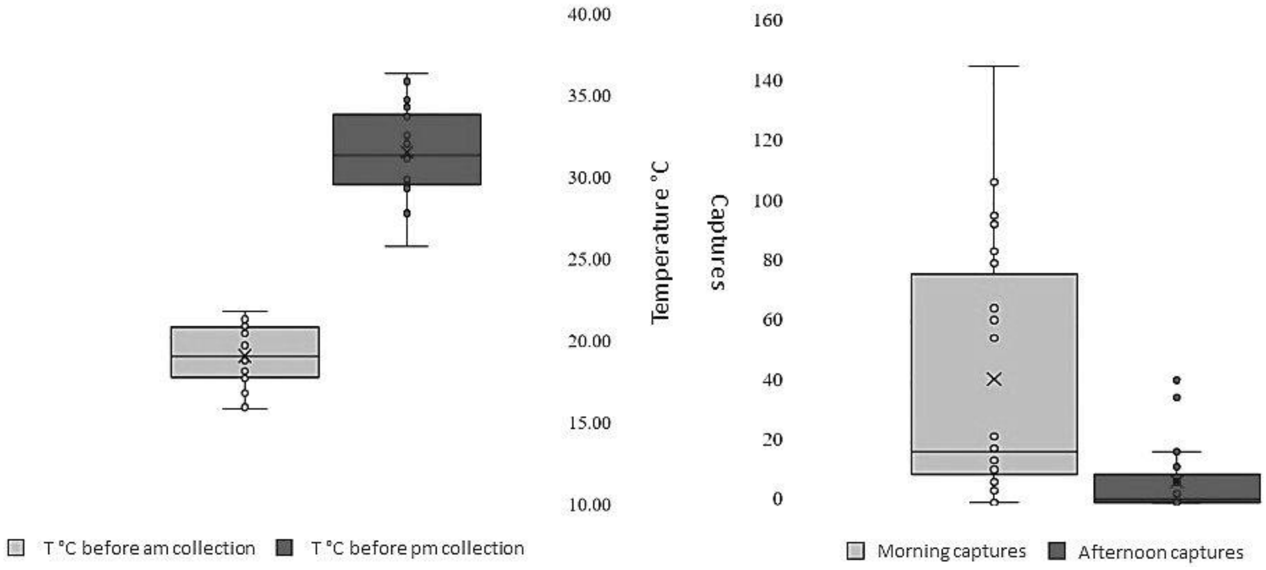

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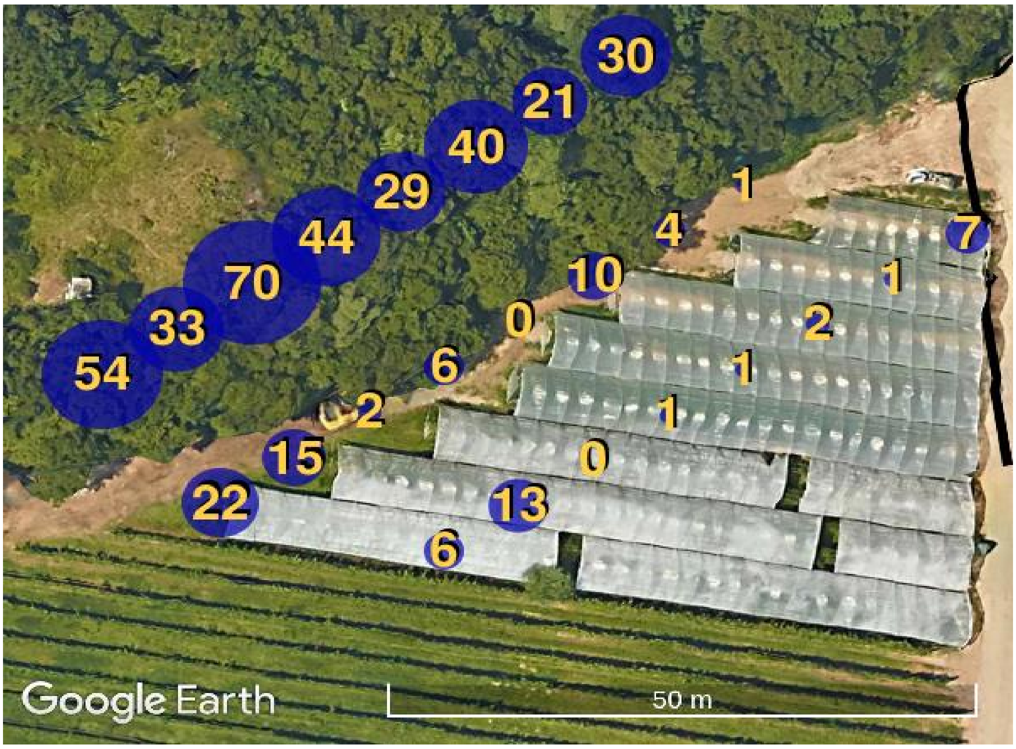

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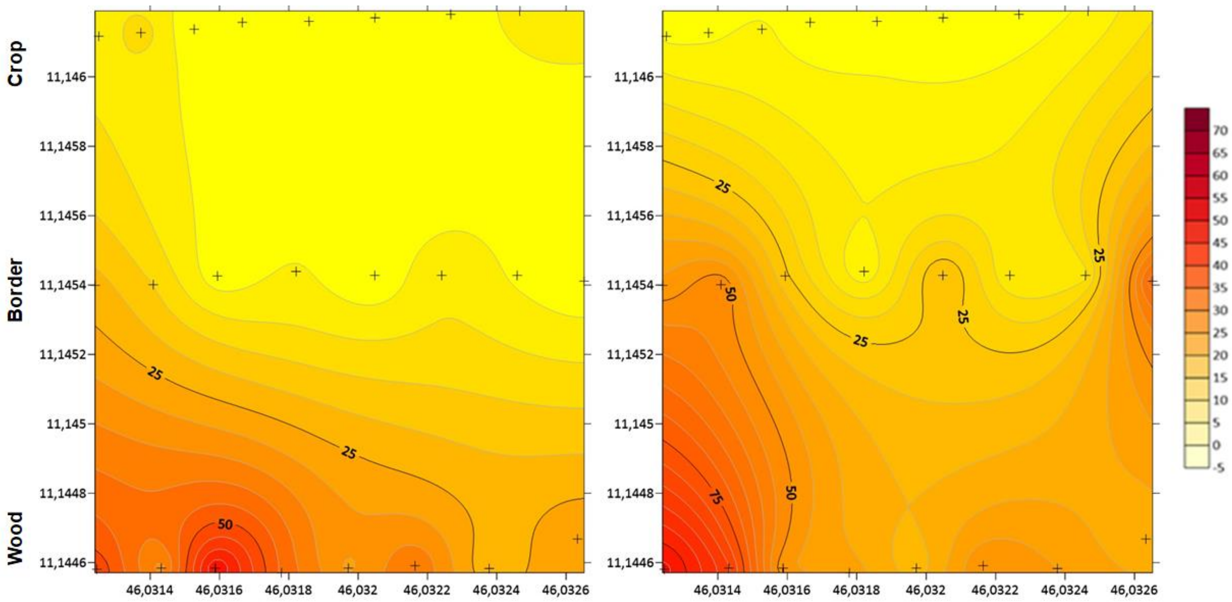

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CHAPTER 5.

Oviposition behavior of *Drosophila suzukii* with evidence of an oviposition marking pheromone

Paper in preparation. To be submitted to Journal of Pest Science

Oviposition behavior of *Drosophila suzukii* with evidence of an oviposition marking pheromone

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Abstract

Drosophila suzukii Matsumura (Diptera: Drosophilidae) is an invasive fly native to Eastern Asia that has successfully invaded Europe and the Americas since its first detection in 2008 outside its native range. Its rapid spread and establishment is largely attributed to its ability to exploit for oviposition multiple intact host fruits ripening on plants; this ability is due to the presence of a sharp, enlarged ovipositor. However, the mechanisms of the oviposition site selection of *D. suzukii* are still matter of hypothesis. The aim of this work was therefore to elucidate behavioral and chemical aspects of short-range site selection of this species. We first analyzed whether *D. suzukii* preferred to lay eggs of on already egg-infested or artificially-pierced fruits instead of intact ones. Video recording evidenced several discrete and repeated steps during oviposition. In particular, the final step was the release of an anal secretion over the fruit surface near the oviposition site. Gas chromatographic analysis revealed the presence of 14 compounds peculiar to the infested fruit, including methyl myristate and methyl palmitate, which have been known for being pheromone components in the model species *Drosophila melanogaster*. Further electrophysiological experiments of six of these compounds showed that they were all perceived by both males and females *D. suzukii* antennae. Finally, a synthetic blend composed by the six compounds in a ratio similar to that found in the post-oviposition secretion (marking liquid) increased the oviposition rate of conspecific females, suggesting that this secretion play a role in promoting oviposition and possibly aggregation in *D. suzukii*. The knowledge gained with this study may accelerate establishment of control strategies based on the interference and disruption of the *D. suzukii* communication during the oviposition processes.

1 INTRODUCTION

An alien species is an organism that lives beyond its natural range and is often combined with the connotation of being invasive, continuing to spread and thrive extensively. An alien species occurs in both natural and agricultural systems, and affects many aspects of human well-being, as well as the integrity of the receiving environments, and the survival of many of the species they contain (New, 2016). Historically, alien pest invasions result in severe production losses and efforts deployment, in order to mitigate their environmental disturbances wrought by the invader (Andreazza *et al*., 2017). *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) or Spotted Wing Drosophila (SWD) is a vinegar fly that originated from South East Asia and recently invaded western countries, threating soft fruits production there (Cini *et al*., 2012; Asplen *et al.*, 2015). Adult females lay eggs on a variety of fruits by means of an enlarged and serrated ovipositor that permits penetration of the intact skin of ripening fruits (Cini *et al*., 2012; Atallah *et al*., 2014; Poyet *et al*., 2015). Larvae develop inside the fruit, making them unmarketable. The ability to select the best oviposition sites is fundamental to ensure progeny survival and maximize fitness in polyphagous insects, and especially in insect species such as *D. suzukii* whose offspring have limited or no capabilities of relocating themselves *(*Jaenike, 1978).

Knowledge of the mechanisms regulating the oviposition behavior of this pest may be of great importance to consider alternative methods based on the interference with these behavioral processes, such as oviposition disruption and/or mass trapping, to control this invasive species.

Despite its economic relevance, little information is available concerning the egg-laying behavior of *D. suzukii*. From an evolutionary point of view, egg-lay decision is critical for insect species (Guedes *et al*., 2016). Egg production is costly and therefore the capacity to weigh different options might have been selected to ensure better survival of progeny (Yang *et al*., 2008). Up to now, what is known is that females of *Drosophila* species demonstrate preferences toward egg-laying site characteristics (Takamura *et al*., 1980; Allemand, 1989; Yang et al., 2008; Fountain *et al*., 2018). In particular, Fountain et al. (2018) has showed how *D. suzukii* females are strongly attracted by isoamyl acetate a well-known yeast volatile, suggesting that yeast developing on the surface of the berry can influence the attraction and therefore the egg-oviposition choice.

The aim of this work is therefore to elucidate the chemical ecology behind the oviposition behavior of *D. suzukii* with the following goals: 1) assess the effect of prior conspecific infestation and population density of *D. suzukii* 2) find out whether the oviposition behavior of *D. suzukii* includes a marking phase; 3) analyze by means of GC-MS the marking liquid for a complete characterization of the compounds; 4) test the antennal responses (EAG) of *D. suzukii* to the compounds identified in the marking liquid; 5) evaluate behavioral responses of *D. suzukii* mated females to a synthetic blend resembling the marking liquid.

2 MATERIALS AND METHODS

2.1 Insects stocks

D. suzukii cultures were established either at Edomund Mach Fondation (FEM) from a wild strain collected from multiple locations in the Adige valley (Italy) in 2016, or at OSU from wild strains collected from multiple locations in the Willamette Valley (Oregon) in 2016. Flies were reared in cubic cages (Bugdorm-1, 30x30x30 cm) on an artificial cornmeal diet (Prokopy *et al*., 1972), that served as both food source and oviposition medium. Insects were held under constant laboratory conditions (23±2 °C; 70±10 % RH; 16:8 L:D cycle).

2.2 Intraspecific factors affecting *D. suzukii* oviposition activity

Three experiments were carried out in order to assess the effect of prior conspecific infestation (experiments 1 and 2) and population density (experiment 3) on the egg-laying activity of *D. suzukii*. All experiments were carried out under controlled conditions using 7-15 day-old adult *D. suzukii*. In all experiments, a water-soaked cotton ball was added to the arenas as a moisture source.

2.2.1 Effect of prior D. suzukii conspecific infestation

The effect of prior conspecific infestation was tested in both no-choice (experiment 1) and choice (experiment 2) conditions. For the no-choice test, four sets of 30 vials each were prepared. The first set of vials contained one intact blueberry and one 7-15 day old mated *D. suzukii* female (negative control, NC). The second set of vials contained one infested blueberry (3-5 eggs) and one *D. suzukii* female (treatment 1, T1). Vials from the third set contained one intact blueberry and two *D. suzukii* females (treatment 2, T2). Vials from the fourth set contained one artificially pierced blueberry (3-5 holes) and one *D. suzukii* female (positive control, PC). Berries were checked for oviposition after 2, 6 and 24 hours. For the choice test, a set of 30 plastic cylindric boxes was prepared (15 x 10 cm). One *D. suzukii* female was placed in a single box with three blueberries each with a different degree of infestation: an intact blueberry, a low infested blueberry (1-2 eggs) and a highly infested blueberry (8 eggs). Berries were checked for oviposition after 6 and 24 hours.

2.2.2 Effect of population density

To assess the effect of population density on *D. suzukii* oviposition activity (experiment 3), three sets of 30 boxes were prepared. All boxes contained three intact blueberries (fixed factor), whereas the number of *D. suzukii* was 1, 3 and 6 for the first (1 female), the second (3 females) and the third (6 females) set of boxes, respectively. Berries were checked for oviposition after 6 and 24 hours.

2.3 *Drosophila suzukii* marking behavior

To determine whether the oviposition behavior of *D. suzukii* includes a marking phase, two experiment were carried out. The first consisted of direct observations of ovipositing *D. suzukii* females, using a high resolution camera (Canon). Blueberries were exposed for 8-10 days to mated females in mixed gender cages for 15 minutes. Then, blueberries on which SWD were resting were moved in the camera frame (about 36 mm^2). The oviposition behavior of 26 females was recorded from the moment in which they began to oviposit (i.e., insertion of the ovipositor into the blueberry) until the fly walked out of frame (mean ± SD = 205.63 ± 51.80 s). For each video, the occurrence and length in seconds of the following behaviors were noted: walking, marking (i.e. the female bends the abdomen’s tip and touches the substrate), and probing (i.e., proboscis extends and touches the substrate). The time in seconds between the ovipositor’s extraction and the first behavior performed immediately after it was measured. The second experiment was designed to detect marking signs on the oviposition substrate. Mated *D. suzukii* females (3 to 4 days old) were fed with a sugar solution containing 46% water, 46% honey and 8% fluorescent dye (Polyment GmbH, Pinneberg, Germany) for 24 hours. Two females, one green-marked and one red-marked, were transferred into plastic cups (Fisherbrand), and provisioned with a single blueberry, where they remained for two hours. Ten cups were prepared. After the flies were removed, the blueberries were checked for marking signs under a stereomicroscope using UV light.

2.4 Characterization of the markings as aggregation pheromone.

2.4.1 Markings collection and identification

Collection of the markings were performed by washing 50 highly infested blueberries in 5 mL of methanol (Keesey *et al*., 2016). Each berry was individually immersed in the solvent and gently shaken for 15 seconds. A second collection was obtained by repeating the same process on artificially pierced blueberries. Marking candidates were identified by direct comparison of the two washings. Methanol was used as control solution. Collections were reduced to 100 µL via evaporation under a slow air stream. Chromatographic analyses were carried out with a Trace GC Ultra gas chromatograph coupled with a TSQ Quantum XLS Tandemmass spectrometer (Thermo Electron Corporation, Waltham, Massachusetts) and equipped with a PAL Combi-xt autosampler (CTC Analytics AG, Switzerland). The separation module consisted of a ZB Wax PEG capillary column (30 m × 0.25mm inner diameter × 0.25 µm film thickness; Phenomenex, Italy) programmed to increase from 60 °C (held for 3min) at 8 °C/min to 220 °C (held for 10 minutes) and, finally, to 250 °C at 10 °C/min for 5 min. Helium was used as the carrier gas at a flow-rate of 1.2 mL/min. The temperature of the transfer line was 250 °C. The electron impact energy was 70 eV and the filament current was 50 µA. The compounds were characterized by a comparison with synthetic standards, taking into account their GC retention indices and with mass spectra via the Wiley Registry of Mass Spectral Data.

2.4.2 Antennal response to the selected volatile compounds.

The response of *D. suzukii* adult antennae to the compounds identified as putative markings, was recorded by electroantennography (EAG, Syntech, Hilversum, NL). Ten females and ten males were recorded. The EAG connected to the antenna through two glass microcapillaries containing the ground and the recording silver electrodes and filled with Kaissling solution [NaCl (7.5 g/L); $CaCl_2$ (0.21 g/L); KCl (0.35 g/L); and $NaHCO_3$ (0.2 g/L) plus 5g/L polyvinylpyrrolidone]. The protocol included that the head of the insect was mounted on the ground electrode via the occipital aperture, while the recording silver electrode was brought in contact with the distal tip of one antenna. Each compound was delivered to the antenna through a glass tube (12cm x 8mm) via a constant humidified air stream (0.5 l/min) filtered with charcoal. The air tube was located 4-5 mm away from the antenna. The test cartridge was connected to a stimulus controller (CS-55, Syntech) that generated the air puffs. Stimuli were diluted in hexane to four different concentrations (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} µg/µl) and 10 µl were put on 1.5 cm^2 piece of filter paper (Albet 400: Sparks Lab Supply Limited) inside Pasteur pipettes. Cartridges were replaced after fifteen puffs. To avoid biases in the response amplitude, stimuli presentation was randomized among individuals. For each individual we delivered odorants in ascending order of concentration at 30-40 s intervals. Each odorant and concentration was presented only once to each individual. As control stimuli, we used pure hexane and blank air at the beginning, at the middle and at the end of each recording. Data obtained were analyzed with EAG2000 software (Syntech, Hilversum, NL), by measuring the maximum amplitude of depolarization (mV) elicited by each stimulus and then subtracting the amplitude of the response to the hexane control.

2.6 Behavioral response to the selected volatile compounds.

To verify the effect on ovipositional activity of the compounds identified as putative markings, a double-choice behavioral assay was performed (n=40). The compounds were mixed according to the ratio observed in the collection analyzed by GC-MS. The mix was then dissolved in hexane (i.e. marking solution; 100 mg/30 mL). Arenas were prepared using 2-L transparent Griffin-style graduated low-form plastic beakers (Nalgene, Rochester, NY). For each beaker, 9 ventilation holes (1 cm diameter) were cut along the circumference approximately 6 cm from the base and covered with fine mesh in order to prevent *D. suzukii* escapees. The top of each beaker was drilled and connected to a 0.5 cm diameter plastic tube providing a vacuum in order to create a constant and uniform air flow (1.5 L min^{-1}) within the containers. Beakers were placed upside down on a flat work surface covered with white paper. Two petri dishes (3 cm diameter) containing a single blueberry each were placed at opposite sides of each arena. A treated blueberry was marked with 15 droplets of the marking solution (2 uL per droplet), and a control blueberry was marked with 15 droplets of solvent. After marking, berries were left at room temperature for 10 minutes to permit the solvent to evaporate. A single *D. suzukii* female was released in each arena. Every 15 minutes the position of the female within the arena was noted (treated berry, control berry or no choice). After 2 hours, flies were removed and the number of eggs on each berry was counted.

2.5 Statistical analysis

The effects of intraspecific factors on *D. suzukii* oviposition activity (experiments 1-4) were tested with the Mann-Whitney U test or the Kruskal-Wallis test followed by Dunn's post hoc test. The behavioral response of *D. suzukii* to the marking solution was tested with the Wilcoxon matched pairs test when comparing the oviposition on treated and control fruit. A skew index was used to assess whether there was a preference either berry. In particular, for each 15-minute interval, the skew index was estimated as (C-T)/(C+T), where C is the number of females on the control berry and T is the number of females on the treated berry. This index varies between 1 (100% preference for the control berry) and -1 (100 % preference for the treated berry). Differences in EAG responses were first evaluated using Student’s t-test, to assess differences between male and female responses. Parametric one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc multiple comparison test were used to assess the effect of each compound dosage on the amplitude of *D. suzukii* antennal responses. All statistical analysis were performed using Statistica 64© 12 (StatSof. Inc., Tulsa, OK). All mean values are presented as averages ± standard deviation (SE).

3 RESULTS

3.1 Intraspecific factors affecting *D. suzukii* oviposition activity

In the no-choice test (experiment 1), oviposition (total eggs) generally and gradually increased over the 24 hours in all treatments (T1, T2) and controls (NC, PC). In T2 and PC, a significantly greater number of eggs were laid within 2 hours ($H = 17.04$; $p < 0.001$; figure 1a). After 6 hours, both treatments and PC showed a significantly greater number of eggs than NC ($H = 27.35$; $p < 0.001$), and the same trend was maintained for 24 hours ($H = 22.06$; $p < 0.001$; figure 1a). In experiment 2, oviposition after 6 hours was significantly different between treatments and control ($H=6.53$; $p=0.033$; figure 1b). The same trend was observed at 24 hours ($H = 10.62$; $p < 0.01$; figure 1b).

In experiment 3, the female density did not affect oviposition rates and no significant difference in the number of eggs laid was observed, after 6 or 24 hours (figure 1c).

3.2 *Drosophila suzukii* marking behavior

Immediately after extraction of the ovipositor from the berry, most of the females walked away from the oviposition site (85%), although they remained in the recording frame. The remaining 15% of females performed probing (3/26) or marking (1/26) before walking away. The time from the ovipositor extraction to each behavior was 1.88 ± 2.56 s for walking, 60.49 ± 103.62 s for probing, and 2.12 s for marking. During the entire observation period, marking behavior was observed at least once in 77% of females. Considering the females that marked the berry, the number of markings per female was on average 3.30 ± 1.92. For each marking, the abdomen of the female was in contact with the berry's surface for 0.26 ± 0.16 s. The observation of the marking signs under fluorescence light revealed three different marks on the oviposition substrate: large spots, small spots and scrapings (figure 2). The first two types of marks were observed on all blueberries, whereas scraping only occurred on berries in which eggs were laid. The large spots were 200-300 µm in diameter and showed different level of fluorescence intensity (figure 2c,e). Small spots were 25-50 µm in diameter and always appeared to be highly fluorescent (figure 2c,e). Scrapings consisted of drop-shaped stains (lenght 150-400 µm) aligned over trails surrounding oviposition sites (figure 2d,e).

3.3 Markings collection and identification

The GC-MS analysis of the surface washings revealed the presence of several peaks (Figure 3a). A total of 19 compounds from infested fruit and 9 compounds from artificially pierced fruit were identified (table 1). Five compounds were common to both collections and were not considered for the subsequent electrophysiological experiments. Among the remaining compounds identified in the infested fruit collection, six were selected for further electrophysiological and behavioral analysis, since they corresponded to active volatiles previously identified in the *D. melanogaster* aggregation pheromone (Keesey *et al.*, 2016). These compounds were: palmitic acid, palmitoleic acid, myristic acid, lauric acid, methyl myristate and methyl palmitate.

3.4 Antennal response to the markings.

Figure 3b shows the changes in EAG response in males and females due to the varying pure compound dilutions. An initial t-test was performed to assess the presence of a potential sex-bias in the response amplitude. For each of the six compounds tested at all concentration, no statistical difference was detected ($p > 0.05$). Females did not make statistically different responses to the six compounds at all the four concentrations. By contrast, males responded significantly to the greatest and least concentrations of palmitic and palmitoleic acid. This difference palmitic acid was ($F = 5.089$, $df = 14.63$, $p = 0.039$), and for palmitoleic acid, ($F = 5.659$, $df = 13.35$, $p = 0.032$).

3.5 Behavioral response to the markings.

The volatiles ratio used for the marking solution was: 43.10% palmitic acid, 41.11% palmitoleic acid, 14.74% myristic acid, 0.66% lauric acid, 0.32% methyl palmitate and 0.07% methyl myristate. The number of eggs laid was significantly greater on treated berries than on control berries (Wilcoxon Matched Pairs Test: $T=144.5$, $Z=2.028$, $p<0.05$; figure 4a). The skew index was always less than than 1 (figure 4b), suggesting that female *D. suzukii* spent more time on berries treated with the marking solutions than on control berries.

4 DISCUSSION

Our study aimed to elucidate the chemical ecology behind the oviposition behavior of *D. suzukii*. This is the first evidence of the presence of an aggregation pheromone in *D. suzukii*. The research allowed us to demonstrate that *D. suzukii* oviposition behavior is affected by multiple factors as happens for other species (Frati *et al*., 2016; Zhang *et al*., 2015; Wallingford *et al*., 2016). We performed a set of experiments (choice and no-choice tests) to investigate whether infested fruits are more attractive compare to the uninfested one. Results consistently showed that highly infested and artificially pierced fruit were the preferred treatments, resulting in higher oviposition rates by *D. suzukii* females (experiment 1 and 2). Even more interesting was the fact that high female densities did not negatively affect the oviposition rate (experiment 3). Some hypothesis may be outlined as contributing factors for the evolutionary adaptation of *D. suzukii* to be attracted by wounded fruit. Firstly, the loss of sugary liquids from the egg holes over the fruit surface may provide an easy food source for egg-laying females, which may be attracted at a relatively long range by the release of volatile substances from the wounded fruit surface (Abraham *et al*., 2015; Revadi *et al*., 2015; Mazzetto *et al*., 2016). Secondly, start of potential alcoholic fermentations may occur on the wounded fruits, thus leading to bacteria and fungi development, which would stand as additional food sources, and attract conspecifics (Fountain *et al*., 2018). Indeed, in such substrates, with respect to healthy fruit, which is a relatively poor diet (Jaramillo *et al*., 2015), growing fungi and other microorganisms can represent a very protein rich diet to *Drosophila* adults and larvae limiting problems of food source competition among individuals. Thirdly, a role of the liquid released by females as a marking pheromone can by hypothesized. This substance would likely attract other females and stimulate them to lay eggs. The marking could be exploited by other females as indication of a good oviposition substrate, hence providing a reproductive advantage.

Video recording analysis showed a peculiar behavior of egg-laying females with a release of a liquid from the abdomen tip over the fruit surface after oviposition. This behavior was observed in the majority of ovipositions (77%), thus suggesting that it is a widespread behavior rather than occasional. The observation of the fruit surface under fluorescent light also supported the hypothesis of a marking pheromone, revealing the presence of several different mark types. Some of these marks occurred on all the observed berries and could be related with the probing activity made with the proboscis tip (large spots), or with the feces produced through the normal excretory activity (small spots). On the contrary, the scraping marks were only observed in those berries where eggs where laid, and their remarkable shape suggests the intent of the fly to create a trail around the oviposition site.

Proof of the existence of a marking pheromone requires demonstrating that a chemical compound (or mix of compounds) is deposited by a female insect on its host in association with oviposition and that the compound influences the behavior of females visiting the host subsequently (Nulfio and Papaj, 2001). Here we showed the presence of 19 peaks in the surface washing of infested fruit. Most of these peaks (14) were absent in the surface washing of the artificially pierced fruit (control), hence they are indicative of compounds originated from the insects rather than from the fruit. In particular, we found six carboxylic acids, which were reported to interfere with oviposition behavior of several insects (Thiery et al., 1995; Navarro-Silva et al., 2009; Bobera and Rafaelib, 2010). Butyric acid was proven to have repellent effect on oviposition of Mosquitoes (*Culex* p. *quinquefasciatus* and *Culex tarsalis*) (Hwang et al., 1980). Palmitic acid and palmitoleic acid were shown to have deterrent effect as host-marking pheromone on oviposition of *Lobesia botrana* (Gabel and Thiery, 1996) and *Cydia pomonella* (Thiery et al., 1995). On the other hand, palmitic and palmitoleic acid together with other 4 compounds that we found in the infested fruit surface washing (myristic acid, lauric acid, methyl myristate and methyl palmitate) have been previously identified by Keesey et al. (2016) in *D. melanogaster* frass and described as aggregation pheromones.

EAG tests revealed that the female antennal responses to the six compounds at all the four concentrations were not statistically different, while males showed significantly higher EAG responses to the highest and lowest concentrations of palmitic and palmitoleic acid. It has been demonstrated that these two acids together with myristic acid strongly activate olfactory receptor Or47b, which is a specific pheromone detector in *D. melanogaster* (Dweck *et al.*, 2015). The last experiment performed spraying the synthetic blend of the six major compounds resembling the ratio found in the extract over intact blueberry surface, gave us the final determining proof about the role of the released anal liquid as an attractive marking pheromone. Results indeed indicate that marked fruits attract more flies, which lay more eggs on it. In support of this, the skew index was always less than than 1, suggesting that females *D. suzukii* spent more time on berries treated with the marking solutions than on control berries.

In future, our findings may give important contribution in the development of environmentally friendly control methods by interfering with *D. suzukii* communication during the phase of oviposition.

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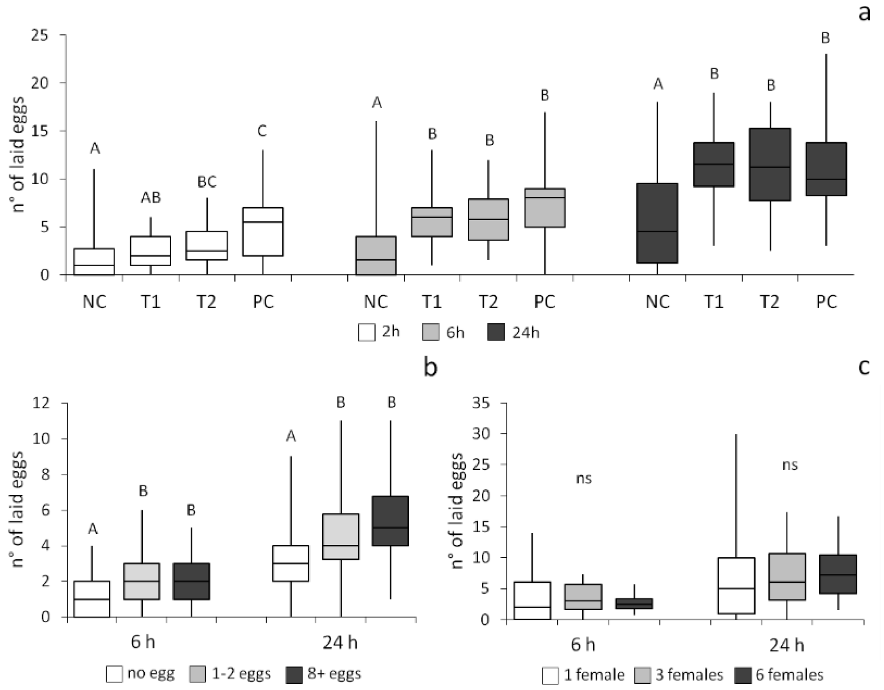

Figure 1. Results of the oviposition experiments. Experiment 1 (a) and Experiment 2 (b) were carried out to assess the effect of prior conspecific infestation in no-choice and choice conditions respectively. Experiment 3 (c) was carried out to assess the effect of female population density. Different letters indicate significant differences between treatments after Dunn's post hoc test. NC= negative control; PC= positive control; ns= not significant.

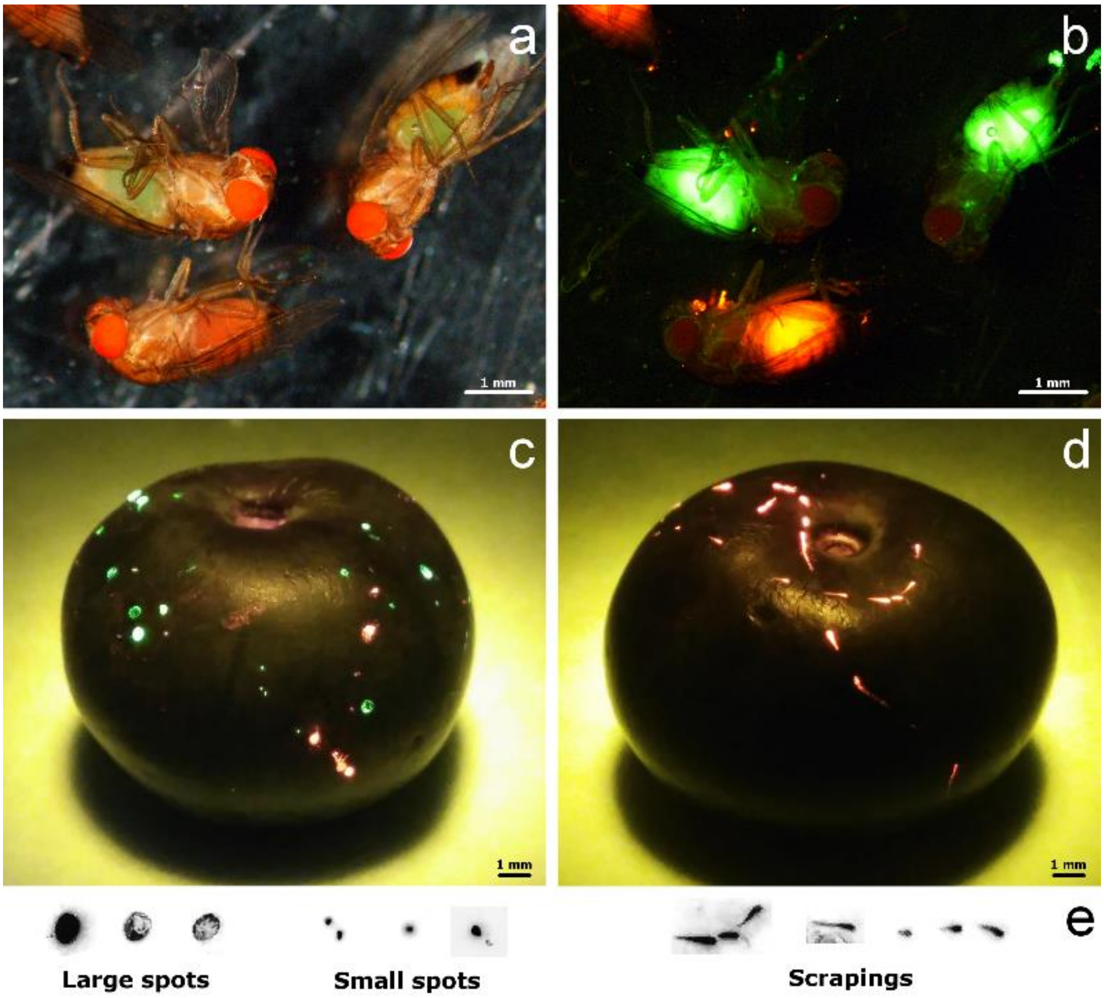

Figure 2. Marked females of *D. suzukii* under normal (a) and fluorescent (b) light. c-d) Blueberries exposed to marked *D. suzukii* females under fluorescent light: in the absence of oviposition punctures (c) only large and small spot marks are visible, when oviposition occurred (d) scraping marks can be observed. e) Negative pictures of the different mark types.

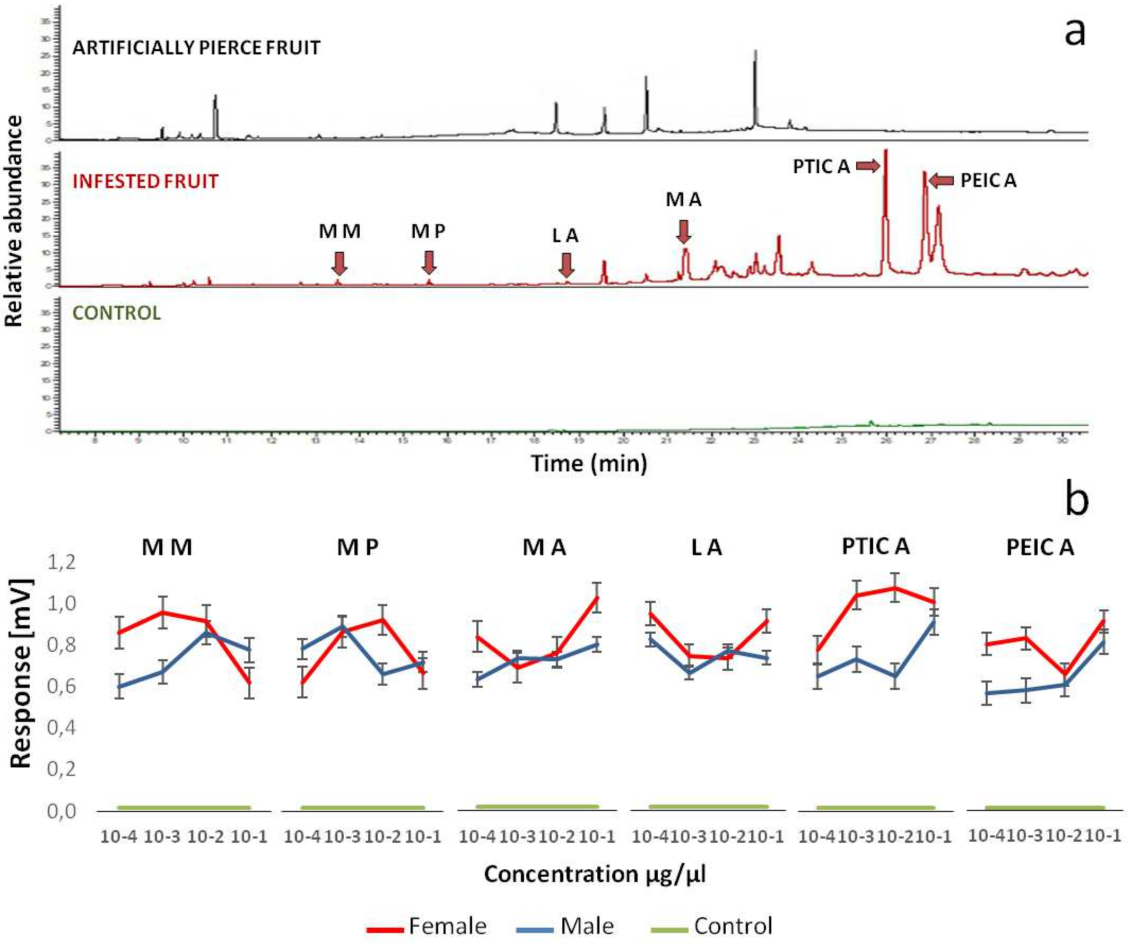

Figure 3. a) GC/MS analysis of the surface washings from the artificially pierced (blue line) and the infested fruit (red line), and the control injection (pure hexane, green line). b) EAG absolute responses (Mean±SE, MinmV) to different compounds as a function of Stimulus and Concentration. Compounds tested are indicated on top of each plot: **MM**= myristic acid, **PTIC A**= palmitic acid, **MP**= methyl palmitate, **MA**= myristic acid, **PEIC A**= palmitoleic acid, **LA**= lauric acid. The green line shows the baseline response for the control stimulus (pure hexane).

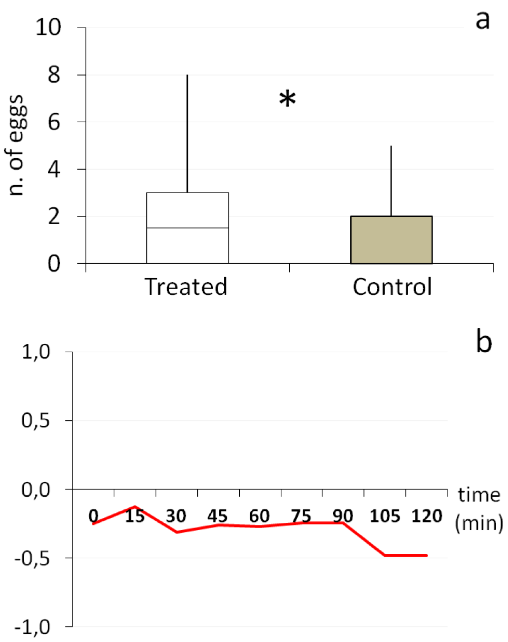

Figure 4. a) Behavioral responses to the selected volatiles. a) Number of laid eggs and b) berry choice throughout the duration of the experiment. Asterisk indicate significant difference between treatments after Wilcoxon Matched Pairs Test.

RT	Egg-Infested blueberries	RT	Pierced berries
9.25	Acetol	9.51	Acetol
10.57	Acetic Acid	9.85	Furfural
11.54	Propionic Acid	10.73	Acetic Acid
12.68	Butyric Acid	11.48	Pyranone
13.03	2-furylmethanol	11.68	Propanoic Acid
13.19	Methyl myristate	13.09	2-furanmethanol
14.12	Methyl salycilate	18.47	2-propanone 1,3-dihydroxy
14.35	?m/z 133,151	19.57	Azepan-2-one
15.58	Methyl Palmitate	20.52	2,3-dihydro-3,5dihydroxy 6 methyl 4H
18.5	Lauric Acid		
19.55	Azepan-2-one		
20.52	Pyranone		
21.26	1-tricosanol		
21.41	Myristic Acid		
22.1	Pentatonic Acid		
25.96	Palmitic Acid		
26.87	Palmitoleic Acid		
27.16	Squalene		
34.12	Octadecenoic Acid		

Table 1. In the table are reported the identified compounds in the marking liquid and in the control. RT is retention time.

CHAPTER 6.

A food-grade gum as a management tool for *Drosophila suzukii*

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A food-grade gum as a management tool for *Drosophila suzukii*

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Abstract

Drosophila suzukii is an insect pest of worldwide distribution on soft-skinned fruit. This species is able to utilize different habitats and substrates for nutrition and reproduction, a capacity that can be attributed to olfactory cues. The first aim of the current study was to create and evaluate a novel gum matrix as a management tool for *D. suzukii* in a commercial cropping systems. Next, we identified a biologically important volatile from an important ingredient within the matrix. The efficacy of the proof of concept matrix as a management tool was assessed in laboratory and semi-field conditions. The detection of active volatile compounds was performed using gas chromatography (GC) coupled with electroantenna detection (EAD) techniques. Volatiles significantly modified response of *D. suzukii* in controlled electrophysiology and orientation studies, resulting in 46.7% mean oviposition reduction in controlled laboratory trials on five susceptible fruit types. Field trials were thereafter conducted over periods of 72 ± 2 to 96 ± 2 hours on commercial-standard blueberry bushes. Fruit on bushes exposed to predetermined numbers of *D. suzukii* displayed 50 to 76% reductions of fruit infestation and total eggs laid, respectively, in gum treatments. Up to 40% of fruit on untreated plants were targeted by *D. suzukii*, whereas less than 20% of fruit on treated bushes were targeted. These results indicate that the insecticide-free gum matrix significantly reduces *D. suzukii* damage under commercial production conditions. This reduction may be due to a combination of altered behaviour and the division of reproductive resources. The current work will likely expand integrated pest management options to control *D. suzukii* populations in commercial field settings.

Key words: Spotted-wing drosophila, oviposition, insect bait, behaviour manipulation, Integrated Pest Management.

Introduction

The spotted-wing drosophila, *Drosophila suzukii* (Matsumura), indigenous to Southeast Asia, is an invasive pest species causing direct damage to soft-skinned fruits (Lee *et al*., 2011; Cini *et al*., 2012). The first description of the economic damage caused by this species was recorded in Japan in 1916 (Kanzawa, 1939). *D. suzukii* has since spread to become a pest of worldwide importance (Hauser *et al*., 2011; Asplen *et al*., 2015). Following its detection in 2008 in both Europe and USA, its damage caused estimated economic losses of €3 million in Trentino province (Italy) (Cini *et al*., 2012) and can cause an estimated $511 million in losses annually in western production regions of North America (Bolda *et al*., 2010; Farnsworth *et al.,* 2017). Current integrated pest management (IPM) practices for *D. suzukii* include trapping and fruit monitoring (Lee *et al*., 2011; 2013; Evans *et al*., 2017; Kirkpatrick *et al*., 2017), biological control (Chabert *et al*., 2012; Kasuya *et al*., 2013; Rossi-Stacconi *et al*., 2013; 2017; Gabarra *et al*., 2015; Nomano *et al*., 2015; Woltz *et al*., 2015; Daane *et al*., 2016), chemical control (Beers *et al*., 2011; Bruck *et al*., 2011; Van Timmeren and Isaacs, 2013; Wise *et al*., 2015; Murphy *et al.,* 2015), and cultural control (Lee *et al*., 2015; Tochen *et al*., 2016a; 2016b). The majority of these control methods significantly increases production costs. Pesticides controls result in 1-7 days of effect, needing up to twenty applications per season.

Oviposition levels of *D. suzukii* are affected by multiple factors, including environmental conditions (Kinjo *et al*., 2014; Tochen *et al*., 2014; 2016b; Enriquez and Colinet, 2017), sexual maturity (Zerulla *et al*., 2015; Rossi-Stacconi *et al*., 2016; Ryan *et al*., 2016; Wiman *et al*., 2016; Grassi *et al*., 2018), presence of essential food resources (Mitsui *et al*., 2006; Briem *et al*., 2016; Tochen *et al*., 2016a), and fruit susceptibility (Bellamy *et al*., 2013; Burrack *et al*., 2013; Ioriatti *et al*., 2015; Lee *et al*., 2015). Pest detection is one of the foundations of effective IPM. To this end, several attractants have been tested and are used to bait *D. suzukii* traps (Landolt *et al*., 2012; Cha *et al*., 2013; 2017; Tonina *et al*., 2018). Several of these baits are composed of a mix of volatiles of red wine, cider wine and sugars and are focused on attraction (Landolt *et al*., 2012; Cha *et al*., 2017), but little focus to this date have been given to manipulation of oviposition. In particular, work has been done on oviposition deterrents, whereas none tried to encourage oviposition on non-crop substrates (Renkema *et al*., 2016; Wallingford *et al*., 2016a, 2017). The oviposition behaviour of *D. suzukii* is not well characterized (Mitsui *et al*., 2006). Oviposition includes multiple steps during which the insect tests the quality of fruit (Karageorgi *et al*., 2017). One of the important steps includes probing of the substrate with the proboscis based on the volatiles associated with ripening fruit (Karageorgi *et al*., 2017; Tait, unpublished), and a decision is made whether to feed and oviposit or not. It is believed that leaves may contribute to volatile signals resulting in *D. suzukii* ovposition (Keesey *et al*., 2015). Currently, however, little information is available as to the specific role that each of these aspects plays in oviposition by *D. suzukii*.

Behavioural manipulation of insects has historically been an important component of the IPM toolkit (Dent, 1995). Volatiles emitted from the plant surface are received by olfactory receptor systems in phytophagous insects and act as a chemical message, that helps them orient toward hosts (Visser, 1986; Agelopoulos *et al*., 1999). This concept is often used in 'push-pull' control tactics. Such a strategy may also hold potential for the control of *Drosophila* species, many studies have examined the odour preference of *D. suzukii* which may be of use in push-pull tactics (Cha *et al*., 2012; 2014; Hamby *et al*., 2012; Keesey *et al*., 2015; Abraham *et al*., 2015; Revadi *et al.*, 2015; Hamby and Becher, 2016; Kirkpatrick *et al*., 2016, 2017; Renkema *et al*., 2016; Wallingford *et al*., 2016a; 2016b; 2017; Frewin *et al*., 2017; Huang *et al*., 2017; Rice *et al*., 2017). It is possible that *D. suzukii* may maximize survival of offspring by selecting optimal hosts for oviposition (Minkenberg *et al*., 1992; Gripenberg *et al*., 2010) by having the ability to select the best option from a myriad of acceptable ones (Yang *et al*., 2008). Females should be more willing to accept alternative hosts, however, as egg load and search time for an oviposition site increase (Jaenike, 1990). By providing attractive alternative non-fruit hosts, it may be possible that *D. suzukii* may divert resources toward these alternate resources.

The goal of this work was to develop proof of concept. We believe that a behaviour disruptor will ultimately modulate the *D. suzukii* oviposition in commercial crops. Based on results of previous field trials on *D. suzukii* aggregation behaviour (Tait, unpublished), we identified novel compounds that ultimately affect oviposition. Starting from these ingredients, two attractive food-grade and biodegradable matrices were produced by varying the water content. The one with less water, the solid matrix (SM), had a gelatinous consistency, which allowed spotted applications even on sloped surfaces without drippings. The one with more water, the liquid matrix (LM), had a creamy consistency and could be applied using standard spray equipment on wider areas. Here we describe a series of experiments aimed to: 1) evaluate the matrices as a management tool for *D. suzukii* in a commercial-standard cropping system and 2) verify the presence of active volatile compounds. The efficacy of the matrices to reduce oviposition was assessed in both laboratory and field conditions. The detection of active volatile compounds was performed using gas chromatography (GC) coupled with electroantenna detection (EAD) techniques.

Materials and methods

Insects

D. suzukii used for the laboratory and field trials were direct offspring of individuals collected in Oregon, USA (Willamette Valley, 44°55'58.2"N 123°03'50.6"W) in 2009. A second *D. suzukii* population originating from individuals collected in northern Italy (Trentino Province) 46°04'24.3"N 11°07'17.2"E) was used for GC-EAD experiments in addition to Oregon *D. suzukii*. Laboratory colonies from both geographic locations were regularly supplemented with locally collected flies to prevent inbreeding. Flies were reared in a 24 × 24 cm cage (Bugdorm-1, MegaView Science Co., Ltd., Taichung, Taiwan) and provided with a water wick and an artificial corneal diet that served as both an oviposition medium and food source. Insects were maintained in the laboratory at 22 °C, 65% RH and a photoperiod of 8:16 (L:D). In all tests, *D. suzukii* were circa 8 days old and previously mated.

Laboratory choice assays

Constant and uniform airflow was created within each of the containers using a vacuum at 1.5 L min^{-1} from the base and through the upper portion of each of the respective containers. Attraction to the active ingredient A was verified in double-choice behavioural experiments (figure 1). Arenas were prepared using 2-L transparent Griffin-style graduated low-form plastic beakers (Nalgene, Rochester, NY). For each beaker, 9 ventilation holes (1 cm diameter) were cut along the circumference approximately 6 cm from the base. The holes were covered with fine white mesh in order to prevent *D. suzukii* individuals from escaping. The top of each beaker was drilled and connected to a 0.5 cm diameter plastic tube providing a vacuum in order to create a constant and uniform air flow (1.5 L min^{-1}) within the containers. Beakers were placed upside down on a flat work surface covered by white paper sheets. Two 25 mL plastic cups (Dart Container Corporation, Mason, MI) containing ~10 mL of water and 5 g of the selected ingredient A were placed at the base of each beaker. Both cups were covered with a wax film (Parafilm® M, Pechiney, Chicago, IL).

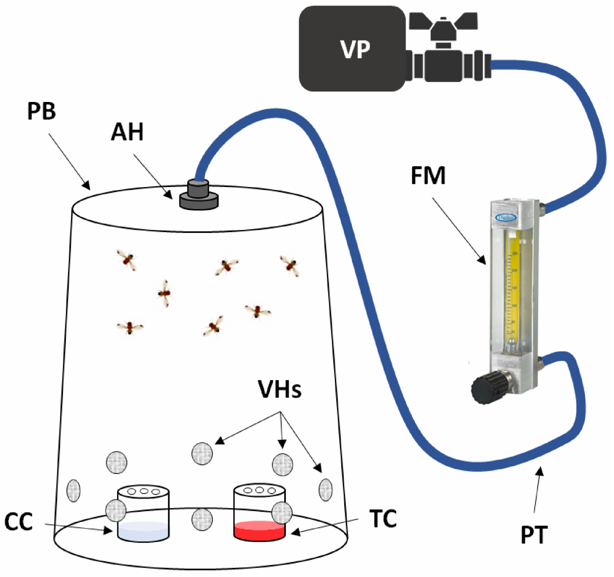

Figure 1. Schematic representation of the setup used for the laboratory double-choice assays. AH: aspiration hole; CC: control cup; FM: flow meter; PB: plastic beaker; PT: plastic tube; TC: treatment cup; VHs: ventilation holes; VP: vacuum pump.

3 holes (~0.3 cm diameter) were cut on the film coverings in order to allow the flies to enter the cups. Twenty mated females and males were released within each arena, allowing them to orient and eventually enter the cups. After 24 h, the number of *D. suzukii* caught within each cup was counted, as well as flies that did not make any choice.

Oviposition tests

The principal ingredient A was mixed at a 1.5% rate with other proprietary food-grade components (US patent appl. 62/697,296) under laboratory conditions at 22 °C, 65% RH. The matrix also contained between ~84% (SM) and 89% (LM) deionized water. The resultant matrices were used for *D. suzukii* efficacy trials within 1-2 hours of preparation in both laboratory experiments and field trials.

Laboratory oviposition experiments

Experiments were performed on blackberries, blueberries, cherries, strawberries, and raspberries under controlled conditions. Ventilated arenas were prepared as described for the double-choice experiments. Two 25-ml Deli cups (Dart Container Corporation, Mason, MI) were placed inside each arena, one containing circa 6 ± 0.1 g of the SM, and the other containing fresh fruit in treated repetitions. The control treatments only contained fruit. For each fruit type, we exposed an approximate surface area of 32 mm^2, corresponding to the exposed area of the matrix within the Deli cup in the treated repetitions. 15 *D. suzukii* individuals, 10 females and 5 males, were released into each arena. After 24 h, the number of eggs laid on the berries and into the matrix was counted. The experiment for each fruit type was replicated ten times. Initial trials looking at increase in cherry fruit per arena did not result in a significant reduction in the number of eggs laid per berry between treatments (single cherry = 36.5 eggs/cherry; three cherries = 49.25 eggs/cherry; $F_{4,15} = 4.18$, $P = 0.82$).

Field oviposition trials

Oviposition trials were conducted from 8 August to 17 October 2017 at the Lewis-Brown Farm at Oregon State University (44°33'13"N 123°13'07"W) in an organic 11-year-old drip irrigated cv. Elliott blueberry field. Plants were spaced approximately 0.76 m apart within rows with 3.05 m between rows and were not treated with insecticides. The width and height of plants were approximately 1 m and 1.5 m, respectively. Two drip irrigation lines, one on either side of the blueberry plant, were placed under sawdust mulch cover within standard raised beds and provided irrigation at levels of 100% (255 ± 5 mm water per growing season), of the estimated crop evapotranspiration (ET_c) requirement (Bryla *et al.*, 2011).

Two series of trials were performed. In the first, single fruit clusters were isolated in mesh bags, while the second trial was carried out using isolated whole bushes. Field oviposition trials including fruit were conducted by covering fruit clusters (10-23 berries) with 20 cm × 30 cm white organza mesh bags (Uline, Pleasant Prairie, WI). All mesh bags were placed approximately 1 mm apart on the north side of the bush within the shade of the canopy and 0.2 to 1.3 m above ground level. Each mesh bag contained 10 *D. suzukii* adults, 5 females and 5 males. Either SM or LM were added to the treatment mesh bags. Solid matrix was provided at 40 ± 0.5 mL in 10 cm diameter petri dishes (figure 2a) on 8 and 31 August; 13, 15 and 25 September; and 11 October 2017. Liquid gum matrix was applied by spraying 5 mL on the surface of the mesh bags using an all-purpose spray bottle (Home Depot, Atlanta, GA) (figure 2b) on 13 September, 15 September, and 11 October 2017. Every trial date contained 10 replicates for treated and control clusters each. All trials were started between 15:00 and 17:00 and were collected 72 ± 2 hours later to determine the levels of oviposition on fruits and within the gum matrix. All damaged berries were excluded when assessing the egg-laying levels in the laboratory under a dissecting microscope. Experiments based on these treatments were replicated 10 times on each of the treatment dates and were conducted on 10 separate days.

Whole-bush *D. suzukii* exposure trials using both SM and LM were conducted in order to determine efficacy when the matrix is applied at the base of the bush. Entire bushes were covered using 80 g Tek-Knit netting (Berry Protection Solutions, Stephentown, NY) which extended to the soil surface (figure 2c). In total, 100 *D. suzukii* (50 females and 50 males) were released within each netted bush. Solid and liquid gum matrices were respectively trialled using 100 ± 2 mL SM placed within 5 Petri dishes (10 cm diameter), or by spraying 100 ± 2 mL of LM at the base of the bush on a 15 × 30 cm microfiber cloth (Costco, Kirkland, WA). Replicates of both treated and control plants were exposed between 15:00 and 17:00 and fruit were collected 72 ± 2 to 96 ± 2 hours after initial placement of flies. Solid matrix experiments were replicated twice, i.e. on 22 and 24 September 2017. Liquid matrix experiments were repeated 3 times, i.e. on 27 September, 3 October and 9 October 2017. On each date, 5 replicates (bushes) per treatment were set. After the *D. suzukii* exposure period, 20 firm berries were collected from each area of the plant, designated as the top (~1.3 m above soil level), middle (~0.8 m above soil level) and bottom (~0.3 m above soil level) for a total of 60 berries collected per bush. Assessments of oviposition were determined by calculating the number of eggs laid per berry and percent of infested berries.

Weather data

Weather data including temperature (°C), humidity (%) and rainfall (mm) were obtained from the Corvallis, Oregon Agrimet weather site (Oregon State University Hyslop Farm 44°38'03"N 123°11'24"W) (https://www.usbr.gov/pn/agrimet/agrimetmap/crvoda.html). In this way it was possible to verify daily the weather conditions during the field trials.

Volatile collection

To extract volatiles from the principal ingredient A, 50 mL of water were mixed with 2 g of it in order to obtain a paste. 10 g of paste were placed into a glass tube

Figure 2. *D. suzukii* blueberry exposure trials using 20 cm by 30 cm white organza mesh bags and covering fruit clusters containing 10-23 berries (**a**). Solid and liquid gum matrices were respectively trialed using ~40 mL solid gum within 10 cm diameter petri dishes (**b**), or by spraying 5 mL of liquid gum on the surface of the mesh bags. Whole-bush *D. suzukii* exposure trials using both solid and liquid gum were conducted by covering whole bushes using netting, which extended to the soil surface (**c**).

(length 25 cm, diameter 3 cm) acting as a collection chamber. Air from the headspace of the tube was extracted at 150 mL min^{-1} and forced through a sorbent cartridge (75 mg Super Q; Sigma-Aldrich, Germany) by means of a vacuum pump (DC 12/16 FK, Aersistem, Milan, Italy). Charcoal-filtered air was pushed simultaneously into the tube by the same pump to maintain constant pressure. Multiple replications of volatile collection were carried out over 72 h in a climatic chamber at 25 ± 2 °C and 70 ± 5% RH. Trapped volatiles were eluted from the sorbent cartridge using 1 mL of dichloromethane (>99% purity, Sigma-Aldrich) at room temperature. Collections were reduced to 200 µL via solvent evaporation under a low-velocity air stream. The final extract was then kept at −20°C until use in GC- EAD trials.

GC-EAD analysis

Adult individuals of *D. suzukii* were anesthetized by refrigeration at −20°C for 2 min. The response of the insects was recorded with an EAG apparatus (Syntech, Hilversum, NL) connected to the antennae through microcapillary glass electrodes filled with Kaissling solution [NaCl (7.5 gL^{-1}); $CaCl_2$ (0.21 gL^{-1}); KCl (0.35 gL^{-1});

$NaHCO_3$ (0.2 gL^{-1})] containing 5 gL^{-1} polyvinylpyrrolidone (PVP, Sigma-Aldrich). The protocol called for the head of the insect to be mounted on a microcapillary connected to a grounded silver electrode, while the recording silver electrode was brought in contact with the distal tip of one antenna. Two µL of the concentrated principal ingredient A extracts were injected into a Clarus 500 GC (Perkin Elmer Inc., Waltham, MA) in splitless mode, with a polar Elite-VMS column (30 m × 0.32 mm; Perkin Elmer Inc., Waltham, MA) programmed from 60 °C (hold 3 min) at 8 °C min^{-1} to 250 °C (hold 10 min) with hydrogen as the carrier gas and interfaced with the EAG apparatus (Revadi *et al.*, 2015). The GC column effluent was combined with nitrogen make-up gas and then a 1:1 ratio between the flame ionization detector (FID) and an antenna of a *D. suzukii* female. Compounds eluted from the capillary column were delivered to the antenna through a glass tube (12 cm × 8 mm) via a constant humidified air stream (0.5 L min^{-1}) filtered with charcoal. The air tube was located 4-5 mm from the antenna. Both the antennal and the FID signals were amplified and recorded simultaneously using GC-EAD Syntech software (Ockenfels Syntech GmbH, Buchenbach, Germany). Headspace extracts were tested on 2 different *D. suzukii* populations, one originated from individuals collected in northern Italy and one from individuals collected in Oregon. From each population, 5 females were tested. The graphical area under the peaks was used for analysing the chromatograms. The relative quantity of each compound was calculated in relation to the most abundant compound, which was set at a value of 100. The EAD responses were calculated by Autospike software (Syntech) measuring the maximum amplitude of negative deflection (mV) elicited by *D. suzukii* antennae.

Statistical analysis

Data from laboratory double-choice experiments and oviposition trials were analysed using one-way ANOVA, and the Tukey's test was applied to separate difference at $\alpha < 0.05$. Mesh bags field trials data were analysed using one-way ANOVA tests. Whole-bush field trials data were analysed using factorial ANOVA test followed by Tukey's HSD test. Differences in volatile perception between Oregon and Italian *D. suzukii* were tested with the Mann-Whitney U-test (Mann and Whitney, 1947). All analyses were run using Statistica 64©12 (StatSoft Inc., Tulsa, OK) (Hill and Lewicki, 2007).

Results

Laboratory choice assays

The number of flies selecting Deli cups containing attractant ingredient A was significantly higher ($F_{2, 33}$ = 188.99, $P < 0.001$, figure 3) than both flies selecting deionized water and flies not making any choice.

Laboratory oviposition experiments

Overall, the presence of gum matrix resulted in a significant reduction in egg laying in all fruit types compared to untreated control treatments under controlled laboratory conditions (mean reduction = 48.3%, $F_{1, 98}$ = 19.13, $P <$ 0.001). When flies were presented a choice between gum matrices and fruit, a reduction of 46.5%, 46.28%, 51.5%, 43.5% and 48.5% of eggs in fruits compared to the control treatments were recorded for blackberry, blueberry, cherry, raspberry and strawberry respectively (table 1). The numbers of eggs laid in fruit

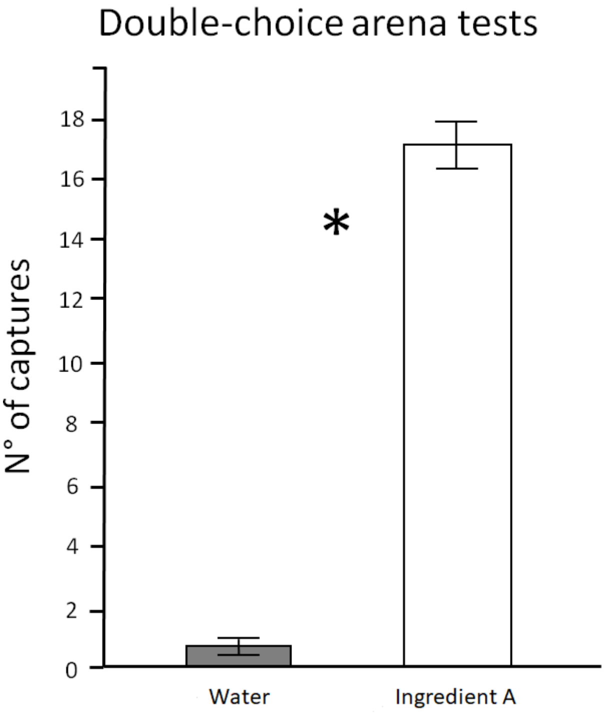

Figure 3. The number of *D. suzukii* flies selecting Deli cups containing ingredient A compared to flies selecting deionized water under controlled laboratory conditions within ventilated choice arenas.

Table 1. Mean number of *D. suzukii* eggs in control compared to berries with liquid matrix (LM). Laboratory oviposition experiments included a choice test between a water control and fruit type within an inverted modified 2-L plastic beaker. Numbers of deposited eggs were recorded after 24 hours of exposure to *D. suzukii.* For each F-values the degrees of freedom are (1, 19).

Fruit type	Eggs in control fruit	Eggs in the LM	Eggs in fruit next to LM	F-value	*P*-Value
Blackberries	11.6 ± 2.0	0 ± 0	6.2 ± 1.2	5.42	0.032
Blueberries	17.2 ± 1.1	11.8 ± 3.12	8.4 ± 1.1	32.27	<0.001
Cherries	58.1 ± 8.9	2 ± 0.66	28.2 ± 6.2	7.60	0.013
Raspberries	46.0 ± 4.7	10.2 ± 3.82	26.0 ± 2.6	13.73	0.002
Strawberries	46.4 ± 4.8	1.1 ± 0.48	23.9 ± 4.1	12.93	0.002

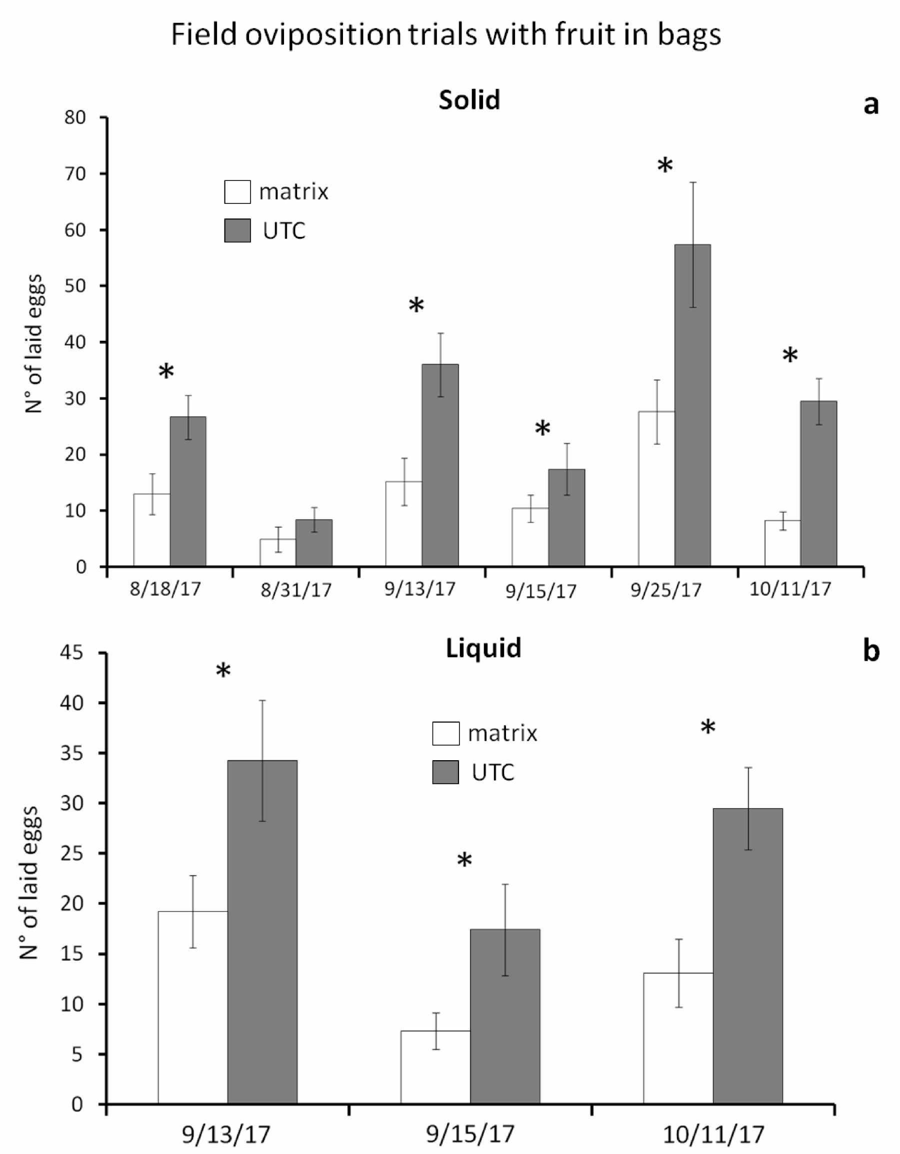

Figure 4. Effect of solid (a) and liquid gum (b) on *D. suzukii* egg laying in fruit within mesh bags on eleven-year-old drip irrigated 'Elliot' blueberry in Corvallis Oregon during 2017. Bars with an asterisk indicate a significant reduction in egg laying.

were statistically higher than in the matrices ($F_{1,98} = 32.85$, $P < 0.001$), with 18.54 ± 2.0 eggs in fruit compared to 5.02 ± 1.2 eggs in the matrices.

Field oviposition trials

Field experiments using mesh bags to cover branches containing fruit indicated a consistent reduction of eggs laid using both SM and LM, with an overall reduction of eggs laid on fruits of 51.2% ($F_{1,99} = 20.76$, $P < 0.001$). The fruit exposed to *D. suzukii* in the mesh bag field experiments showed a significant reduction of eggs laid on berries for the solid gum formulation on 5 out of 6 of the dates when this formulation was trialled (mean eggs in fruit in absence of the matrix = 28.7 ± 3.7, mean eggs in fruit in presence of the SM = 14 ± 1.7, $F_{1,99} = 20.76$, $P < 0.001$, figure 4a). The reduction of oviposition ranged from 41.5 to 72.2% during the *D. suzukii* exposure periods, which ranged from 72 to 96 ± 2 hours. On 31 August 2017, the reduction of egg-laying was not statistically significant at 41.5% reduction in egg laying. The fruit exposed to *D. suzukii* in the mesh bag field experiments displayed a significant reduction of eggs laid on berries for the LM on all 3 dates when this formulation was tested (mean eggs of control = 26.8 ± 3.7, mean eggs in fruit in presence of LM = 13.2 ± 3.0, $F_{1,53} = 17.56$, $P < 0.001$, figure 4b). The reduction of egg-laying ranged from 43.8 to 58.1% over each of the ~86 h exposure periods to *D. suzukii*.

The whole-bush field experiments using the solid formulation resulted in a significant reduction (61.1%) of eggs laid on berries on both of the dates (figure 5a). The reduction of egg-laying ranged from 39 to 75% over the exposure periods to *D. suzukii*. Treatment, location, interaction between treatments, date and interaction between the treatment and the location within blueberry bushes significantly affected the *D. suzukii*

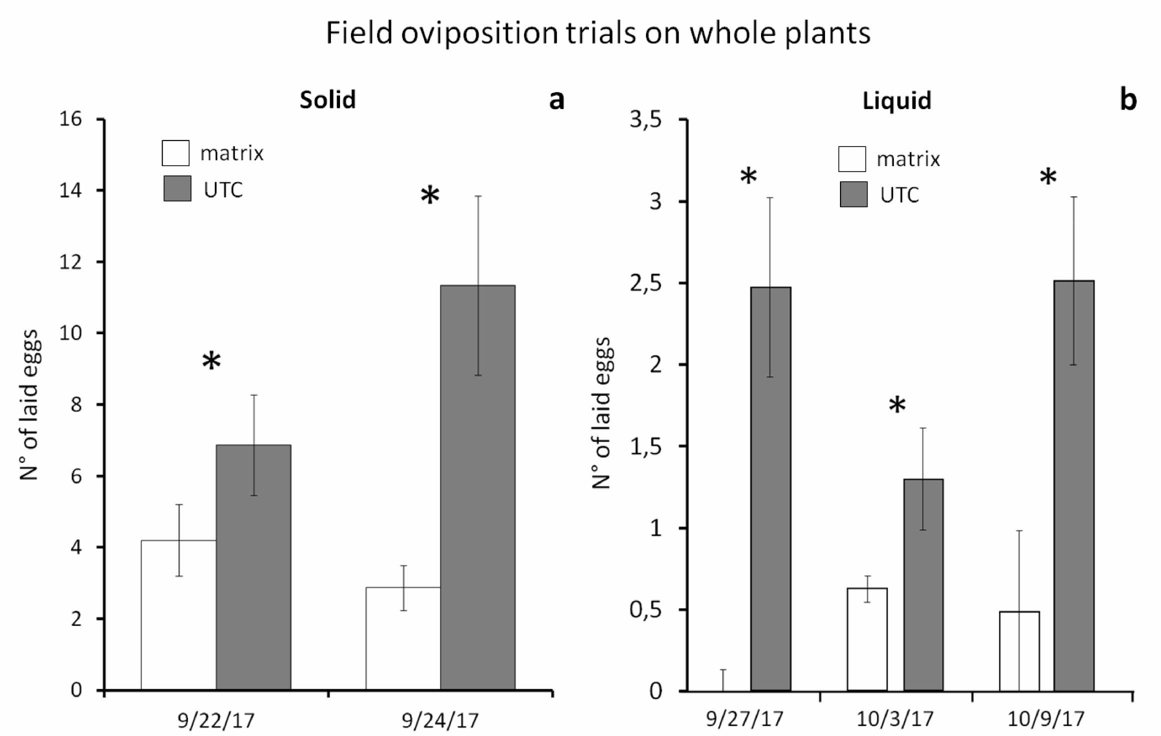

Figure 5. Effect of solid (a) and liquid gum (b) on *D. suzukii* egg laying in fruit on whole plants on eleven-year-old drip irrigated 'Elliott' blueberry in Corvallis Oregon during 2017. Bars with an asterisk indicate a significant reduc- tion in egg laying.

oviposition and also the fruit infestation level (table 2). When the liquid formulation was used, we observed a significant oviposition reduction (60.1%) on all the dates when the experiment was performed (table 2, figure 5b). The reduction of egg-laying ranged from 44 to 75% over the exposure periods to *D. suzukii*. The treatment, the date and the location significantly affected the

Weather data

The temperature, humidity and precipitation from 15 August to 15 October varied significantly during the respective trial events. The days during which the field experiments were performed are indicated (figure 6). The hottest day with the lowest humidity on which experiments were conducted occurred on 1 September, (T_{max} 34.2 °C, mean 49.5% RH) with no precipitation recorded. The coldest day with the highest humidity on

Table 2. Results of the factorial ANOVA analysis performed on the whole-bush field trials data.

Factors	Wilks value	F-values	df	P-values
Solid matrix				
Treatment	0.547	19.43	2, 47	***
Date	0.932	1.71	2, 47	0.193
Location	0.350	16.20	4, 94	***
Treatment*Date	0.854	4.03	2, 47	*
Treatment*Location	0.731	3.99	4, 94	**
Date*Location	0.867	1.73	4, 94	0.15
Treatment*Date*Location	0.930	0.86	4, 94	0.488
Liquid matrix				
Treatment	0.697	15.46	2, 71	***
Date	0.846	3.10	4, 142	*
Location	0.610	9.95	4, 142	***
Treatment*Date	0.904	1.83	4, 142	0.126
Treatment*Location	0.898	1.95	4, 142	0.105
Date*Location	0.816	1.90	8, 142	0.064
Treatment*Date*Location	0.929	0.67	8, 142	0.718

* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

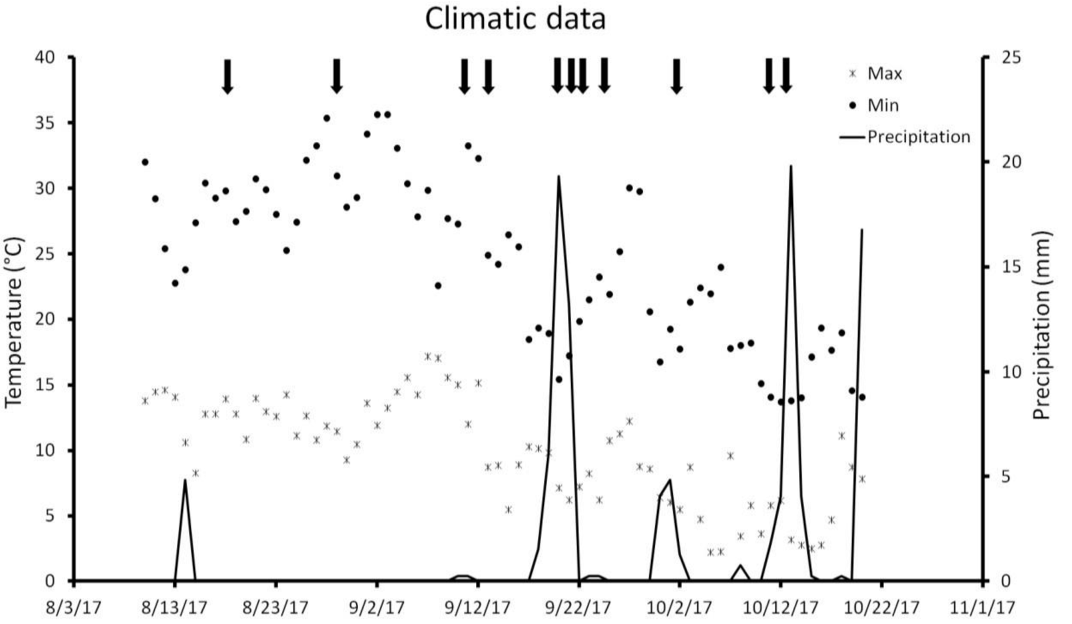

Figure 6. Daily minimum and maximum temperatures and precipitation during the experimental period on 'Elliott' blueberry in Corvallis, Oregon during 2017. Arrows indicate the days during which field experiments were initiated.

which experiments were conducted occurred on 12 October, (T_{max} 13.7 °C, a mean of 91.3% RH) with 18.9 mm precipitation. Generally, it appeared as if more eggs were laid by *D. suzukii* during periods when temperatures were below 30 °C and above 20 °C.

Electrophysiological response to attractive volatiles

GC-EAD analyses from the headspace extracts of the principal ingredient A revealed the presence of 2 distinct peaks (figure 7) named from here onward as Compound 1 (CP1) and Compound 2 (CP2). Both compounds and

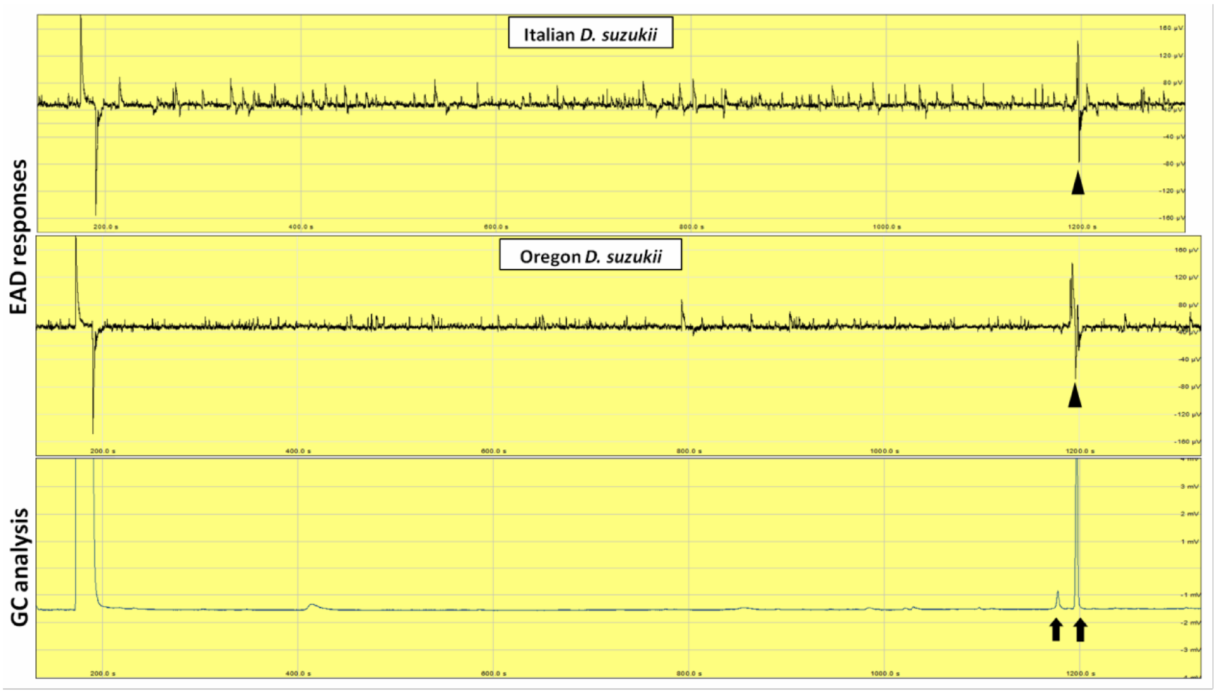

Figure 7. Coupled gas chromatographic-electroantennographic (GC-EAD) analysis of headspace extracts tested on two different *D. suzukii* populations, one northern Italy and one from individuals collected in Oregon. The upper traces are the antennal response from a *D. suzukii* female antenna, the lower trace is the chromatogram monitored with flame ionization detector (FID). The EAD responses were calculated by Autospike software (Syntech) measuring the maximum amplitude of negative deflection (mV) elicited by *D. suzukii* antennae. The headspace extracts were collected from the principal ingredient A.

their relative quantities of the corresponding volatiles were 14.47 and 100 for the first CP1 and the second peak CP2 respectively. *D. suzukii* individuals elicited consistent antennal response only to CP2. No difference in the antennal response amplitude was observed between Italian (0.062 ± 0.096 mV; mean ± SD) and Oregon (0.058 ± 0.094 mV) *D. suzukii* females (U = 11; *P* = 0.834).

Discussion

The data presented provide a novel and proof of concept approach to the management of *D. suzukii* egg-laying behaviour through behavioural manipulation. We demonstrated significant reductions of egg-laying on blueberries, cherries, strawberries, raspberries and blackberries under controlled laboratory conditions. Field experiments were carried out on blueberry clusters, each containing a predetermined number of *D. suzukii*. Egg-laying was significantly reduced on 8 out of 9 experimental dates. These results were obtained using both SM and LM formulations over periods of 72 to 96 ± 2 hours. Then, whole-bush field experiments exposing fruit to *D. suzukii* populations over 3-4 days included the location within the experimental bush as an additional factor in the experimental design. Here, oviposition was significantly reduced on all 5 of the experimental dates. The location on the bush had the largest impact on the reduction of egg-laying, with the largest reductions occurring in the middle and lower portions of the blueberry bush. Overall, the reduction of the absolute number of eggs and the number of infested berries was largely similar. In all cases, when egg-laying was reduced, there was also a reduction in the number of infested berries.

The data were collected under varying environmental conditions over a 2-month period for the field experiments on blueberry. Considering weather conditions, it appeared as if egg-laying was higher under conditions where temperatures were above 20 °C and below 30 °C; however, it appeared that temperature, humidity and rainfall had minimal overall impact on the efficacy of the applied treatments. Finally, the data generated from the EAG trials indicate a similar response in both the Italian and Oregon populations of *D. suzukii* using volatiles originating from carbohydrates within the matrix. Subsequent experiments (Rossi-Stacconi *et al*., in preparation) conducted under controlled laboratory conditions showed significant attraction to the key volatile identified in the EAG trials.

The fact that the treated fruit consistently displayed significant reductions of *D. suzukii* damage provides a strong impetus of future commercial implementation. Research on the control of *D. suzukii* has predominantly focused on attractants (Landolt *et al*., 2012; Cha *et al*., 2017; Frewin *et al*., 2017; Tonina *et al*., 2018). Alternative methods looked at the use of sugar, a phagostimulant mixed with pesticides and sprayed on the canopy to improve efficacy of insecticides (Cowles *et al*., 2015). Push-pull strategies proposing aversive and attractive stimuli in order to modify *D. suzukii* pest distribution in the crop resulted in encouraging results (Renkema *et al*., 2016; Wallingford *et al*., 2016a; 2016b; 2017; 2018). In the current study, the attractive gum matrices for *D. suzukii* shows comparatively favourable results, where treatments using the substrate lead to significant reductions of fruit infestation over relatively extended periods. It is possible that these reductions are the result of altered egg-laying behaviour toward susceptible fruit. These data indicate a strong, consistent and competitive attraction of the substrate when presented together with berries. The current study provides evidence that *D. suzukii* crop damage can be reduced by using attractive volatiles in commercial field settings. The compound as tested contains no conventional insecticides and provides an alternative and environmentally friendly management tool for *D. suzukii.* Since *D. suzukii* has become an economically important pest in both Americas and Europe (Cini *et al*., 2012; Asplen *et al*., 2015; Tait *et al*., 2017), the number of tools and techniques to manage *D. suzukii* commercially has increased. Currently, several trap designs (Lee *et al*., 2011; 2013), synthetic volatiles (Cha *et al*., 2014) and different types of baits (Landolt *et al*., 2012; Cha *et al*., 2013; Renkema *et al*., 2014; 2017; Grassi *et al*., 2015) are available, aiding in the control of this important pest. It is acknowledged that the currently available tools have many limitations (Haye *et al*., 2016). Moreover, the prevailing problem with synthetic volatile compositions is that they do not mimic natural circumstances. Even with all available options, effective management of the pest is a challenge and requires a more efficient method of control.

The matrices described in this study focuses on manipulation of pest behaviour. In addition to be an alternative ovipositional medium, this matrix may be providing *D. suzukii* a substrate where several additional activities of biological significance may be observed (Tait, unpublished) including feeding and mating. Our findings support the use of the matrices as a biologically-based product to handle problematic *D. suzukii* infestations in an environmental friendly way (Alnajjar *et al*., 2017; Lanouette *et al*., 2017; Woltz and Lee, 2017). Despite this, it must be considered that trials show between 50% and 76% reduction in eggs laid in fruit that received the treatments, suggesting a pairing of this method with additional approaches available in an IPM program (Cossentine and Ajjanath, 2017; Frewin *et al*., 2017). One of the characteristics of the gum is that it is water-soluble on account of its organic composition and can be applied without limitations of time or quantity. Considering the work of Tochen *et al*. (2014; 2016a) and Grassi *et al*. (2018), a consistent application of the substrate could be particularly useful during the late dormant period, and when fly activity increases. This is because during these 2 key bottleneck periods of the year, few ovipositional sites and limited food sources are available, with the exception of berries on secondary plant species including *Hedera helix* L. and *Viscum album* L. berries during winter/early spring (Grassi *et al*., 2018), and *Sarcococca* species (Lee *et al*., 2015; Kenis *et al*., 2016). Providing flies with an alternative substrate for oviposition could possibly reduce the chances of infestation of early-ripening susceptible commercial fruits at the beginning of the growing season (Little *et al.*, 2017). Moreover, larvae do not survive because of the dehydrating effect of both matrix formulations (Tait, unpublished), providing a constant reduction of the population. Future avenues of research are needed to address these concerns.

Additional laboratory experiments should also focus on understanding the mechanisms of the gum, including Gas Chromatography-Mass Spectrometry (GC-MS), Electroantennography (EAG) dose-response curve, olfactometer tests, wind tunnel experiments, longevity studies in the field and primarily find a natural toxicant to include into the matrix in order to reduce the natural adult wild population in the field. Future goals may include testing the product during early and late winter conditions with the aim to assay the efficacy of attraction of the lure using winter morph *D. suzukii*.

Although our data provides information on a non-commercial proof of concept, we believe that the matrix provides an alternative to conventional control techniques and is not directly applied onto fruit, potentially resulting in the opportunity of reductions of pesticide residues. Ultimately a market-ready product would be applied similar to multiple other commercially available behaviour disruptors also in the border areas where the surrounding vegetation offers refuge to the pest (Ometto *et al.*, 2013; Kenis *et al.*, 2016; Enriquez and Colinet, 2017). Specifically, this lure is able to attract both females and males of *D. suzukii* and induces females to lay eggs on the substrate. All field trials were conducted for a total of 3 to 4 days, within the time span of field longevity periods of many currently used conventional pesticides (Van Timmeren and Isaacs 2013; Wise *et al.*, 2015). We observed no phytotoxicity associated with the matrices on plant parts. Additional studies regarding the persistence of attraction of the substrate in different climatic regions is suggested.

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CHAPTER 7.
Discussion and Conclusion

The goal of my thesis was to scrutinize the ecology, genetics, chemistry and behavior of *D. suzukii* in search of potential targets for integrated pest management programs.

I am confident that with this work I contributed in expanding the knowledge about this invasive species, giving more inputs to create alternative and sustainable strategies of control.

Chapter 2: Genetic variability of Italian population of Drosophila suzukii

With the first work we showed how human transportation is the most probable explanation for the extensive spread of *D. suzukii* (Rota-Stabelli *et al*., 2013; Cini *et al*., 2012). When an alien species is introduced into an environment outside its native range, expansion can be identified not only by analysing genetic diversity indices, but also by analysing the genetic flow between populations, which is a direct proof of rapid distribution (Adrion *et al*., 2015; Tait *et al*., 2017). In particular, in the last 40 years, the risk of biotic invaders has increased significantly because of levels of international trade not seen before (Hulme *et al*., 2009). Our population genetic analysis at Italian level supports the hypothesis that human activities facilitated the genetic flow between groups located in different areas. Indeed, the level of heterozygosity (F_{st}) increases rather than declining from the likely spreading source of the invasive Italian population (Cini et al., 2012), suggesting that there is a high migration rate among localities and that *D. suzukii* moves extensively across most of the Italian peninsula. Interestingly, the case of Sicily region is peculiar. The majority of the Sicilian production of vegetables and fruits, including fruits highly susceptible to *D. suzukii* damage, is exported to central and northern Italy. While this could suggest a high probability of flies being transported between Sicily and the rest of the peninsula, our results indicate that there was no gene flow from Sicily to other regions. This is probably due to the fact that ripe fruits are exported from Sicily mostly during the cold season, when moderate temperatures allow the production of soft fruits in Sicily, but not in the rest of Italy. Therefore, any *D. suzukii* accidentally moving from Sicily to the rest of Italy would arrive at a time when the winter temperature would cause high mortality rate and the local population is made up of a few individuals in reproductive diapause. A second interesting piece of information revealed by our results is related to the scenario in Sardinia. To satisfy local demand for berry fruits, this region imports fruit from Italy and northern Europe, Spain, USA and South America. The flies used in this study were collected in Arborea, a town 13 km away from the port of Oristano, one of the most important commercial ports in Italy. Thus, it is likely that the Sardinian population is made up of immigrants from other regions, as suggested by the low differentiation between this population and those on the mainland. This research represents the first study investigating the pattern of genetic variability for *D. suzukii* following its introduction to Italy. Defining the population structure of a species, in particular of an invasive species, it is necessary to improve our knowledge of its genetic architecture and evolution. Furthermore, understanding the current genetic structure of *D. suzukii* has significant implications in relation to geographical and economic impact. The evaluation of the genetic status of the *D. suzukii* populations in newly invaded areas and their expansion or reduction phases during defined periods of the year may provide valuable information for predicting population spread, outbreaks, and thus improve integrated pest management programmes. Proper genetic management practices for *D. suzukii* and constant monitoring are critical for maintaining populations under control. The information obtained can be applied in particular to the management of coastal areas; one important action could be to increase monitoring control with the use of traps and other early warning tools in order to limit either multiple reintroductions of the same species or new introductions of exotic organisms.

Chapter 3 Large-scale spatial dynamics of Drosophila suzukii in Trentino, Italy

Once defined the large-scale distribution patterns taking Italy as a model area, we decided to focus on a smaller scale movement, with the aim to investigate *D. suzukii* dispersal ability during different times of the seasons, and along an elevational gradient in a mountain valley in Trentino Province, Italy. The findings of this study add to the scientific understanding of *D. suzukii* seasonal migration patterns, suggesting that this species moves and selects environmentally suitable sites. Overall, we provided new information on the magnitude of seasonal long-distance movement of *D. suzukii*. Our data are the first to document *D.*

suzukii dispersal over distances of up to 9 km and further suggest that during spring and summer, flies move from low to higher elevations. The difference in temperature between these elevations may play a role in the migration of *D. suzukii* populations toward more suitable temperature regimes. Migration towards higher elevations in spring might be exploratory movement to search for additional oviposition sites and food resources in favorable environments (Mitsui *et al*., 2010; Tochen *et al*., 2014; Tochen *et al*., 2016; Wiman *et al*., 2014; Evans *et al*., 2017). Summer migration may additionally be a strategy to avoid suboptimal high temperatures at lower elevations. Conversely, it is possible that migration in autumn towards lower elevations might be driven by suitable climatic conditions during the latter portion of the season here (Evans *et al*., 2017). We suggest that the dispersal of *D. suzukii* from a low to a high elevation during spring and summer could be facilitated by diurnal mass movement of air due to fluctuations in temperature from the day to the night. Genetic analysis provided additional data to support our hypothesis. As a fact, they revealed that insects sampled along the valley and across the season belonged to the same population. This small-scale genetic homogeneity is not only attributable to transport mediated by human activities, but it is likely due to the recorded natural migration towards different elevations during different times of the year (Mitsui *et al*., 2010). Strategies to control this destructive species must hence take into account its high dispersal potential and patterns. For example, our results suggest that *D. suzukii* populations are present at lower altitudes during the early portion of the season. No *D. suzukii* are found at high altitudes during the early season. Factors contributing to this finding include suboptimal winter conditions and the lack of wild fruits (Tonina *et al*., 2016). Considering these observations, a valid approach could be to plant early-producing berry varieties in high-elevation sites. Such crops might benefit from temporal escape from the onset of seasonal migration. Knowledge concerning the capacity of *D. suzukii* dispersal is crucial to better-forecast development of pest populations, to validate and optimize population models, and to more effectively design management strategies.

Chapter 4: Drosophila suzukii daily dispersal between crop and surrounding vegetation

The third project of my thesis reports the studies of the dispersal capacity of *D. suzukii* at a significantly shorter range, from the crop to the surrounding vegetation in strictly relation of the change of abiotic factors like temperature and humidity, which are the most important parameters that influence the survival of *D. suzukii* species. Our data, together with earlier studies, suggest that temperature and humidity influenced the migration between these distinct habitats. Temperature and humidity values were respectively higher and lower within the crop compared to the surrounding wood. This was likely due to the nylon coverings of the crop tunnels, that trap the heat in the crop. In contrast, the trees of the vegetation area provide shade to traps deployed in it. In both the experimental trials (11 to 14 July; 31 July to 3 August 2017), the daily temperatures of the wood were closer to 20 °C (the optimal *D. suzukii* temperature) compared to the cultivated areas. In the same daylight periods, the average humidity values recorded in the wood were the highest for three out of eight days and the middle value for two out of eight days (high humidity condition are the optimal ones for *D. suzukii* in warm climates). These temperature and humidity values suggest that the wood itself is an optimal shelter for the fly (Tochen *et al.*, 2014; Tochen *et al.*, 2016; Enriquez & Colinet, 2017). Particularly remarkable is the ability of the wood to maintain level of humidity higher than that of the crop while temperature values were still well under 30 °C. We were not expecting this condition, given the fact that the nylon coverings of the crop should trap humidity inside the tunnels. An additional factor contributing to environmental suitability of surroundings vegetation is the presence of alternative host plants, on which *D. suzukii* can feed and oviposit before reaching the crop. Previous works have explained how such non-crop hosts may provide appropriate ovipositional media as well as energy in the form of nectar (Lee *et al.*, 2016; Klick *et al.*, 2016). In the studied surrounding area, two known *D. suzukii* non-crop hosts were found: *Cornus sanguinea*, one of the most European common shrub, and *Taxus baccata*, a host tree also found in other surveyed areas (Lee *et al.*, 2015; Kenis *et al.*, 2016; Poyet *et al.*, 2016). In particular, Kenis et al. (2016) reported that these two species were frequently found infected from *D. suzukii*. Two other plants found in the area, sometimes reported as *D. suzukii* hosts, have not been considered in this analysis, because their presence was infrequent and because the literature on their relations with the pest is not clear i.e., *Rosa canina* and *Viburnum lantana*. Finally, *Rubus idaeus*, red raspberry, was reported in the surveyed area: this plant is one of the main hosts of *D. suzukii*, and it may have increased the fitness of the forest area, even if it was found only sparsely. The presence of vegetation surrounding cultivated crop fields can therefore undoubtedly increase the number of *D. suzukii* flies infesting nearby crops (Klick *et al.*, 2016). Our results indicate that the insects move from the crop to the wood; this information could lead to more optimized IPM approaches (Kenis *et al*., 2016; Diepenbrock *et al.*, 2016a; Diependbrock *et al*., 2016b), possibly making this vegetation area a key factor in control strategy (Rossi Stacconi *et al*., 2017). The information in this study underlines the important role played by the combined abiotic and biotic landscape to provide ideal conditions for *D. suzukii*. On this final topic there are some considerations to make: first of all, given the fact that *D. suzukii* move back and forth between the wood and the crop, there could be the possibility to apply insecticides in specifically times and days of the year. Applying pesticides or attractants in climatically more moderate environments in specific times of the year, may also enhance their persistence, partially mitigating the negative effect that direct rainfall has on it (Van Timmeren and Isaacs, 2013). Other types of controls could also be used in the wood area nearby crops, like “attract and kill” traps, in order to kill a higher number of flies. In conclusion, these distribution and spatial analysis could help in different ways to guide management investments concerning the spread of *D. suzukii*, as well as to identify areas where coordinate actions should be focused to reduce the infestation and the damage caused by this threatening pest. Future studies should be aimed at verifying if applying control strategies within the vegetation area could increase their efficiency in decreasing the number of *D. suzukii* flies infesting nearby crops.

Chapter 5: Oviposition behavior of Drosophila suzukii with evidence of an oviposition marking pheromone

This project is the first study that evidences the presence of aggregation pheromone in *D. suzukii*. The research permitted us to demonstrate that *D. suzukii* female after oviposition release an aggregation marking pheromone easily distinguable from other signs on the surface of the fruit. Results of choice and no-choice test consistently showed that highly infested and artificially pierced fruit were the preferred treatments, resulting in higher oviposition rates by *D. suzukii* females. Some hypothesis may be outlined as: 1) the loss of sugary liquids from the egg holes over the fruit surface may provide an easy food source for egg-laying females, which may be attracted at a relatively long range by the release of volatile substances from the wounded fruit surface (Abraham *et al*., 2015; Revadi *et al*., 2015; Mazzetto *et al*., 2016); 2) start of potential alcoholic fermentations may occur on the wounded fruits, thus leading to bacteria and fungi development, which would stand as additional food sources, and attract conspecifics (Fountain *et al*., 2018); 3) a role of the liquid released by females as a marking pheromone can by hypothesized. This substance would likely attract other females and stimulate them to lay eggs. In support of this, video recordings and pictures analysis showed a peculiar behavior of egg-laying females with a release of a liquid from the abdomen tip over the fruit surface after oviposition. GC-MS anaylsis allowed us to characterize the peculiar compounds emitted in the liquid. Most of these peaks (14) were absent in the surface washing of the artificially pierced fruit (control), hence they are indicative of compounds originated from the insects rather than from the fruit. In particular, we found some carboxylic acids, which were described as aggregation pheromone by Keesey et al. (2016) in *D. melanogaster* frass. EAG tests revealed that the female antennal responses to the six compounds at all the four concentrations were not statistically different, while males showed significantly higher EAG responses to the highest and lowest concentrations of palmitic and palmitoleic acid. It has been demonstrated that these two acids together with myristic acid strongly activate olfactory receptor Or47b, which is a specific pheromone detector in *D. melanogaster* (Dweck *et al*., 2015). The last experiment performed spraying the synthetic blend of the six major compounds resembling the ratio found in the extract over intact blueberry surface, gave us the final determining proof about the role of the released anal liquid as an attractive marking pheromone. Results indeed indicate that marked fruits attract more flies, which lay more eggs on it. In future, our findings may give important contribution in the development of environmentally friendly control methods by interfering with *D. suzukii* communication during the phase of oviposition.

Chapter 6: A food-grade gum as a management tool for Drosophila suzukii

The last chapter (6) of this thesis is focused on the development of a new food-grade matrix (patent pending) able to manipulate the behavior of *D. suzukii* and therefore to reduce egg-infestation on berries. We demonstrated that using the new gum matrix developed at the Oregon State University, there is significant reductions of egg-laying on blueberries, cherries, strawberries, raspberries and blackberries under controlled laboratory and field conditions. The fact that the matrix preparation consistently resulted in significant reductions of *D. suzukii* damage on fruit provides a strong impetus of future commercial implementation. Data collected this current year (Rossi-Stacconi et al., unpublished data) demonstrate how this approach is significant valid in strawberry, blueberry and cherry field trials. Research on the control of *D. suzukii* has predominantly focused on attractants (Landolt *et al*., 2012; Cha *et al*., 2017; Frewin *et al.,* 2017; Tonina *et al*., 2018). In the current study, the the attraction is towards the gum matrices which shows comparatively favorable results, where treatments using the substrate lead to significant reductions of fruit infestation over relatively extended periods. These data indicate a strong, consistent and competitive attraction of the substrate when presented together with berries. The compound as tested contains no conventional insecticides and provides an alternative and environmentally friendly management tool for *D. suzukii.* Our findings support the use of the matrices as a biologically-based product to handle problematic *D. suzukii* infestations in an environmentally friendly way (Alnajjar *et al.,* 2017; Lanouette *et al.,* 2017; Woltz and Lee 2017). One of the characteristics of the gum is that it is water-soluble on account of its organic composition and can be applied without limitations of time or quantity. Moreover, larvae do not survive because of the dehydrating effect of the paste (Tait unpubl. data), providing a constant reduction of the population. Despite this, it must be considered that trials show between 50% and 76% reduction in eggs laid in fruit that received the treatments, suggesting a pairing of this method with additional approaches available in an IPM program (Frewin *et al.*, 2017). Considering the work of Tochen et al. (2014), (2016a) and Grassi et al. (2017), a consistent application of the substrate could be particularly useful during the late dormant period, and when fly activity increases. This is because during these two key bottleneck periods of the year, few ovipositional sites and limited food sources are available, with the exception of berries on secondary plant species including *Hedera helix* and *Viscum album* L. during winter/early spring (Briem *et al*., 2016; Grassi *et al.,* 2017), and *Sarcococca* species (Lee *et al.,* 2015; Kenis *et al.,* 2016). Providing flies with an alternative substrate for oviposition could possibly reduce the chances of infestation of early-ripening susceptible commercial fruits at the beginning of the growing season (Little *et al.,* 2017). The matrix provides an alternative to conventional control techniques and is not directly applied onto fruit, potentially resulting in the opportunity of reductions of pesticide residues. The product can also be applied during any time of year, including in the border areas where the surrounding vegetation offers refuge to the pest (Ometto *et al.,* 2013; Kenis *et al*., 2016; Enriquez and Colinet 2017, Tait et al., unpublished). Specifically, this lure is able to attract both females and males of *D. suzukii* and induces females to lay eggs on the substrate.

In conclusion, we can say that all together the information collected during three years of PhD program, definitely increase the knowledge about this destructive species, which is the fundamental prerequisite for any applied future investigation. Furthermore, my research leads to valid and concrete indications about the development of new alternative and environmentally-friendly methods against *D. suzukii*.

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