UNIVERSITÀ DEGLI STUDI DEL MOLISE

Department of Agricultural, Environmental and Food Sciences

Ph.D. course in:

AGRICULTURE TECHNOLOGY AND BIOTECHNOLOGY
(CURRICULUM: Sustainable plant production and protection)
(CYCLE XXXIV)

Related Disciplinary Scientific Sector: AGR / 11 (General and Applied Entomology)

Ph.D. thesis

Development of New Tools for an Agroecological Management Of Spotted Wing Drosophila, *Drosophila suzukii*

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Academic Year 2021/2022

INDEX

SUMMARY

Invasive alien species are animal or plant species that are very often unintentionally introduced into non-native ecosystems, with negative impact for the environment and human activities.

Drosophila suzukii Matsumura (Diptera: Drosophilidae), also known as Spotted Wing Drosophila, is an invasive alien species native to East Asia, which has widely established in Americas and Europe, where it has become a serious pest of fruit crops, causing considerable economic losses.

In Trentino Province the first oviposition on crop hosts was reported in 2009.

The rapid worldwide spread is attributed to peculiar features of *D. suzukii*, such as the serrated ovipositor that allows it to oviposit into healthy ripening fruits, broad host plants range, high fecundity potential and tolerance of wide climatic conditions.

Current approaches for controlling *D. suzukii* rely primarily on integrated pest management strategies, including chemical control, insect-proof netting, mass trapping and cultural practices, such as canopy pruning, drip irrigation, and field sanitation.

Similarly to other invasive pest, *D. suzukii* lacks of an effective suppression by natural enemies in the recently invaded areas, enabling it to reproduce quickly and spread unlimitedly, increasing pest pressure on cropping systems. Augmentative and classical biological control are still under investigation as promising candidates for limiting *D. suzukii* seasonal outbreaks and related damage on crops.

However, despite the remarkable steps forward in the knowledge on biology and ecology of *D. suzukii*, it is still a serious threat to crops and further investigations are urgently required in order to update the availability of management tools to cope with this crop pest.

During our faunistic surveys in Trentino province, we found and reported for the first time in Europe, the presence of the Asian larval parasitoid *Leptopilina japonica* Novkovic & Kimura (Hymenoptera: Figitidae), one of the most effective natural enemies of *D. suzukii* in the native range.

The following extensive field monitoring based on fruit sampling revealed the high capacity of this parasitoid to parasitize its host on a complex of several plant species, belonging to Moraceae, Rosaceae, Glossulariaceae, Rhamnaceae, Cornaceae, Ericaceae, Phytolaccaceae, Dioscoreaceae, Adoxaceae and Solanaceae. These findings offer new perspective for biological control of *D. suzukii* in the introduced areas, also in relation to the classical biological control programmes with *Ganaspis brasiliensis* Ihering (Hymenoptera: Figitidae).

Biological control by means of parasitoids or predators can be easily integrated with sterile insect technique (SIT), synergizing the control effects on pest population.

Nevertheless, the success of SIT is largely influences by mating system of target pest, thus deepen reproductive traits is a fundamental step forward.

Paternity analysis by means of microsatellite loci genotyping of the progeny of wild-caught females, detected high levels of multiple paternity in genotyped broods, stating the polyandrous behaviour of *D. suzukii*, but no found evidence of a strong paternity skew in sperm allocation, in terms of prevailing male.

Beyond biological control and SIT, habitat manipulation strategies may further help local farmers to deal with *D. suzukii* in addition to common applied cultural practices.

Our field trials have shown that intercropping with Mentha x piperita (Peppermint, Lamiaceae) *Origanum vulgare* L. (Oregano, Lamiaceae), *Thymus vulgaris* L. (Thyme, Lamiaceae), or *Ocimum gratissimum* L. (African basil, Lamiaceae), has no effect in limiting the damage of *D. suzukii* on blueberries, whilst hedges of *Prunus padus* (European bird cherry, Rosaceae) was able to reduce the infestation on raspberry, having a potential role as dead-end trap plant for crop protection against *D. suzukii*.

Furthermore, conservative biological control by means of the *augmentorium* technique may be further explored, as we observed that our prototype has shown to be well adapted to *D. suzukii* and its indigenous parasitoids, favouring crop sanitation from infested fruits and sustaining the most common *Drosophila* pupal parasitoids.

This research aims to give new insights for a more sustainable management of *D. suzukii*, offering new solutions that, by combining biological control, both conservative and classical, SIT and habitat manipulation, may move towards an agroecological approach for controlling this invasive pest.

RIASSUNTO

Le specie aliene invasive sono specie animali o vegetali che molto spesso vengono introdotte involontariamente in ecosistemi non autoctoni, con impatto negativo per l'ambiente e le attività umane.

La *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), nota anche come Spotted Wing Drosophila, è una specie aliena invasiva originaria dell'Asia orientale, che si è ampiamente diffusa nelle Americhe e in Europa, dove è divenuta un grave parassita delle colture frutticole, causando notevoli perdite economiche.

In Trentino la prima segnalazione di questo insetto su ospiti coltivati è avvenuta nel 2009.

La sua rapida diffusione in tutto il mondo è attribuita a delle caratteristiche peculiari di *D. suzukii,* come l'ovopositore seghettato che gli consente di deporre le uova in frutti sani in maturazione, l’ampia gamma di piante ospiti, l’elevata fecondità e la tolleranza nei confronti di un ampio spettro di condizioni climatiche, fattori che ne ha aumentano significativamente l'invasività.

Gli attuali approcci per il controllo di *D. suzukii* si basano principalmente su strategie di gestione integrata dei parassiti, tra cui il controllo chimico, le reti anti-insetto, la cattura massale e pratiche culturali, come la potatura delle chiome, l'irrigazione a goccia e il sollecito allontanamento della frutta infestata dai campi nel corso della raccolta.

Analogamente ad altri parassiti invasivi, *D. suzukii* non viene efficacemente controllata da parte dei nemici naturali nelle aree invase, consentendogli di riprodursi rapidamente e diffondersi illimitatamente, aumentando la pressione dei parassiti sulle coltivazioni.

Il controllo biologico inondativo e quello classico, sono in corso di valutazione e potrebbero diventare dei promettenti candidati per limitare i focolai stagionali di *D. suzukii* e i relativi danni alle colture.

Tuttavia, nonostante i notevoli passi in avanti sulle conoscenze della biologia e l'ecologia di *D. suzukii*, questo insetto rappresenta ancora una seria minaccia per le colture e vi è la necessità di ulteriori studi al fine di aggiornare gli strumenti di gestione a disposizione per far fronte a questo importante parassita delle colture.

Nel corso dei monitoraggi faunistici in provincia di Trento, abbiamo riscontrato e segnalato per la prima volta in Europa la presenza del parassitoide larvale asiatico *Leptopilina japonica* Novkovic & Kimura (Hymenoptera: Figitidae), uno dei più efficaci nemici naturali di *D. suzukii* nell’areale nativo.

L’esteso monitoraggio in campo successivo al primo ritrovamento ha permesso di stabilire l'elevata capacità di questo parassitoide di parassitare il suo ospite su un ampio spettro di specie vegetali, appartenenti a diverse famiglie tassonomiche come Moraceae, Rosaceae, Glossulariaceae,

Rhamnaceae, Cornaceae, Ericaceae, Phytolaccaceae, Dioscoreaceae, Adoxaceae e Solanaceae. Questi risultati offrono nuove prospettive per il controllo biologico di *D. suzukii* nelle aree di recente introduzione, anche per quanto concerne i futuri programmi di controllo biologico classico con *Ganaspis brasiliensis* Ihering (Hymenoptera: Figitidae).

Il controllo biologico per mezzo di parassitoidi o predatori può essere facilmente integrato con la tecnica dell'insetto sterile (SIT), potenziando il controllo sulle popolazioni di parassiti.

Tuttavia, il successo della SIT è in gran parte influenzato dal sistema riproduttivo del parassita bersaglio, per cui l'approfondimento dei tratti riproduttivi è un prerequisito fondamentale per verificare l'effettiva applicabilità di questa tecnica di controllo.

L'analisi della paternità mediante la genotipizzazione di loci microsatellite della progenie di femmine catturate in natura, ha rilevato elevati livelli di paternità multipla nelle covate genotipizzate, stabilendo il comportamento poliandro di *D. suzukii,* sebbene non sia stata rilevata alcuna distorsione della paternità nell'allocazione dello sperma, ovvero in termini di maschio prevalenza.

Oltre al controllo biologico e alla SIT, le strategie basate sulla manipolazione dell'habitat potrebbero utilmente integrare le comuni pratiche culturali messe in atto dagli agricoltori per il controllo di *D. suzukii*.

Le nostre prove in campo hanno dimostrato che la consociazione con *Mentha x piperita* (Menta piperita, Lamiaceae) *Origanum vulgare* L. (Origano, Lamiaceae), *Thymus vulgaris* L. (Timo, Lamiaceae) o *Ocimum gratissimum* L. (Basilico africano, Lamiaceae), non risulta avere un impatto positivo nel limitare i danni causati da *D. suzukii* su colture di mirtillo, mentre la realizzazione di una bordura di *Prunus padus* (ciliegio europeo, Rosacee) si è dimostrata in grado di ridurre l'infestazione su di una coltura di lampone, agendo come pianta trappola.

Inoltre, il controllo biologico conservativo mediante la tecnica dell'*augmentorium* può un'ulteriore integrazione degli strumenti di controllo. Abbiamo infatti potuto osservare come il nostro prototipo si è dimostrato adatto per una sua applicazione su *D. suzukii* e sui relativi parassitoidi indigeni, favorendo la sanificazione delle colture dai frutti infestati e sostenendo i più comuni parassitoidi pupali.

Con questa ricerca abbiamo inteso verificare l'efficacia di nuovi strumenti di controllo di *D. suzukii,* quali il controllo biologico, sia conservativo che classico, la SIT e la manipolazione dell'habitat, la cui opportuna integrazione offrirebbe la possibilità di implementare un approccio agroecologico di gestione di questo parassita invasivo.

INTRODUCTION

Overview

Drosophila suzukii Matsumura (Diptera: Drosophilidae), also known as Spotted Wing Drosophila, is a species native of East Asia and in recent years is become a serious invasive pest of soft and stone fruit crops (Hauser et al., 2011).

This insect species belongs to the genus *Drosophila*, subgenus Sophophora and to the *melanogaster* group, particularly to the *suzukii* subgroup (Ometto et., 2013).

Compared to the other close relatives Drosophila, which lay their eggs inside damaged, overripe or decay fruits, *D. suzukii* females prefer to lay their eggs on healthy ripening fruits. After egg-laying, fruit damage is caused by the larvae feeding inside fruit pulp, leading to the collapse of the infested fruit (Walsh et al., 2011). Furthermore, secondary infections by fungi or bacteria may further contribute to fruit decay (Ioriatti et al., 2018).

The most peculiar features that have allowed this species to derive this new ecological niche are morphological adaptations, such as the development of a serrated ovipositor enabling to penetrate the fruit skin (Atallah et al., 2014), and the evolution of specific neuroethological processes to find the breeding sites (Keesey et al., 2015).

The first report and detailed information of this specie dates back to the early twentieth century as pest of cherry orchards in Japan (Kanzawa et al., 1935, 1939).

Outside its native range D. suzukii was firstly reported in Hawaii islands in 1980 (O'Grady et al., 2002) and about thirty years after, in 2008, it was detected both in North America (California) receiving notable attention due to the crop’s losses (Bolda et al., 2010).

In Europe, first adult catches were reported in Spain (Rasquera Province), and in Italy (San Giuliano Terme, Pisa, Italy) (Raspi et al., 2011; Calabria et al., 2012), and first crop infestation again in Italy (Trentino province) the following year (Grassi et al, 2009).

Following these initial records, *D. suzukii* rapidly spread worldwide during the last decade (**Fig.1**).

Morphology and life cycle

Immature stages of *D. suzukii* cannot be reliably distinguished from other common *Drosophila* species using morphology, whilst the identification based on diagnostic characters of the imago is the most pursued method (Hauser et al, 2011).

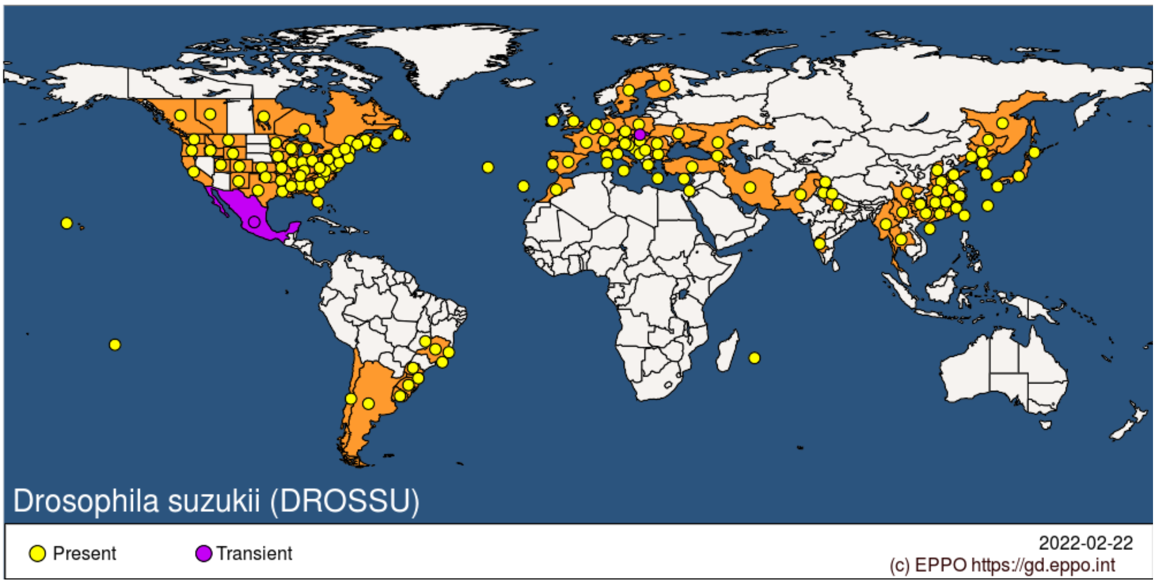


Figure 1. *D. suzukii* global reports by European and Mediterranean Plant Protection Organization (EPPO) database. (https://gd.eppo.int)

Adults are yellowish brown flies with red eyes and unbroken black bands on the abdominal tergits. *D. suzukii* males are approximately 2.0-3.5 mm long, possessed a large black spot on the wing at the top of vein R_{2+3} (**Fig.2a**) and two sets of tarsal sex combs on the forelegs, one row on the first and one row on the second tarsomere. Females are slightly larger than males, 2.5-4.0 mm long, having a strongly sclerotized and serrated ovipositor (**Fig.2b**).

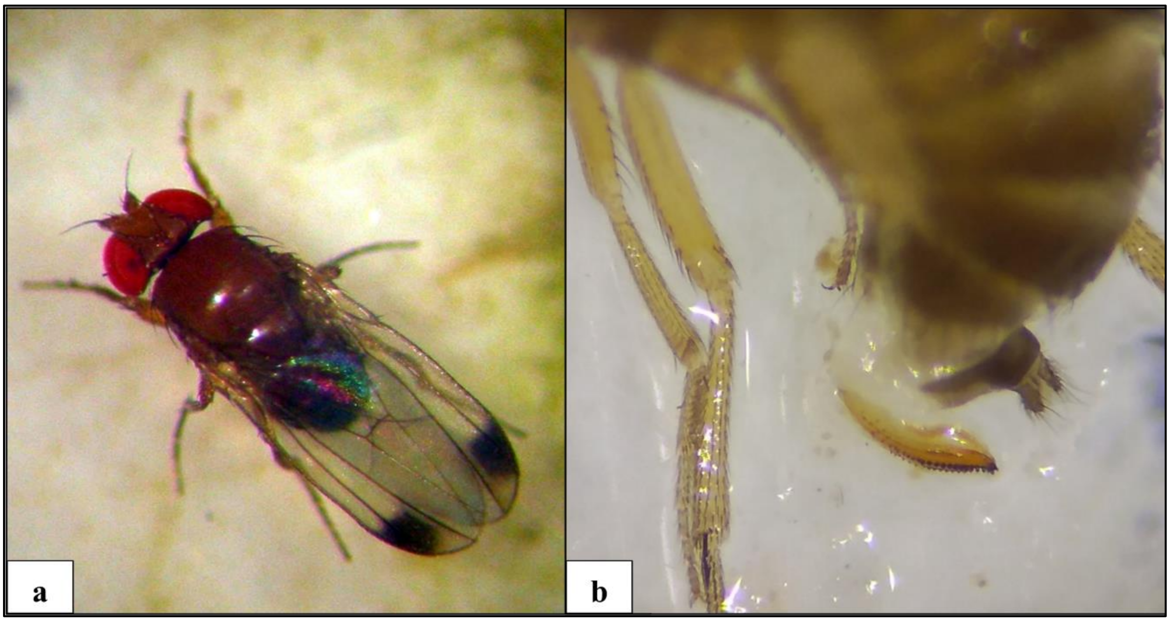


Figure 2. a) Male with black spots on wings (A. Grassi). b) Detail of the serrated ovipositor of the female (S. Puppato).

Although these morphological criteria are commonly used for *D. suzukii*, differentiating the pest from other European and North American *Drosophila* species, several identification issues may arise from species that are sympatric with *D. suzukii*, among which *D. subpulchrella*, *D. pulchrella* and *D. biarmipes*, thus other diagnostic characters should be added (Hauser et al., 2011, Atallah et al., 2014). Furthermore, morphological analysis may be further confirmed by DNA barcoding based on mitochondrial cytochrome c oxidase I (COI) gene.

The life cycle of *D. suzukii* begins with the deposition of an egg within the host fruit by means of the serrated ovipositor. Eggs' hatching is followed by three larval instars that develop within the fruit pulp, then a pupal stage occurred. Wandering larvae can pupate inside the fruit or drop off the fruit and pupate in the soil (Walsh et al., 2011, Woltz et al., 2017).

Eggs are oval, milky-white and glossy with an average size of 0.62 mm and 0.18 mm, length and width respectively, with two respiratory spiracles. Spiracles usually protruding out the fruit skin and are visible under magnification (**Fig.3a**). Larval stages are milky-white with black mouth hooks. Their size ranging from the 0.67 mm long first instar larvae to the 3.94 mm of the third instar larvae. Pupae is golden brownish, about 4 mm long with anterior respiratory tubes (Walsh et al., 2011) (**Fig.3b**)

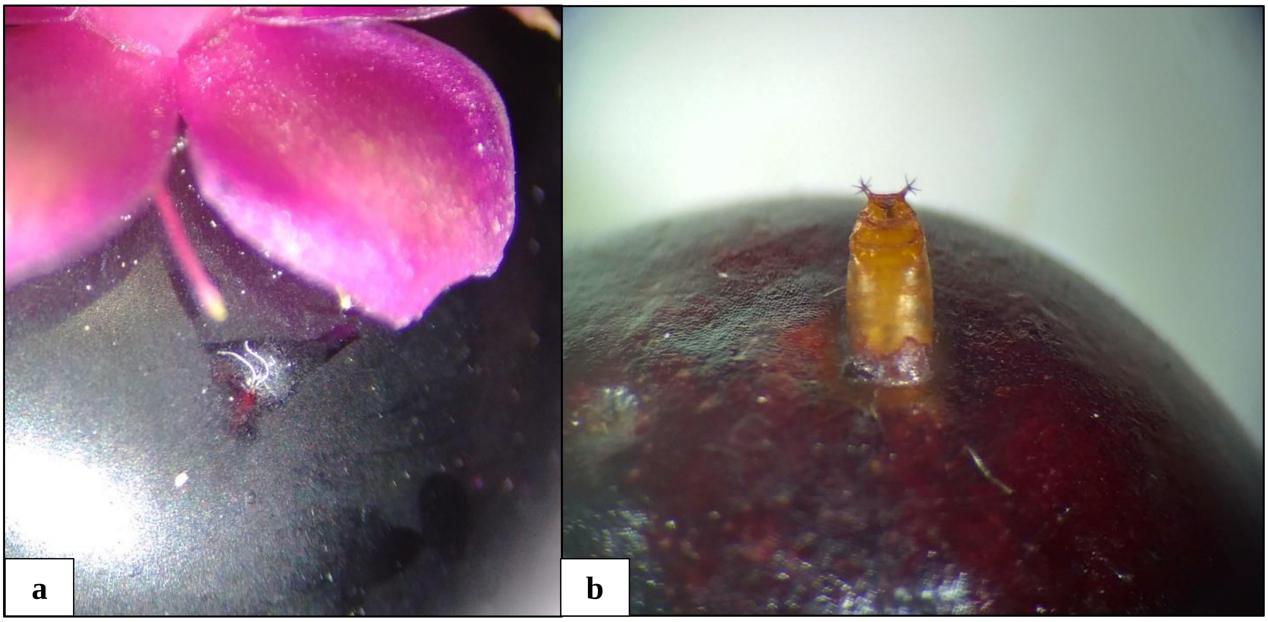


Figure 3. a) Respiratory spiracles on the surface of *Phytolacca americana* fruit. **b)** *D. suzukii* pupae. (S. Puppato)

At 22°C, the life cycle from egg to adult lasts approximately 14 days on cherry and blueberry (Tochen et al., 2014). According to climatic conditions *D. suzukii* can complete 3 to 13 generations per year with an adult lifespan of 20-60 days (Kanzawa, 1939, Winkler et al., 2020), even though longer times have been observed (Panel et al., 2020).

Reproductive behaviour, host plants and seasonal polyphenism

Sexual maturation of *D. suzukii* adults is complete within the three-four days after the emergence from the pupae, a process that is accompanied by an increase in chemical signals deriving from cuticular hydrocarbon compounds, as known in other *Drosophila* species (Revadi et al., 2015). In *D. melanogaster*, males produce the pheromone cis-vaccenyl acetate, that is essential during the interaction between the sexes. Nevertheless in *D. suzukii* this compound is not synthetized, moreover the ejaculatory bulb is atrophic (Dekker et al., 2015).

Beyond chemical communication, the courtship behaviour of male towards the female assumes a key role for mating success. *D. suzukii* males courts the females through a series of body and wings movements, involving substrate-borne vibrations (Mazzoni et al., 2013, Revadi et al., 2015).

During mating sperm is delivered to the females, in which is maintained in two specific reproductive organs, that are the seminal receptacle and in the paired spermathecae (**Fig. 3**), until used for the egg fertilization in the uterus (Avanesyan et al 2017).

Figure 3. a) A whole spermatheca isolated from a *D. suzukii* female in which is visible the sclerotized capsule and the spermathecal duct. **b)** Crushed spermatheca with sperm mass (S. Puppato).

A female oviposited with a random distribution on fruits, producing clutches of single eggs (Mitsui et al., 2006) and her lifetime fecundity may reach up to 400 eggs (Winkler et al., 2020).

The high reproductive potential of *D. suzukii* is realized on a wide host plant range, including several cultivated species of stone fruits, particularly cherries, soft fruits, such as blueberries, strawberries, raspberries, blackberries, cranberries and some varieties of grape (Bellamy et al, 2013, Ioriatti et al., 2015a). Furthermore, a huge number of wild and ornamental non-crop hosts can be used by *D. suzukii* as breeding sites, covering the whole reproductive season (Lee et al., 2015, Poyet et al., 2015, Kenis et al., 2016). In addition to healthy fruits, *D. suzukii* is able to explore other fruit substrates (Bal et al., 2017).

In temperate regions, *D. suzukii* tolerates the diverse climatic condition exhibiting a seasonal phenotypic plasticity, modulated by temperatures and photoperiodism (Shearer et al., 2016).

Figure 4. Seasonal morphotypes of *D. suzukii*, a winter morph female (left) and a summer morph female (right) (A. Grassi)

A summer morphotype is present from late spring to autumn, whilst a larger and darker winter morphotype become evident from September onwards when the coldest season approaches, inducing physiological changes and preparing the insect for the overwintering period (Shearer et al., 2016, Grassi et al., 2018) (**Fig.4**). This phenotype is strategic to induce a diapause and reproductive dormancy, directing a large part of the metabolic processes towards an improvement of the resistance to abiotic stress (Stockton et al., 2019, Rendon et al., 2019a). The recovery following the cold season is a slow and critical period that begins during late winter and early spring, in which the insect exploits the few resources available for its nutrition, maturing its reproductive system in order to rise the new generations of summer morph (Briem et al., 2016, Tochen et al., 2016, Grassi et al., 2018, Panel et al., 2018)

In addition to the plasticity given by the polyphenism, the strong adaptation of *D. suzukii* to environmental changes is due to its high dispersion capacity which allows to avoid stressful abiotic stimuli that impact its survival, exploring as much as possible the resources available for expanding its populations (Mitsui et al., 2010a, Tait et al., 2018).

Current integrated pest management in Italy

Adults of *D. suzukii* are detected by using red traps (Droso-trap®) filled with 200-250 ml of liquid bait, composed of apple cider vinegar, wine and sugar (Droskidrink®, Grassi et al., 2015) (**Fig.5**).

Traps are usually hanged on plants at about 1.5 m from the ground level inside crop or at woodland border nearby crop. Traps are service once a week, inspecting under magnification the presence of the insect pest. Although fermenting attractants may be not very reliable to predict fruit infestation on crops, to date more effective lures are not available for *D. suzukii* management (Tonina et al., 2018a). For this reason, monitoring by traps requires to be coupled with fruit inspection to better establish the timing of insecticide application. By increasing the traps density along the crop hedge, a mass trapping strategy could be employed to limit population pressure on crop, even though as reported for the monitoring purpose the effectiveness may be reduced during the ripening period of crops due to the strong competition of fruits towards the food bait (Ioriatti et al., 2015b).

The chemical control of *D. suzukii* relies on the application of conventional insecticides belonging to the chemical class of organophosphate, pyrethroid, spinosyns, diamides and neonicotinoids, which mainly affect the adult stage, but some have shown to reduce egg hatching (Shawer et al., 2018). Among these spinosad is the only active ingredient admitted in organic crop production.

In growing seasons with high pest pressure, the management of *D. suzukii* requires several repeated applications of insecticide with notable repercussion on chemical residue on fruits, risk to facilitating the onset of resistance to pesticide and ecotoxicity to the environment.

Figure 5. Typical red trap used to monitor *D. suzukii* population dynamics. (S. Puppato)

It has been shown that the number of pesticide spraying can be significantly reduced by covering crops with insect-proof net with a mesh size less than 1 mm^2, obviously taking care to avoid flies' entry from the outside during the various agronomic practices (Grassi et al., 2012).

The physical barrier of the insect-proof netting should be activated after flower blooming, with the aim of ensure the ecosystem service of pollinators, but before fruit colouring (**Fig.6**).

Figure 6. Insect-proof nets in a cherry orchard. (A. Grassi)

Furthermore, canopy pruning, drip irrigation and mulching are useful cultural practices to maintain adverse environmental conditions for the survival and oviposition of *D. suzukii*, particularly reducing humidity and increasing temperature in crop system.

Rapid harvest schedule and sanitation by removing the infested fruits from the field further contribute to reducing damage on crop by *D. suzukii* (Ioriatti et al., 2020).

Future perspective: biological control and sterile insect technique

During past years faunistic surveys in Europe have found that few parasitoid wasps of Drosophila species were able to parasitize *D. suzukii*. Most of these parasitoids were ecto- and endoparasitoids that target the pupal stage and with a wide host range (Miller et al., 2015, Knoll et al., 2017).

Recent studies have clarified their effectiveness as potential biocontrol agent for controlling *D. suzukii.* Among these parasitoids, *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae) has been shown interesting life history traits. Field trials have confirmed that *T. drosophilae* may be used for augmentative releases against *D. suzukii* (Rossi Stacconi et al., 2019).

Since *T. drosophilae* is commercially available, growers may carry out several releases starting early in the season to limiting pest outbreaks during the ripening period of crop. Dose suggested is 0.3-0.5 parasitoids/m^2.

Parallel to the investigations conducted on indigenous parasitoids, field monitoring in East-Asia has revealed a complex assemblage of larval parasitoid on *D. suzukii* (Daane et al., 2016, Girod et al., 2018a, Giorgini et al., 2018), where *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae), *Leptopilina japonica* Novkovic & Kimura and *G. brasiliensis* Ihering (Hymenoptera: Figitidae) were the most abundant species attacking *D. suzukii*.

G. brasiliensis, lineage-G1 (Nomano et al., 2017), has proved to be the most specialist parasitoid towards *D. suzukii*, potentially having no detrimental effects on non-target insect species, thus resulting a potential candidate for classical biological control (Girod et al., 2018b, Biondi et al., 2021, Daane, 2021).

In Italy, a detailed benefits and risks assessment dossier has been submitted to institutional bodies with aim to obtain the issue permit for the environmental release of *G. brasiliensis* starting from 2021.

Sterile Insect Technique (SIT) is a promising approach to maintain pest population below an economic threshold, or even leading to local eradication in some environmental setting. Moreover, involving pest control by means of reproductive aspects is highly species-specific and can be combined with biological control methods.

SIT relies on mass rearing of the target pest species, sterilization of males using ionizing radiation, followed by repetitive overflooding releases of sterile males on large scale. Once released, sterile males search and mate with wild females, leading to unviable embryo production and lowering offspring population (Nikolouli et al., 2017). Concerning *D. suzukii*, irradiation parameters assessment and mass rearing protocols are still ongoing, but first outcomes seems very encouraging (Lanouette et al., 2017, Krüger et al., 2018, 2019, Sassù et al., 2019a,b).

The field success of this technique depends on several aspects of the biology and sexual behaviour of insect pest and despite the remarkable value that this technique may offer to pest suppression, several knowledge gaps on reproductive strategies of *D. suzukii* must be clarified, in order to improve pest management programs and do not make research effort ineffective.

An area-wide implementation of cultural practices, combined with biological control strategies integrating indigenous and exotic parasitoids with SIT releases, is expected to contribute significantly to lowering pesticide input in crops management with positive long-term effect on agroecosystems.

AIMS OF THE RESEARCH

Drosophila suzukii, originally from East Asia, is the major invasive pest of stone and soft fruit crops in Trentino. The economic losses due to this alien species pose a serious risk to the fruit production and farm sustainability. To date, repeated agrochemicals spraying remains the first line of defense against *D. suzukii*; therefore any side effects on environment and human health must be considered and effective alternative solutions need to be also implemented.

Population size of this pest has shown a continuous increase over the years since its arrival, indicating that locally applied control practices were poorly successful to limit the area-wide dynamics of this highly dispersive species. Consequently, new perspectives for its management on large-scale appear to be urgent. Recent studies have highlighted interesting themes that need to be explored for a potential field implementation and beside the already integrated pest management strategies, agroecological principles may be useful to outline a new approach that can guide future practices.

My PhD project addressed three main topics:

- **Biological control:** by means of fruits sampling and monitoring tools I realized a faunistic surveys with the aim of evaluate the parasitoids assemblage associated with *D. suzukii* in Trento province ten years after the first report of this pest in this area. Surveillance was carried out in different habitat types and on a wide range of host plants known to support *D. suzukii* with special regard to the main hosts following the overwintering period.

  Furthermore, the ecology and spatiotemporal dynamics of plant-host-parasitoid interaction was further evaluated.
- **Sterile insect technique:** by means of field sampling and microsatellites markers analysis I carried out an assessment of the reproductive behaviour of wild-caught *D. suzukii* females. Parentage analysis was focused on the evaluation of the polyandry extent and paternity skew in an Italian population during early summer, with the aim of clarifying this fundamental aspect of the mating system of *D. suzukii,* which may play a key role for a successful application of the sterile insect technique for controlling this invasive pest.
- **Habitat manipulation:** by means of field trials I tested several agroecological techniques with potential top-down and bottom-up effects on *D. suzukii*. The aims of these research were the development of sustainable practices that could be easily implemented by farmers at local scale to reduce pest infestation on soft-fruit crops, by enhancing the ecosystem services provided by intercropping, trap plants and natural enemies.

CHAPTER 1

First Report of *Leptopilina japonica* in Europe

Publishing: Puppato, S.; Grassi, A.; Pedrazzoli, F.; De Cristofaro, A.; Ioriatti, C. First Report of *Leptopilina japonica* in Europe. *Insects* **2020**, *11*, 611. https://doi.org/10.3390/insects11090611

Abstract

Drosophila suzukii (Matsumura; Diptera: Drosophilidae) is a key pest of sweet cherry and small fruits worldwide. Biological control remains unutilized in the framework of *D. suzukii* management. Nonetheless, natural enemies may play an important role in regulating this pest. We report for the first time the presence of *Leptopilina japonica* Novković and Kimura (Hymenoptera: Figitidae) in Europe. Two specimens emerged from ripened fruits and one was collected after direct observation on a cherry tree in June 2019. They showed the distinctive morphological traits already described and shared more than 99% sequence similarity with specimens of *L. japonica* collected in Asia. This first finding was confirmed by a wider survey carried out in 2020; *L. japonica* emerged from cherry fruit samples collected in five other sites across the Trentino region, suggesting that L. japonica has already colonized a wide area. Detection of this Asian species is relevant to the future direction in managing *D. suzukii*, both in Europe and North America. In fact, *L. japonica* showed similarity with *Ganaspis brasiliensis* (Ihering) (Hymenoptera: Figitidae), the most promising candidate for the classical biological control, in terms of developmental time, egg maturation, host age preference and lifetime fecundity.

Keywords: Figitidae; biological control; larval parasitoid; cherry; *Drosophila suzukii*

Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), also known as the spotted wing drosophila (SWD), belongs to the ever-expanding group of invasive alien species (IAS) (Westphal et al., 2008). Native to East Asia (Kanzawa, 1939), *D. suzukii* has rapidly spread both in the Americas and in Europe, where it has become a serious pest of stone and soft fruit crops, causing huge annual crop losses (Asplen et al., 2015).

In Italy, it was recorded for the first time on raspberries in the Trentino region in 2009 (Grassi et al., 2009), and it became an economically important pest on several species of cultivated berries of the region, generating up to €3 million in losses in 2011 (De Ros et al., 2012). Economic costs of *D. suzukii* include crop loss in the field, increased labor, chemical input costs for monitoring and management, cost of the secondary selection of fruits in the storage facilities after harvest, as well as losses due to the shorter shelf life of fruit containing eggs of *D. suzukii* (Lee et al., 2011a). The peculiar evolution of a serrated ovipositor (Atallah et al., 2014), as well as of specific behavioral traits (Ometto et al., 2013, Keesey et al., 2015, 2019, Ramasamy et al., 2016, Karageorgi et al., 2017, Keesey et al., 2019), allowed *D. suzukii* to exploit healthy ripening fruits as substrates for egg-laying, creating a new ecological niche, thus reducing interspecific competition with other *Drosophila* species (Markow et al., 2006, Dancau et al., 2017, Shaw et al., 2018). Polyphagia towards a broad range of cultivated and wild hosts (Lee et al., 2015, Poyet et al., 2015, Kenis et al., 2016), in addition to high reproductive potential (Emiljanowicz et al., 2014, Tochen et al., 2014), has increased its invasiveness in the colonized regions. Finally, the lack of an effective suppression action operated by natural enemies in the newly invaded areas makes possible a rapid building up of the pest population since the beginning of the season. Current control methods rely on integrated pest management (IPM) strategies, among which exclusion netting and chemical insecticides remain the first lines of defense (Haye et al., 2016, Ioriatti et al., 2020). However, reiterated applications of pesticides due to *D. suzukii* lifecycle and behavior (Tait et al., 2018, 2019, Tonina et al., 2018b) may bring some negative side effects (Desneux et al., 2007, Shaw et al., 2019, Van Timmeren et al., 2019).

Generalist predators, pathogens and two cosmopolitan pupal parasitoids, *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae) and *Pachycrepoideus vindemmiae* Rondani (Hymenoptera: Pteromalidae), proved able to use *D. suzukii* as prey/host (Lee et al., 2019). However, their generalist behavior raised several doubts about large-scale effectiveness (Miller et al., 2015), unless conservation practices (Blaaw et al., 2015, Haro-Barchin et al., 2018) and optimal releasing time (Rossi Stacconi et al., 2019) are integrated with advanced control strategies still under development (Kruitwagen et al., 2018, Woltering et al., 2019).

Host–parasitoid interaction is a complex phenomenon of co-evolution influenced by multiple ecological and genetic factors, which have become key elements in biological control process (Vet et al., 1992, Godfray et al., 1994, Prévost et al., 2009). Elegant studies have investigated the biological basis of the immune response of *D. suzukii*, clarifying the physiological and molecular mechanisms determining failure (Chabert et al., 2012, Kacsoh et al., 2012, Poyet et al., 2013, Iacovone et al., 2018) or success (Poyet et al., 2013, Tetsuo et al., 2015, Poirié et al., 2018), in the development of parasitoid wasps. Field studies in the newly invaded areas by *D. suzukii* completed the picture (Miller et al., 2015, Gabarra et al, 2014, Mazzetto et al., 2016, Knoll et al., 2017).

An alternative approach might be classical biological control (CBC), defined as the authority regulated introduction of a biological control agent of exotic origin aiming at permanent control of the invasive pest. In past years, faunistic surveys in East Asia revealed an assemblage of parasitoid wasps able to efficiently parasitize and develop on *D. suzukii* (Mitsui et al., 2007, Mitsui et al., 2010a, Kimura et al., 2012, Daane et al., 2016, Girod et al., 2018a, Giorgini et al., 2019). Among these, larval parasitoids *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae), *Leptopilina japonica* Novković and Kimura and *Ganaspis brasiliensis* (Ihering) (both Hymenoptera: Figitidae) were the most abundant species attacking *D. suzukii*. Therefore, in-depth studies have been carried out in quarantine facilities in order to establish the most promising candidate for CBC. Special attention has been given to the risk assessment towards non-target species, native to the ecosystem where the foreign candidate will be released (Van Lenteren et al., 2006, Hajek et al., 2016, Girod et al., 2018b,c). Results identified *G. brasiliensis* lineage G1 (Kasuya et al., 2013, Nomano et al., 2017) as the most specialized parasitoid and a petition to release it for the biological control of *D. suzukii* in the United States was submitted to the United States Department of Agriculture (USDA) in 2019. However, as is the case for alien crop pests, their natural enemies might also be unintentionally introduced in new areas where they presumably were not present. Parasitoid wasps of the invasive *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) are well known examples (Talamas et al., 2015). Recently, an exotic parasitoid of *D. suzukii* was also found in Mexico (Gonzalez-Cabrera et al., 2020).

Here, we report the presence of the Asian larval parasitoid, L. japonica, in the Trentino region. This parasitoid shows local adaptation to some sympatric *Drosophila* species and has been demonstrated to be highly virulent to those considered as its major hosts, included *D. suzukii* (Kimura et al., 2015). On the other hand, due to its ecological fitting, it can form novel associations with some allopatric *Drosophila* species in novel environments that can have unpredictable consequences on the biological control of *Drosophila* populations.

Materials and methods

Specimens of presumptive parasitoids of *D. suzukii* were collected from April to July 2019 during a field survey in the Adige Valley (Trento, Italy), at 186 m a.s.l., with the aim of investigating the parasitoids' complex of the pest. Several sampling points (SPs) (**Table 1**), near isolated cherry trees, were set across an agricultural area of around 20 hectares, where apple orchards and vineyards represent the main crops.

Table 1. Sampling points and habitat location.

Sampling point (SP).	Habitat type description
SP1	*Prunus avium* nearby vegetable garden
SP2	Vineyard
SP3	Backyard cherry tree
SP4	Apple orchard
SP5	*Prunus avium* in a lawn surrounded by apple orchards
SP6	Domestic compost nearby henhouse

Surveys were carried out weekly by means of four different sampling methods as follows.

(1) Fresh ripening fruits: a total of 50 fruits were collected from isolated cherry trees found in each SP and were checked under stereomicroscope for *D. suzukii* eggs. Infestation level was expressed as percentage of infested fruits on total fruits inspected. Fruits were considered infested when carrying at least one egg. Infested fruits were placed in plastic boxes equipped with a fine mesh netted lid for ventilation and sand and paper towel as substrates to dry up the juice produced.

(2) Fallen fruits: a total of 30 dropped cherries were sampled from the ground of each identified isolated cherry tree. Fruits were placed onto a plant pot saucer containing a layer of sand and left exposed for seven more days under the tree. After this period, new samples of dropped cherries were collected to replace the old ones, which were brought to the laboratory to recover pupae by sifting the sand and carefully dissecting the fruits. Eventually, each pupa was identified as *D. suzukii* or other drosophilid species based on morphological characteristics and singly incubated in plastic vials.

(3) Sentinel traps: each trap consisted of a small plastic bowl baited with a fresh banana slice infested by thirty third-instar larvae of *D. suzukii*. Each bowl was placed inside a white delta trap and hung at around 30 cm from ground level at each SP.

All samples were kept in plastic boxes at room temperature (22–25 ◦C) in the laboratory and they were daily inspected. Emerged specimens of *Drosophila* spp. and parasitoids were placed in 99% ethyl alcohol for further identification.

(4) Direct visual: observations on the foliage layer were conducted at around 1.5–2.0 m from ground level. Putative parasitoids were collected with an insect aspirator.

In each SP, the population dynamic of *D. suzukii* was monitored by means of a Drosotrap (Biobest, Westerlo, Belgium) filled with 200 mL liquid bait DroskiDrink (75% apple cider vinegar, 25% red wine) mixed with a teaspoon of raw brown sugar and 100 µl Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Traps were hung at 1.2–1.5 m from ground level. Trap bait was weekly renewed and content was inspected under stereomicroscope for *D. suzukii* individual counting.

Temperatures were recorded by means of a data logger placed at the center of the sampling area.

Identification of parasitoid wasps was carried out combining morphological and molecular analysis, in order to achieve robust and informative data. Regarding the first approach, Figitidae species were determined using diagnostic morphological features and identification keys (Nordlander et al., 1980, Schilthuizen et al., 1998, Allemand et al., 2002, Forshage et al., 2008, Novković et al., 2011, Buffington et al., 2016, Lue et al., 2016).

Leptopilina japonica (**Figure 1A**) can be identified from other common *Leptopilina* species known to occur in Europe by the combination of the following diagnostic characteristics reported in Novkovic et al. (2011): female antennal segments, scutellar plate, posterior pit, metapleural ridges and metasoma with the hairy ring (**Figure 1B**). In particular, due to the high relatedness of *L. victoriae* and *L. japonica*, antennal segments of *L. japonica* are darker, 5th and 6th antennal segments more slender and longer compared to *L. victoriae* (**Figure 1C**). Scutellar plate is wider and posterior pit larger than in *L. victoriae*. (**Figure 1D**).

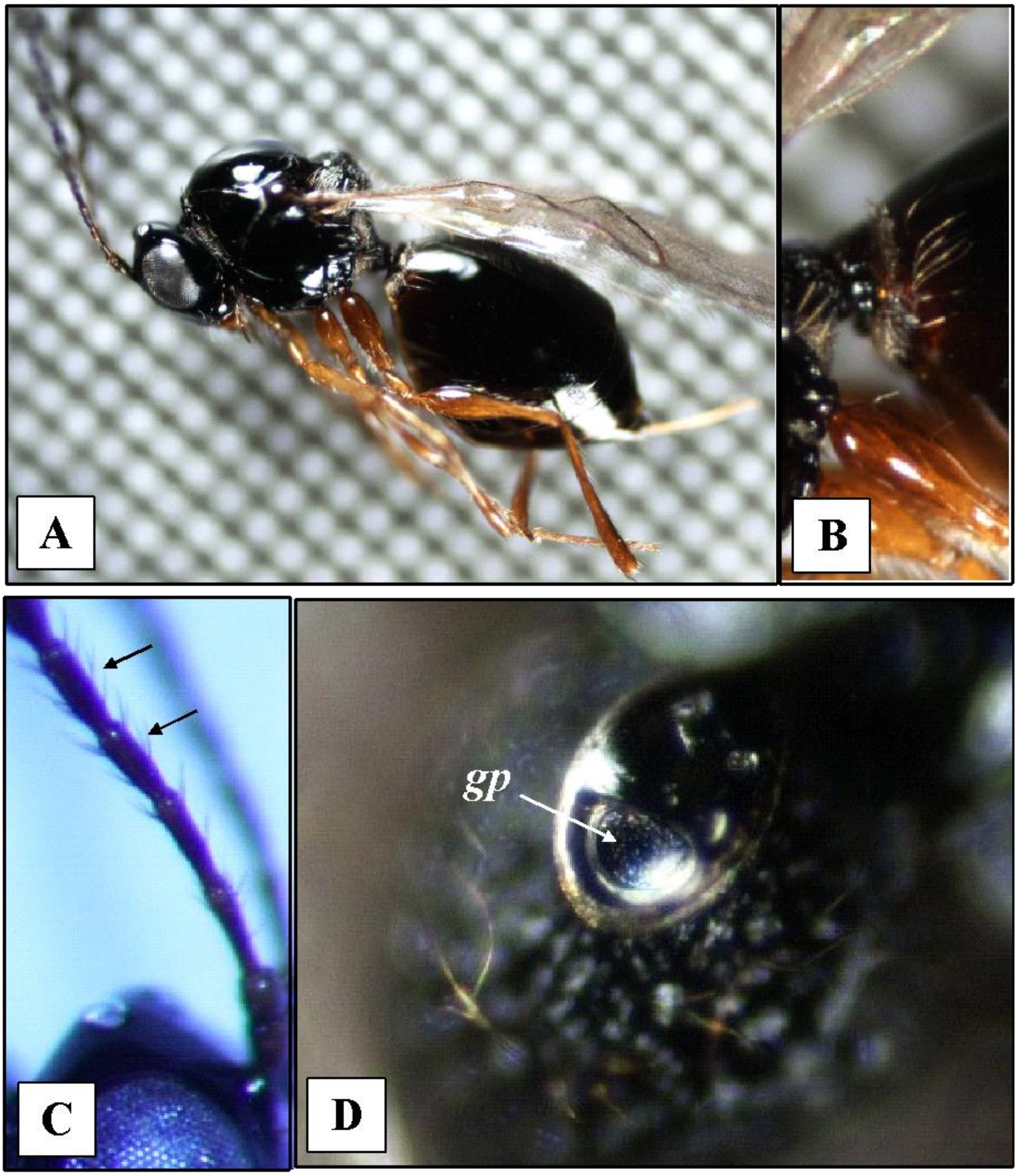


Figure 1. *Leptopilina japonica* female from Trento, Italy: (A) Adult habitus, laterally, (B) Petiole and metasomal hairy ring, lateral view, (C) Female 5th and 6th antennal segments (black arrows) at higher magnification, lateral view (D) Scutellar plate, gp: glandular pit.

For the molecular analysis, the total DNA was isolated from each specimen using a commercial kit (NucleoSpin® Tissue; Macherey-Nagel, Düren, Germany). Amplifications were performed with two primer pairs, amplifying the cytochrome oxidase subunit I (COI) gene (LCO1490: 5’-GGTCAACAAATCATAAAGATATTGG-3’; HCO2198: 5’-

TAAACTTCAGGGTGACCAAAAAATCA-3’) and the internal transcribed spacer 1 (ITS1) (rDNA2: 5’-TTGATTACGTCCCTGCCCTTT-3’; rDNA1.58s: 5’-ACGAGCCGAGTGATCCACCG-3’). Polymerase chain reaction (PCR) conditions were as reported in PM 7/76 (EPPO. PM 7/129 (1), 2016) for COI and in Taylor et al. (2006) for ITS1. PCR products, after purification with illustra ExoProStar1-Step (GE Healthcare, Little Chalfont, UK), were sequenced with the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an 3130 xl Genetic Analyzer (Applied Biosystems; Carlsbad, CA, USA). A Basic Local Alignment Search Tool (BLAST) comparison of the sequences obtained was performed using the NCBI database to confirm the morphological classification.

In 2020, the survey for parasitoids was extended to five additional sites in the Trentino region.

Based on the outcome of 2019, the sampling method was restricted to fresh ripening cherry fruits.

A total of 1750 fruits (50 fruits/site × week) were sampled during the weekly survey between May and June (7 weeks) and checked in the laboratory following the procedure of the previous year.

Results

During the field surveys, a total number of 144 adult parasitoids of different species were collected (**Table 2**): 53 *P. vindemmiae*, 64 *T. drosophilae* and one *Spalangia* spp. emerged from the sentinel traps, 18 *P. vindemmiae*, 3 *T. drosophilae* and 2 *Leptopilina boulardi* (Barbotin, Carton and Kelner-Pillault) emerged from fallen fruits.

Three voucher specimens, one female and two males of Figitidae, were recovered from ripened fruits (two) and by direct observation (one) on a cherry tree (SP 5) in June 2019. All of them showed the distinctive morphological traits already described by Novković et al. (2011) for *L. japonica* (**Figure 1**) and shared more than 99% sequence similarity with specimens of *L. japonica* collected in Asia (2011).

The BLAST search on the NCBI database showed the high sequence similarity of our COI barcode sequence with *Leptopilina* sp. JP COI pseudogene (accession number: AB546877-78). The ITS1 barcode sequence shared the highest similarity score with *Leptopilina* sp. JP gene for internal transcribed spacer 1 (accession numbers: AB546881 and AB583629-31). All specimens showed identical sequences for both molecular markers and a representative sequence was submitted to GenBank (MT840347 and MT840665, respectively). The cherry trees where the specimens were collected were located in the meadow of a farm surrounded by apple orchards (46.006079, 11.120367) (**Figure 2**).

Table 2. Total number of parasitoids collected at the six sampling points during the field survey.

Sampling week	**Total parasitoids**	**Parasitoid species**	**Host (parasitoids number)**	**Sampling method**	**SP (parasitoids number)**
17/18	4	*P. vindemmiae*	*D. suzukii* (4)	Sentinel trap	SP2(3), SP5(1)
18/19	3	*P. vindemmiae*	*D. suzukii* (3)	Sentinel trap	SP2(2), SP5(1)
21/22	18	*P. vindemmiae*	*D. suzukii* (10)	Sentinel trap	SP6(10)
		T. drosophilae	*D. suzukii* (8)	Sentinel trap	SP4(7), SP6(1)
22/23	30	*P. vindemmiae*	*D. suzukii* (4)	Sentinel trap	SP4(1), SP6(3)
		T. drosophilae	*D. suzukii* (25)	Sentinel trap	SP4(10), SP5(15)
		Spalangia spp.	*D. suzukii* (1)	Sentinel trap	SP6(1)
23/24	10	*P. vindemmiae*	*D. suzukii* (1)	Sentinel trap	SP6(1)
		T. drosophilae	*D. suzukii* (9)	Sentinel trap	SP4(9)
24/25	28	*P. vindemmiae*	*D. suzukii* (21)	Sentinel trap	SP3(13), SP4(1), SP5(7)
		T. drosophilae	*D. suzukii* (7)	Sentinel trap	SP4(5), SP5(2)
25/26	16	*P. vindemmiae*	*D. suzukii* (1)	Sentinel trap	SP2 (1)
		T. drosophilae	*D. suzukii* (15)	Sentinel trap	SP2 (15)
26/27	9	*P. vindemmiae*	*D. suzukii* (9)	Sentinel trap	SP3(4), SP4(1), SP5(4)
23	2	***L. japonica***	*D. suzukii* (2)	Ripen cherries	SP5(2)
25/26	7	*P. vindemmiae*	Other drosophilids (4)	Fallen cherries	SP5(4)
		T. drosophilae	*D. suzukii* (2), Other drosophilids (1)	Fallen cherries	SP3(3)
26/27	10	*L. boulardi*	Other drosophilids (2)	Fallen cherries	SP1(2)
		P. vindemmiae	*D. suzukii* (2), Other drosophilids (6)	Fallen cherries	SP1(5), SP3(3)
27/28	4	*P. vindemmiae*	Other drosophilids (4)	Fallen cherries	SP1(3), SP3(1)
28/29	2	*P. vindemmiae*	Other drosophilids (2)	Fallen cherries	SP3(2)
23	1	***L. japonica***	/	Direct observation	SP5(1)

At the time of *L. japonica* findings, the infestation level was around 20% and the mean temperature was around 22 °C (**Figure 3**). Results of the 2020 survey (**Table 3**) confirmed that *L. japonica* is widely established in the region in sites up to around 20 km apart and from 211 to 685 m altitude. A total of 131 parasitoids emerged by the sampled cherry fruits; all of them belonged to the species *Leptopilina japonica*.

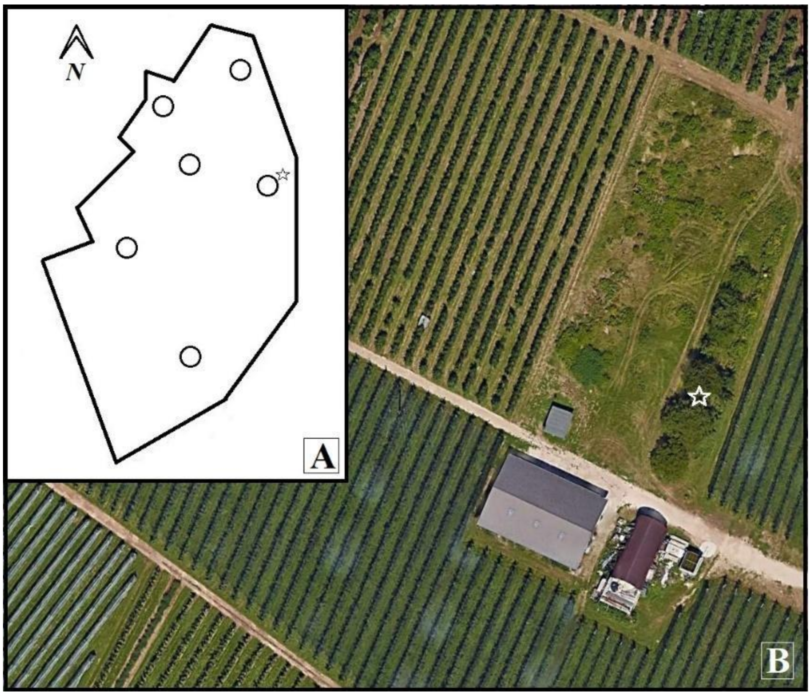


Figure 2. (**A**) Survey area map with sampling points (circles). (**B**) Recovery site of *L. japonica* (star).

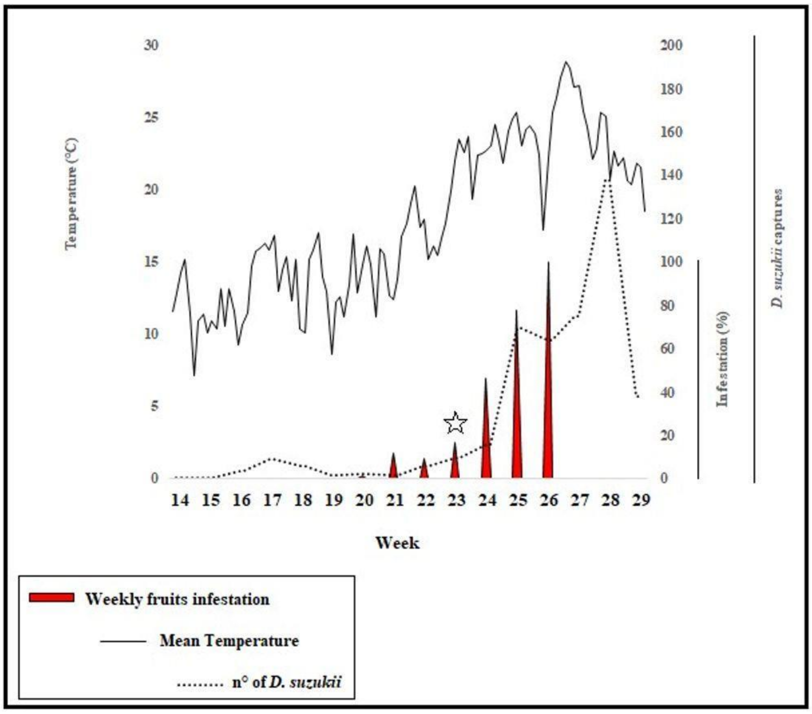


Figure 3. Summary graph of temperature, weekly fruit infestation (% of infested fruits) and *D. suzukii* captures during field survey (flies/trap). The star indicates the week of the *L. japonica* find.

Table 3. Sites and dates of the cherry fruit samplings from which *L. japonica* emerged in 2020 survey.

Sampling Site	Sampling Date	Altitude (m)	Host Fruit	Number of *Leptopilina japonica* Emerged
46.18172, 11.12434	18 May 2020	211	*Prunus avium*	31
46.04736, 11.11347	19 May 2020	200	*Prunus avium*	58
46.09678, 11.11846	25 June 2020	195	*Prunus avium*	10
46.04604, 11.22987	2 July 2020	476	*Prunus avium*	23
46.13564, 11.14373	2 July 2020	685	*Prunus avium*	9

Discussion

Leptopilina japonica is a solitary koinobiont endoparasitoid, consisting of two subspecies, *L. japonica japonica* Novković and Kimura present in Northern and Central Japan and *L. japonica formosana* Novković and Kimura occurring in Taiwan (Novković et al., 2011). These subspecies seem to have distinct characteristics in terms of behavioral ecology (Kimura et al., 2015, Novković et al., 2011, Murata et al., 2013). Based on morphological and molecular identification, our specimens could be associated with *L. j. japonica* subspecies. The similarity of geoclimatic factors between the native range and the Trentino region may support this speculation.

L. japonica was frequently found to parasitize larvae of *D. suzukii* in naturally infested fruits (Novković et al., 2011, Daane et al., 2016, Matsuura et al., 2018, Giorgini et al., 2019). Field surveys in its native areas also reported that this larval parasitoid attacked *D. suzukii* on different wild and commercial fruits, such as *Prunus* spp., *Myrica rubra*, *Solanum nigrum*, *Rubus* spp., *Lonicera maacki*, *Vaccinium* spp., *Fragaria moupinensis* and *Sambucus adnate* (Daane et al., 2016, Girod et al., 2018a, Giorgini et al., 2019). Specimens of *L. japonica* here reported were collected from an unmanaged cherry tree (*Prunus avium*) with 20% of infested hanging fruits. It is noteworthy that this larval parasitoid is foraging at a low infestation level, when the first generations of *D. suzukii* occur on early fruiting non-crop plants.

Furthermore, the field surveys were carried out in a habitat diversity that ranged from inland forest to hedgerow and wild vegetation surrounding cultivations. These ecological features are key aspects for the success of CBC, especially when the target pest exploits a wide range of plant hosts over the season (Mitsui et al., 2010a, Lee et al., 2015, Poyet et al., 2015, Kenis et al., 2016). Understanding habitat and resources preference will improve the experimental design in biological control programs (Tscharntke et al., 2016).

Concerning reproductive biology studies, *L. japonica* showed similarity with *G. brasiliensis* in terms of developmental time, egg maturation, host age preference and lifetime fecundity, while it developed faster than *G. brasiliensis* for both males and females (Wang et al., 2018). Thermal performance of *L. japonica* revealed successful development at constant temperature from 17.2 to 27.3 °C (Hougardy et al., 2019). This thermal range resembles the typical average temperatures occurring in the valley bottom of the Trentino region from the second half of May to September.

Field surveys and pre-release non-target testing with regard to host specificity revealed how *L. japonica* exhibits a wider host range compared to *G. brasiliensis*, leading to exclusion of the former as a candidate agent for CBC (Daane et al., 2016, Girod et al., 2018c). Even though a particular emphasis is now placed on host specificity assessments to avoid the introduction of generalist natural enemies (Kenis et al., 2017), laboratory experiments may be limited in their predictive ability (Jennings et al., 2017), due to the confined experimental set-up (McFadyen et al., 2002) or because of the existence of strains with a narrow/wide host range according to the area of origin (Girod et al., 2018b,c). As for other animal species, parasitoid wasps need to cope with the ecological context in which they are found (Kimura et al., 2015a,b, Matsuura et al., 2018), which provides alternative resources to sustain natural enemies during the season, facilitating biocontrol of target pests (Gillespie et al., 2016). In this sense, if the wider host range of *L. japonica* is confirmed in the open field, it will out-compete the other Asian parasitoids. On the other hand, also an additive effect between *L. japonica* and *G. brasiliensis* (Wang et al., 2019), which would become an essential aspect to be deepened for controlling *D. suzukii*, has been shown.

Moreover, interplay among exotic and indigenous parasitoids need further attention, particularly *T. drosophilae*, being the current candidate for augmentative biocontrol. If no detrimental effects are observed, one might assume an integration of CBC in the future performed with *G. brasiliensis*, with conservative biological control of both resident parasitoids and the newly arrived species for boosting the suppression of *D. suzukii*.

Beyond the abovementioned information, future investigations have to be addressed in order to verify whether *L. japonica* is in its early phases of establishment in Trentino or whether it has already expanded in the Trentino region and even outside this area. A survey is still ongoing in 2020 with the aim to evaluate the spreading of this Asian parasitoid: the first results reveal that *L. japonica* has emerged from cherry fruit samples collected in five other sites across the Province of Trento, suggesting the existence of an adventive population of the parasitoid. This new ecological situation offers the unique opportunity to acquire new insights for future biological control tactics against *D. suzukii*.

Conclusions

Globalization of human trade and travel, along with climate change, are important drivers for the redistribution of the insect species around the world, leading in many cases to new threats for agricultural crops worldwide. There is a growing interest in understanding the pathway by which alien species are introduced into novel environments, but the assessment is difficult due to the lack of comprehensive information.

Similarly, alien arthropod predators and parasites, the majority of which arrived unintentionally through natural extra-range dispersal or as contaminants and stowaways (Wilson et al., 2009), are particularly interesting because of their generally positive impact in controlling crop pests of economic importance. Infrequent are the cases that had negative impacts for their interference with other beneficial insects (Roy et al., 2011).

Even though L. japonica was excluded as a possible candidate for the classical biological control programs due to its potentially wider host range, it remains among the most widespread and effective parasitoids of *D. suzukii* in its native range.

Reproductive biological traits and temperature requirements make possible its rapid dispersal and durable establishment in the Alpine region, with an expected positive outcome on biological control of the spotted wing drosophila.

The coming years will be crucial in order to understand the population dynamics that will evolve between this parasitoid's assemblage and the invasive species in the new ecological setting.

CHAPTER 2

Ecology of the spatio-temporal association of *L. japonica* and *D. suzukii* on a wide-range of plant species in Trentino province

Abstract

Leptopilina japonica Novković&Kimura (Hymenoptera: Figitidae) is a larval endoparasitoid native of East Asia, as well as one of the main and most effective natural enemies of *Drosophila suzukii*, the invasive pest of stone and soft fruit crops.

In 2019, it has been found to parasitize larvae of *D. suzukii* on *Prunus avium* in Trento province (Italy), providing the first report of this parasitoid in Europe.

D. suzukii is known to be a highly polyphagous species, breeding on a wide range of host plants.

For this reason, we carried out an extensive field survey in order to establish the capacity of *L. japonica* of searching and finding its host on several plant families.

During our survey we observed a temporal asynchrony in host-parasitoid interaction, with *L. japonica* parasitism begun about two weeks after first infestation on *P. avium* by *D. suzukii*. On this plant species parasitism rate ranging 1.3-46.2%.

Seasonal long sampling reveals the spread host-parasitoid association throughout non-crop plants collected. On the ubiquitous *Sambucus nigra* and *Rubus ulmifolius* parasitism rate was variable, ranging between 0-25% and 0.6-40%, respectively.

Moreover, this collection has also shown the ability of *L. japonica* to foraging for host on several fruiting species belonging to Moraceae, Rosaceae, Glossulariaceae, Rhamnaceae, Cornaceae, Ericaceae, Phytolaccaceae, Dioscoreaceae, Adoxaceae and Solanaceae.

These results pose the stepping stones for the future evaluation of biocontrol activity on *D. suzukii* by this unintentionally introduced asian parasitoid.

Introduction

Invasive pests, such as *D. suzukii*, can rapidly expand their population size in the colonized areas mainly due to the lack of an effective biological control, according to the “enemy release hypothesis”, that states how the detachment from coevolved natural enemies offers a fitness advantage to the invader in the introduced areas (Wolfe et al., 2002), for this reason classical biological control (CBC) is a common way to cope with the increasing arrivals of invasive species.

Concerning CBC of *D. suzukii*, host specificity and life-history traits of several Asian larval parasitods have been investigated during past years, founding in a specific lineage of *Ganaspis brasiliensis* Ihering (Hymenoptera: Figitidae) the most promising candidate for the biocontrol of the pest in the invaded areas (Nomano et al., 2017, Wang et al., 2018, 2020, Biondi et al., 2021, Daane et al., 2021).

During 2019, North American/Canadian and European research groups concurrently reported the finding of Asian parasitoids of *D. suzukii* outside their native range, likely unintentionally introduced through global trade pathways, as happened in the past for *D. suzukii* (Abram et al., 2020, Puppato et al., 2020).

In Trento province, an adventive population of *Leptopilina japonica* Novkovic & Kimura (Hymenoptera: Figitidae) was probably established in the territory, even though it was unknown the extent of its dispersal and what kind of ecological dynamics were set in the new environment.

D. suzukii invasiveness relies on its capacity of using a wide range of host plants as breeding sites (Poyet et al., 2015), therefore an effective biological control agent should be able to face the pest on most of the fruiting species available in the agroecosystem.

Here, we reported the results of an extensive monitoring on non-crop and crop plant species carried out in order to deepen our knowledge on the spread of *L. japonica* in the new environment with particular attention to the spatio-temporal relationship with *D. suzukii* and their host plant. Host preference of the *L. japonica* in relation to the availability of other drosophilae species along the season was also investigated and consequences on the efficacy of biological control discussed.

Material and methods

Study sites and fruit sampling

This survey aimed to investigate the spatiotemporal dynamics between invasive pest and the adventive population of the Asian larval parasitoid *L. japonica* in Trentino province.

For the above reason, fruit sampling on spontaneous hosts known to be associated with *D. suzukii* has begun in late spring and was extended throughout the breeding season of these species, approximatively from second half of May to early October 2021.

We carried out an extensive sampling on three ubiquitous non-crop host plants, which are the wild cherry (*Prunus avium*, Rosaceae), the European elderberry (*Sambucus nigra*, Adoxaceae) and the wild blackberry (*Rubus ulmifolius,* Rosaceae), known to have a key role on the seasonal build-up of the pest populations.

Furthermore, parallel to *P. avium, S. nigra* and *R. ulmifolius* sampling, we performed additional fruit collection from cultivated and spontaneous plants to investigated the relationship between *L. japonica* and *D. suzukii*.

Early season non-crop host plants sampling

We started sampling ripe fruits of wild cherry trees (*P. avium)* at the end of May, at fruit colouring (BBCH-phenological scale=81), when cherries become susceptible to *D. suzukii* infestation. Samples were collected during three consecutive weeks, until the second half of July (BBCH=89), before fruits became overripe. Fruits were sampled from isolated cherry trees in the two valleys, Adige valley and Valsugana, along an elevation range between 200 m (lowland) up to 700 m.a.s.l. (top level of the hilly zone)

Sampling points were chosen regards to surrounding habitats, in order to evaluate the environmental context in the pest-parasitoids interactions. Habitat types ranging from agricultural areas (apple orchards, vineyards, soft-fruit crops or mixed systems), urban green parks, riparian zone, woodland edges and wetlands.

50-100 fruits from the foliage layer, about 1-3 m above the ground were collected over the whole canopy on each cardinal direction.

Fruit sampling were carried out every 7 days across all sites.

Mid-late season non-crop host plants sampling

Sambucus nigra fruits sampling started in mid-July and ended in mid-September, whilst fruits from *Rubus ulmifolius* were collected beginning in mid-August until October (**Fig.1a**). Overall, fruits sampling was carried out for about two months covering the entire ripening period of these species. Due to the specific growing conditions and rarefaction in highly managed areas, fruits sampling was performed in public parks, riverbanks and semi-natural areas surrounding soft-fruit production in the Adige valley and Valsugana, between 200 m and 800 m.a.s.l.

The different height and shape of elderberry trees and blackberry bushes required a slight adjustment on sampling methods. We collected approximately 10 fruit clusters from elderberry from the bottom up to 2-3 m above the ground, covering the entire vegetation structure. A fruit cluster was sampled if it has at least 50% of ripe berries.

Regards to blackberry, we randomly collected 30-40 ripe berries along the entire linear arrangement of the bushes.

Fruit sampling were carried out every 7 days across all sites.

Collateral non-crop host plants sampling

Due to the challenge of performing standardized sampling and laboratory processing with the three main host species, this collateral sampling was only done to obtain qualitative information on host-parasitoid interaction.

Fruits were collected on the same areas in which the standardized sampling were carried out.

Number of collected fruits were variable due to fruit size and availability at the time of sampling.

The sampled plant species were:

Morus alba L., *Ficus carica* L. (Moraceae), *Fragaria x ananassa, Prunus mahaleb* L., *Rubus idaeus* L., *Rubus fruticosus* L., *Rubus caesius* L (Rosaceae), *Ribes rubrum* L. (Glossulariaceae), *Frangula alnus* L. (Rhamnaceae), *Cornus mas* L., *Cornus sanguinea* L. (Cornaceae), *Vaccinum corymbosum* L. (Ericaceae), *Phytolocca americana* L. (Phytolaccaceae), *Dioscorea (Tamus) communis* L. (Dioscoreaceae), *Viburnum opulus* L. (Adoxaceae), *Solanum nigrum* L. (Solanaceae).

Samples processing, insect rearing and collection

The sampled fruits were placed in plastic bags, identified by means of a reference code and brought to the laboratory.

Fruits were inspected under magnification, searching for typical white egg’s breathing spiracles protruding out of the oviposition hole on the fruit's surface.

Cherries, blackberries and the fruits from the collateral sampling were fully inspected, while for elderberry we checked 20-25 berries for each fruit cluster (200-250 total berries per sample).

Infested fruits were placed inside a modified plastic container (21 x 21 x 8 cm; length, width and height, respectively). The top of the lid was removed and replaced with a 15x15 cm square piece of Nylon netting (150x150 mesh, 160 µm aperture, BugDorm Ltd., Taiwan), for ventilation. Fruits were suspended on a metal grid that kept them raised from the bottom where a thin layer of sand and a sheet of absorbent paper was placed (**Fig.1b**).

Plastic containers were placed on shelves in room equipped with ventilation fan in which temperature, was kept between 15 and 25°C during entire sampling period and monitored by datalogger (**Fig.1c**).

Every 2-3 days each sample was checked for *Drosophila* puparia. Before throwing them away, fruits were carefully inspected for remaining puparia within the second week after sampling (**Fig.1d**). Puparia were singly kept on Eppendorf tubes until flies' or parasitoid-wasps' emergences (**Fig.1e**).
Drosophila and parasitoids emergence was monitored for 35 days (5 weeks). Insects were aspirated and preserved in ethanol absolute.
After insect emergence ceased, dead pupae were dissected under stereomicroscope to check the presence of either flies or parasitoid.

Evaluation of parasitism on non-target Drosophila species

During our last fruits sampling on *P. avium,* we noticed that fermenting fruits were intensively foraged by Drosophila species other than *D. suzukii*, thus with the aim of verify host range of *L. japonica* we carried out a preliminary sampling of these senescent fruits.
The week following the third sampling on *P. avium*, overripe fruits (BBCH≥89) still hung on the plant were collected at different sampling points, for a total of seven samples, including different habitat type, that were *P. avium* in soft-fruit crop areas: *PiV* (46.07878, 11.27154) and *Var* (46.07888, 11.26383), *P. avium* in a private garden: PFT (46.33489, 11.05156), *P. avium* in a biotope BLP (46.07831, 11.22262), *P. avium* in an organic mixed orchard: *Cs* (46.05722, 11.21866), *P. avium* in an abandoned cherry orchard Ca6a (46.04471, 11.22554), *P. avium* in intensive apple orchards system RO3 (46.00235, 11.11519).
Fruits were incubated as reported for ripening fruits until all *Drosophila* larvae pupated.
Puparia were collected from plastic container and kept isolated, checking every 2-3 days for *Drosophila* or parasitoids emergence for a period of 35 days. Insects were aspirated and preserved in ethanol absolute.
Dead pupae were dissected under stereomicroscope to check the presence of either flies or parasitoid.

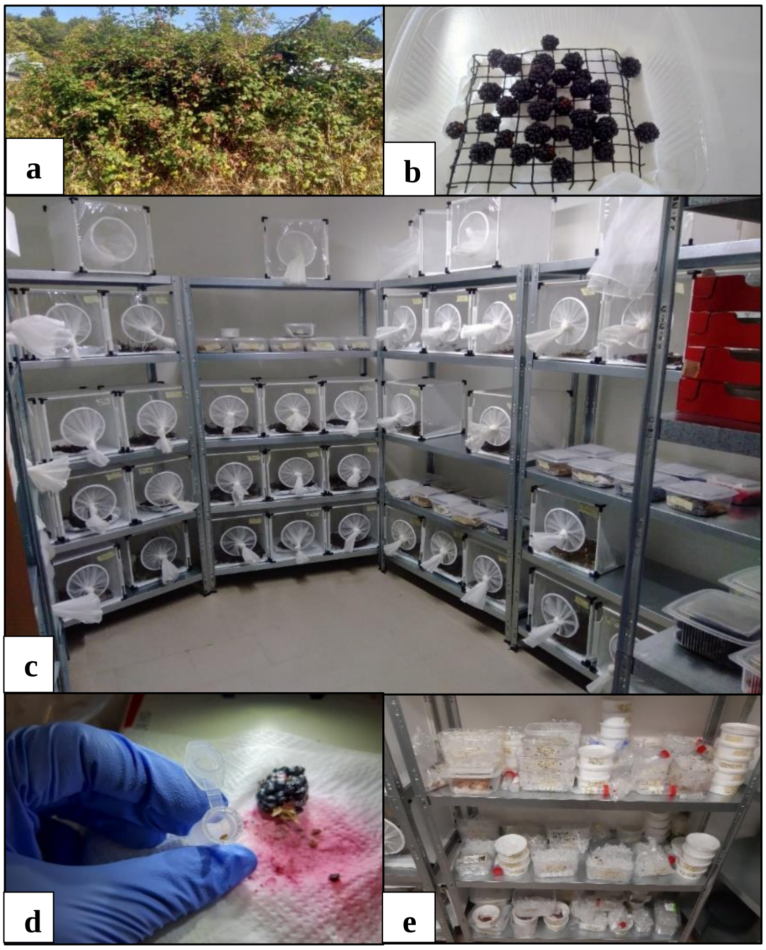


Figure 1. Samples processing phases. a) A shrub of *R. ulmifolius* sampled during the survey. b) Infested blackberries placed inside the plastic container. c) Room used during fruits incubation.
d) Dissection of fruit and isolation of pupae. e) Boxes with Eppendorf vials containing the single pupae.

Insect identification and molecular characterization

Drosophila and parasitoid specimens were identified following morphological diagnostic criteria reported in Novkovic et al., 2011, Markow and O'Grady, 2006, Miller et al., 2017.
Beside morphological identification, some *L. japonica* adults from different sampling point and fruit type have been subjected to molecular analysis with genetic markers, the cytochrome oxidase subunit I (COI) gene and the intertranscribed spacer sequences I (ITS1), in order to carry out a molecular characterization of the adventive population of *L. japonica* in Trentino province (**Tab.1**).

Table 1. List of molecular markers and their primers sequence

Molecular marker	Forward and Reverse primers	Primer sequence
COI "Nancy" (Simon et al., 1994)	*LCO1490*	5'-GGTCAACAAATCATAAAGATATTGG-3'
	C1-N-2191	5'-CCCGGTAAAATTAAAATATAAACTTC-3'
COI "Universal Folmer" (Folmer et al., 1994)	*LCO1490*	5'-GGTCAACAAATCATAAAGATATTGG-3'
	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'
ITS1	*rDNA2*	5'-TTGATTACGTCCCTGCCCTTT-3'
	rDNA1.58s	5'-ACGAGCCGAGTGATCCACCG-3'

Total DNA was isolated from each specimen using a commercial kit (NucleoSpin® Tissue; Macherey-Nagel, Düren, Germany). Amplifications were performed according to conditions reported in PM 7/76 [70] for COI and in Taylor et al. [71] for ITS1. PCR products, after purification with illustra ExoProStar1-Step (GE Healthcare, Little Chalfont, UK), were sequenced with the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on 3130 xl Genetic Analyzer (Applied Biosystems; Carlsbad, CA, USA). A Basic Local Alignment Search Tool (BLAST) comparison of the sequences obtained was performed using the NCBI database. BLAST Tree View was used to compute pairwise alignments.

Data analysis

Infestation and parasitism indexes were calculated as below:

D. suzukii Infestation = (number of fruits containing at least one egg of *D. suzukii* / total number of fruits inspected) * 100

L. japonica Parasitism = (number of *D. suzukii* pupae parasitized by *L. japonica* / total number of *D. suzukii* pupae) * 100

When dead flies or larval parasitoids were identified from unemerged pupae, number was included in the calculation of parasitism.

Very few samples (<1%) of unemerged pupae were degraded to such a point that was not possible to discriminate the content, so they were not included in the calculation of the parasitism percentage. All data are shown as mean ± standard error.

Results

Early season host-parasitoids dynamics on P. avium

A total of 3,823 fruits from *P. avium*, that yielded 2,438 pupae of *D. suzukii*, were collected from 20 sampling points distributed over the entire study area, spanning a diverse heterogeneous landscape from agricultural-dominated area (apple orchards n=3; cherry orchards n=4; vineyards n=1; mixed farms n=2), wetland habitat (n=2), urban zone (green public park n=3; semi-natural n=3), riparian zone (n=2) (**Tab.2**).

Table 2. Early season infestation of *D. suzukii* on *P. avium* and parasitism of *L. japonica* during fruits sampling in May-June 2021. Total fruits collected refer to the sum of cherries over three consecutive weeks of sampling. Total *D. suzukii* pupae included both unparasitized and parasitized pupae

Site GPS coordinates	**Sampling period**	**m a.s.l.**	**Total fruits collected**	**Total *D.suzukii* pupae**	***D.suzukii* Infestation (min-max)**	***L.japonica* parasitism (min-max)**
46.04747, 11.11327	17/05/2021-31/05/2021	188	240	76	9.6-58.8	0.0-4.2
46.07767, 11.13445	26/05/2021-15/06/2021	338	213	79	4.7-79.2	0.0-12.5
46.13289, 11.13203	31/05/2021-14/06/2021	523	162	125	0.0-48.7	0.0-4.5
46.04747, 11.11327	01/06/2021-15/06/2021	277	202	41	0.0-83.1	0.0-16.7
46.05736, 11.13939	01/06/2021-15/06/2021	253	294	73	22.3-43.2	0.0-33.3
46.25079, 11.17204	01/06/2021-16/06/2021	280	224	32	2.0-44.7	0.0-5.6
46.00348, 11.11867	01/06/2021-23/06/2021	187	153	87	7.5-75.6	0.0-4.0
45.92554, 11.07925	03/06/2021-15/06/2021	180	228	38	0.0-27.7	0.0-3.7
46.08578, 11.10829	07/06/2021-21/06/2021	194	139	28	3.8-60.6	0.0-46.2
45.96272, 11.09546	07/06/2021-23-06/2021	196	169	67	0.0-51.7	0.0-1.5
46.07854, 11.22239	10/06/2021-22/06/2021	520	210	66	1.3-33.3	0.0-2.3
46.07596, 11.23312	11/06/2021-23/06/2021	557	227	172	47.4-74.0	0.0-8.9
46.13221, 11.13107	14/06/2021-28/06/2021	502	189	150	11.9-92.3	0.0-7.1
46.09678, 11.11902	14/06/2021-28/06/2021	199	204	125	4.1-89.1	0.0-1.1
46.03588, 11.14248	15/06/2021-28/06/2021	337	177	248	23.1-100.0	0.0-1.3
46.05726, 11.21819	17/06/2021-30/06/2021	514	154	69	23.1-51.3	0.0-19.0
46.06063, 11.22351	17/06/2021-30/06/2021	473	151	174	11.8-74.0	0.0-10.8
46.04617, 11.22506	17/06/2021-30/06/2021	510	187	313	44.1-88.2	0.0-2.0
46.04471, 11.22559	17/06/2021-30/06/2021	499	152	129	21.3-74.5	0.0-6.2
46.04776, 11.2316	17/06/2021-30/06/2021	472	148	346	37.3-100.0	0.0-14.5

Infestation rate and parasitism were variable across altitude profile and habitat type.

Overall, during the three consecutive weeks of sampling the average infestation rate due to *D. suzukii* was 14.0% (±3.7%; 16 out of 20 infested samples) on the first collection date, raised to 40.0% (±5.4%; 19 out of 20 infested samples) on the second collection date and on the last collection date was 66.3% (±5.6%; 20 out of 20 infested samples). (**Fig.2a**).

The earliest sample infested by *D. suzukii* was collected on May 17 with 9.2% of infested fruits, whilst the first *D. suzukii* pupae parasitized by *L. japonica* were found at the same sampling site on May 31. Maximum parasitism by *L. japonica* (46.2%) was found on June 21.

No *L. japonica* parasitism on *D. suzukii* was detected during the first sampling date at any sites. On the second sampling bout, parasitism rate was very low (0.9±0.7%, only 3 out of 20 *D. suzukii* pupae samples),and increased to 10.2% (±2.7%) on the last sampling, in which *L. japonica* emerged from 20 out of 20 *D. suzukii* pupae samples (**Fig.2b**).

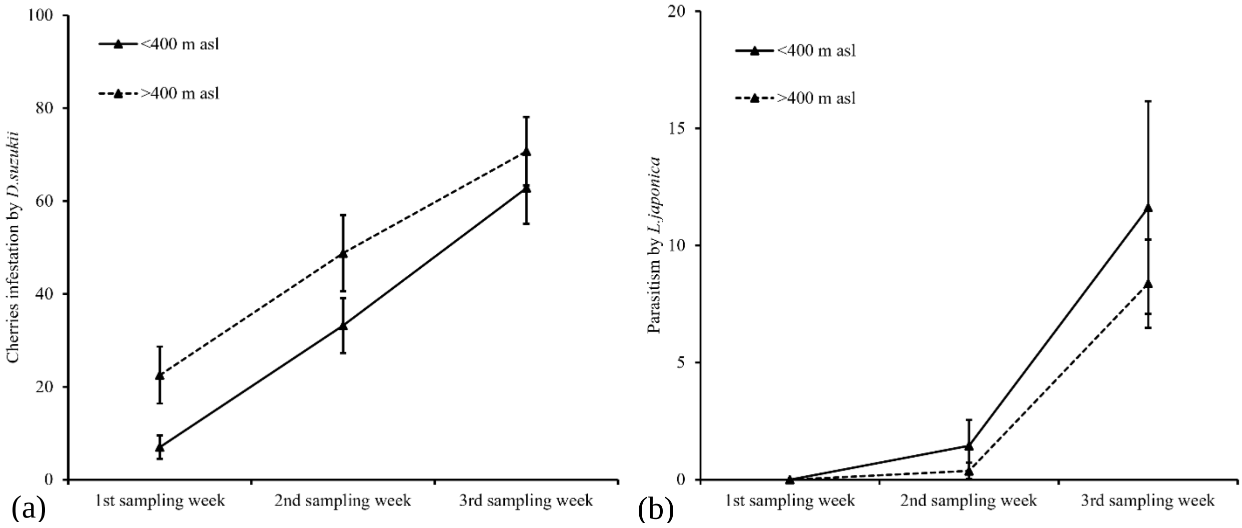


Figure 2. Overall trend of infestation of *D. suzukii* on *P. avium* (**a)** and parasitism rates of *L. japonica (***b)** during the three consecutive weeks of fruits sampling at all sites. The altitudinal threshold value of 400 m a.s.l. was chosen in accordance with the orography of the territory and the ripening stage of the cherry trees, dividing the samples of the lowland zone (<400 m) from those of the hilly zone (>400 m). Error bars represent standard error.

Mid-late season host-parasitoids dynamics on spontaneous host plants

A total of 36 samples of *S. nigra* were collected from July 12 to September 14, obtaining 6,776 fruits, of which 3,372 were infested by *D. suzukii*, yielding 1,167 puparia. In 15 sampling bouts, *L. japonica* parasitism was no detected. Parasitism rate ranged from 0 to 21,3%.

Pooling all samples, the average parasitism of *L. japonica* on *D. suzukii* from *S. nigra* was 5.8% (±1.2%).

Concerning R. *ulmifolius*, a total of 35 samples (1,057 fruits) were collected from August 17 to October 4. 971 berries were infested by *D. suzukii*, providing 4,241 puparia.

L. japonica parasitism was observed in all collected fruit samples, ranging from a minimum of 0.6% to a maximum of 40% of parasitized pupae.

Pooling all samples, the average parasitism of *L. japonica* on *D. suzukii* from *R. ulmifolius* was 13.3% (±1.7%). (**Tab.3**)

Table 3. Mid-late season parasitism of *L. japonica* over the course of fruits sampling period between July and October 2021. Total fruits collected refer to the sum of elderberries or blackberries over the sampling period. Total *D. suzukii* pupae included both unparasitized and parasitized pupae.

Site GPS coordinates	Host plant	Sampling period	Number of sampling	Total fruits	Total *D. suzukii* pupae	*L. japonica* parasitism range (average)
46.19203, 11.12635	*Sambucus nigra* L. (Adoxaceae)	12/07/2021-02/08/2021	4	910	152	3.2-7.7 (4.9)
46.06784, 11.11489	*Sambucus nigra* L. (Adoxaceae)	12/07/2021-09/08/2021	5	1037	82	0.0-25.0 (5.0)
45.96991, 11.0928	*Sambucus nigra* L. (Adoxaceae)	13/07/2021-09/08/2021	5	1104	86	0.0-20.0 (5.7)
46.18978, 11.13555	*Sambucus nigra* L. (Adoxaceae)	19/07/2021-09/08/2021	4	857	148	0.0-5.6 (1.7)
46.05735, 11.14024	*Sambucus nigra* L. (Adoxaceae)	20/07/2021-10/08/2021	4	909	279	0.0-21.0 (8.5)
45.98303, 11.11863	*Sambucus nigra* L. (Adoxaceae)	27/07/2021-10/08/2021	3	687	108	0.0-14.3 (6.8)
46.04786, 11.11297	*Sambucus nigra* L. (Adoxaceae)	27/07/2021-17/08/2021	4	580	71	0.0-7.4 (1.9)
46.03746, 11.14425	*Sambucus nigra* L. (Adoxaceae)	09/08/2021-01/09/2021	4	456	163	0.0-20.9 (10.2)
46.05669, 11.21319	*Sambucus nigra* L. (Adoxaceae)	31/08/2021-14/09/2021	3	236	78	7.1-12 (8.9)
46.04942, 11.50718	*Rubus ulmifolius* Schott (Rosaceae)	17/08/2021-28/09/2021	5	182	138	5.1-40 (17.4)
46.05966, 11.21361	*Rubus ulmifolius* Schott (Rosaceae)	31/08/2021-04/10/2021	6	168	829	3.2-22 (13.9)
46.08809, 11.27835	*Rubus ulmifolius* Schott (Rosaceae)	14/09/2021-04/10/2021	4	126	339	15.9-37.9 (23.8)
46.03778, 11.14518	*Rubus ulmifolius* Schott (Rosaceae)	24/08/2021-29/09/2021	5	138	941	0.6-7.4 (2.5)
46.03649, 11.21559	*Rubus ulmifolius* Schott (Rosaceae)	24/08/2021-29/09/2021	6	180	1034	4.4-15.6 (14.5)
46.07872, 11.51555	*Rubus ulmifolius* Schott (Rosaceae)	24/08/2021-28/09/2021	6	153	542	3.8-21.1 (11.1)
46.07472, 11.23333	*Rubus ulmifolius* Schott (Rosaceae)	24/08/2021-07/09/2021	3	110	418	5.4-16.9 (11.3)

Collateral fruits sampling

From June to October, 1,278 fruits were sampled from 16 plant species, belonging to 10 different taxonomic families (*Moraceae, Rosaceae, Glossulariaceae, Rhamnaceae, Cornaceae, Ericaceae, Phytolaccaceae, Dioscoreaceae, Adoxaceae, Solanaceae*), raising 2,173 *D. suzukii* pupae. (**Tab.4**) Earliest spontaneous berries collected was *Morus alba* L. (White mulberry, *Moraceae*) on June 14 and the last was *Rubus* spp. (*Rosaceae*) on October 4.

Table 4. Parasitism of *L. japonica* on *D. suzukii* in spontaneous and crop host plants. Plant species are listed according to their ripening time during the sampling season, from the one that ripens earlier to the one that ripens later.

Host plant	**Common name**	**Sampling date (number of samples)**	**Total fruits**	***D. suzukii* pupae**	**Parasitism *L. japonica***
Morus alba L. (Moraceae)	White mulberry	14/06/2021 (1)	32	4	25.0
Fragaria x ananassa (Rosaceae)	Strawberry (crop)	28/06/2021 (1)	50	25	4.0
Ribes rubrum L. (Glossulariaceae)	Red currant	04/07/2021 (1)	62	15	46.7
Frangula alnus L. (Rhamnaceae)	Alder buckthorn	15/07/2021-12/08/2021 (2)	201	160	4.7, 16.7
Prunus mahaleb L. (Rosaceae)	St. Lucie cherry	19/07/2021 (1)	64	118	13.6
Cornus mas L. (Cornaceae)	Cornelian cherry	30/08/2021 (1)	54	3	33.0
Rubus idaeus L. (Rosaceae)	Raspberry (crop)	07/09/2021-14/09/2021 (2)	59	314	14.5, 21.5
Cornus sanguinea L. (Cornaceae)	Dogwood	15/09/2021-29/09/2021 (2)	39	37	5.0, 23.0
Vaccinum corymbosum L. (Ericaceae)	Blueberry (crop)	03/08/2021-07/09/2021 (5)	351	375	3.2-18.9
Phytolocca americana L. (Phytolaccaceae)	Pokeweed	21/09/2021 (1)	36	18	11.1
Dioscorea (*Tamus*) *communis* L. (Dioscoreaceae)	Black bryony	21/09/2021 (1)	100	21	4.8
Viburnum opulus L. (Adoxaceae)	Guelder rose	21/09/2021 (1)	53	2	100.0
Ficus carica L. (Moraceae)	Fig	21/09/2021 (1)	5	41	9.8
Rubus fruticosus L. (Rosaceae)	Blackberry (crop)	28/08/2021-04/10/2021 (2)	65	220	15.7, 20.0
Solanum nigrum L. (Solanaceae)	Black nightshade	29/09/2021 (1)	45	3	33.3
Rubus caesius L. (Rosaceae)	European dewberry	29/09/2021-04/10/2021 (1)	62	817	6.2, 11.4

L. japonica parasitism was detected in all samples. Lowest parasitization rate (4%) was found on a sample of cultivated strawberry on June 28 and the highest parasitism (100%) on *Viburnum opulus* L. (Guelder rose, *Adoxaceae*) on September 21.

Evaluation of parasitism on non-target Drosophila species

Concerning overripe cherries, we collected a total of seven samples (total fruits=297) that provided 1,152 *Drosophila* puparia, of which 575 pupae belonging to *D. suzukii* and the remaining 577 pupae to *D. melanogaster/D. simulans*. Regarding these latter species, we did not discriminate them because it is not possible to differentiate based on the puparium morphology, as they are extremely similar being two closely related species.

At all seven sampling sites both *D. suzukii* and *D. melanogaster/D. simulans* were concomitant, although with different ratio between the two species (**Tab.5**).

Table 5. Parasitism of *L. japonica* on *D. suzukii* and other drosophilids in overripe cherries. Fruit samples are listed in downward order of relative abundance of *D. suzukii*.

Overripe sample code	**Total fruits**	**Total pupae**	**(*Dsu*) pupae**	***Dme/Dsi* pupae**	***L japonica* (*Dsu pupae)***	***L.japonica (Dme/Dsi pupae)***	***Dsu* relative abundance**	**Parasitism (*Dsu*)**	**Parasitism (*Dme/Dsi*)**	**Overall parasitism (*Dsu+Dm/Dsi*)**
PiV	65	214	186	28	21	0	0.87	11.3	0.0	9.8
PFT	16	35	29	6	2	0	0.83	6.9	0.0	5.7
VaR	56	122	77	45	2	0	0.63	2.6	0.0	1.6
Cs	30	202	115	87	17	16	0.57	14.8	18.4	16.3
BLP	30	120	44	76	3	3	0.37	6.8	3.9	5
CA6a	55	219	65	154	7	8	0.30	10.8	5.2	6.8
RO3	45	240	59	181	1	1	0.25	1.7	0.6	0.8

L. japonica parasitism on *D. suzukii* was found on seven samples out of 7 (total adult parasitoids=53), whereas *L. japonica* parasitism on *D. melanogaster/D. simulans* was found on four samples out of 7 (total adult parasitoids=28). *L. japonica* parasitism on *D. suzukii* was higher than parasitism on *D. melanogaster* in six samples out of 7, regardless of the relative abundance between the two *Drosophila* species. Maximum parasitism occurred in a sample with approximately equal proportion of the two species (*D. suzukii* pupae/total ratio=0.57), with 14.8% and 18.4% of parasitized pupae of *D. suzukii* and *D. melanogaster/D. simulans*, respectively.

A summary of the pupae species composition and *L. japonica* parasitism on different samples is reported in **Fig.3**

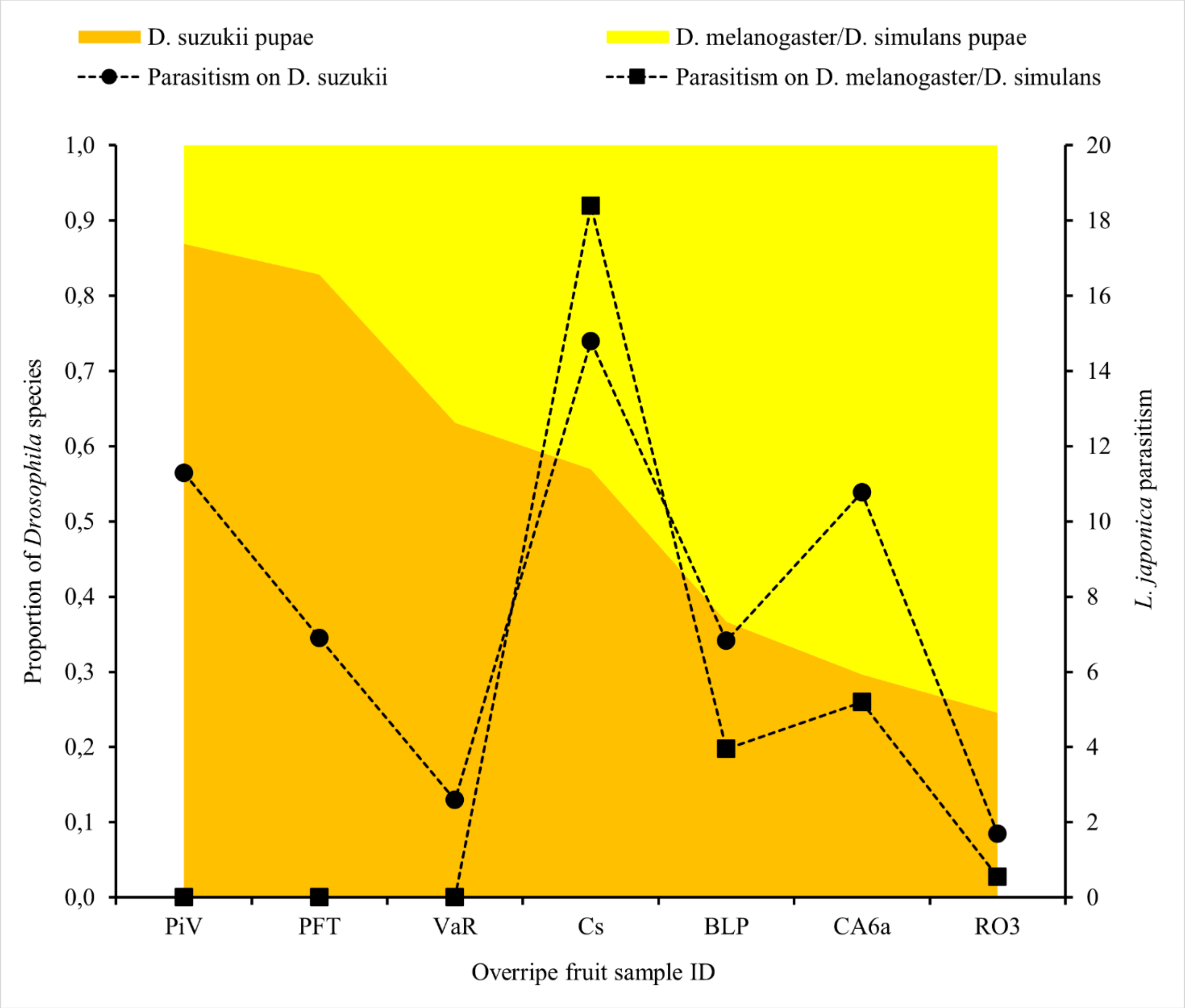
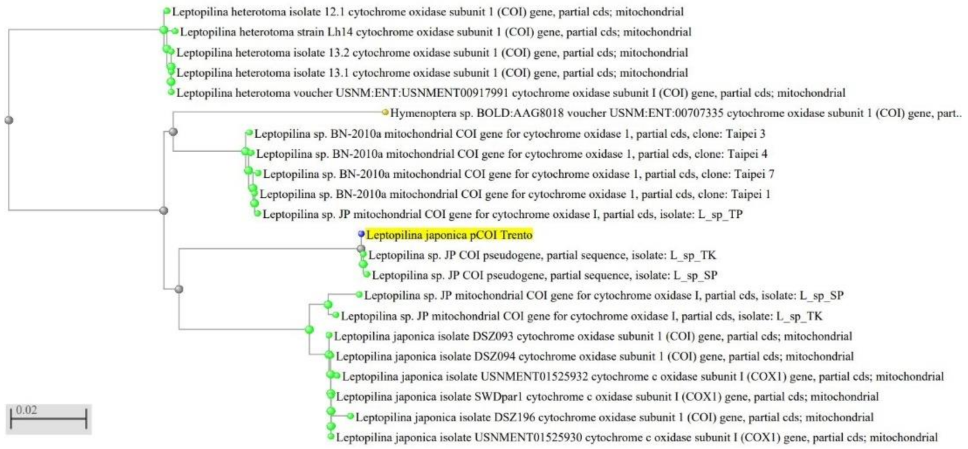


Figure 3. Proportion of *Drosophila* species and parasitism of *L. japonica* encountered on overripe cherries samples during 2021 fruit collections.

Molecular characterization

Neighbor joining trees were performed after BLAST pairwise alignment. COI and ITS1 markers of *L. japonica* of Trento population specimen shown a high similarity with Japan *L. japonica* strains, especially with GROUP III cluster of *L. japonica* subsp. *japonica* from Tokyo and Sapporo. Below are reported the Blast Tree View resulting from pairwise alignment of molecular markers (COI "Universal Folmer" and ITS1).



Discussion

Biological control is actively managed by growers with their agronomic practices aimed at controlling the pest population growth and the consequent crop’s damage.

However, long-term processes required to implement effective biological control plans contrast with the extremely fast movements mediated by global trade and human travel, which lead to the unintentional introduction and spreading of alien species, sometimes reuniting natural enemies with their hosts/preys prior to the man-coordinated actions in the invaded areas (Weber et al., 2021).

When this happens, the most appropriate way to proceed is to deep the interactions that are established in the new environment, evaluating any side effects on non-target species, possibly favoring the ecological link between alien species to gain an advantage in terms of biological control.

We found both temporal and spatial variation in host-parasitoid dynamics.

We observed that parasitism of *D. suzukii* by *L. japonica* is delayed by a couple of weeks with respect to the start of the pest activity.

Phenological asynchrony is a well-known phenomenon involved in the coevolution relationship between pest and natural enemies, balancing population fluctuations, stabilizing community structure and allowing persistence among species (Hassell et al., 2000).

In early season this process could be mediated by abiotic factors, mainly temperature, modifying the trajectories of *D. suzukii*-*L. japonica* system. Exit period from diapause and cold tolerance threshold differ between *D. suzukii* (Ryan et al., 2016, Grassi et al. 2018) and its parasitoid (Murata et al., 2013, Hougardy et al., 2019), most likely resulting the phase shift in reproductive activity that has been observed in the field.

Furthermore, habitat in which these species overwinter may influence metapopulation parasitism; for instance, coincidence in overwintering sites and presence of ripening fruits on the same area early in the season could shape host finding behaviour, thus reducing parasitoid searching time among plant patches, nevertheless this conjecture will require further investigation.

Previous studies have reported how *L. japonica* display a wider host range than more specialized lineages of *G. brasiliensis* (Nomano et al., 2017), parasitizing other phylogenetically related members of the melanogaster group in addition to *D. suzukii*, such as the cosmopolitan species *D. melanogaster* and *D. simulans* (Girod et al. 2018, Daane et al., 2021).

Infestation and transition from healthy to overripe fruits opens a niche for frugivorous *Drosophila* species that breed on fermenting fruits, creating the conditions of coexistence with *D. suzukii* (Rombaut et al., 2017).

Here we confirm the ability of *L. japonica* to exploit these hosts in nature when senescent fruits still hanging on plant are colonized by other generalist *Drosophila* species, although to date it is not known whether this behavior is passively limited to this short time window or is widely and actively adopted also on other decaying substrates (Bal et al., 2017, Biondi et al., 2021).

Differences on parasitization rates of *L. japonica* on *D. suzukii* and other drosophilids among overripe cherries samples should be explored as it may be related to the interaction between spatial and temporal components that affect both parasitoids and Drosophila species assemblage and abundance (Fleury et al., 2009). For instance, habitat type in which insect communities reside and seasonality may have a pronounced effect on the host-parasitoid interaction (Penariol et al., 2013, Gleason et al., 2019). Moreover, abiotic environmental factors can further shape the composition of these species (Poppe et al., 2015).

This ecological fitting process may confer an advantage to *L. japonica*, which would greatly increase its reproductive output compared to the dependence to a single host, especially if the larval parasitoid does not appear to possess a limiting functional response (Wang et al., 2018, 2020). Parasitoid virulence variability and plasticity in host exploitation are well documented in native range (Kimura, M.T. 2015a, b, Matsuura et al., 2018).

Concerning the pest management purpose, accumulating offspring also on alternative hosts in this early phenological phases, may translate in more *L. japonica* adults available for the biological control of *D. suzukii* later in the season (Powell, 1986).

The biological control activity takes place mainly in the semi-natural habitats heterogeneously distributed in the agroecosystem, in which natural enemies such as the parasitoids can find refuge and nourishment. Numerous authors state the key role of these habitats as breeding sites for *D. suzukii*, which being a highly polyphagous species, can rapidly increase its population on a wide range of non-crop hosts before spilling over to crop fields (Klick et al., 2016, Tonina et al., 2018b, Tait el., 2019, Leach et al., 2019a, Urbaneja-Bernat et al., 2020).

During our large-scale monitoring, most of these non-crop hosts were found to grow on remnant vegetation, hedgerows or woodland edges, adjacent to crop hosts. Although parasitism on *D. suzukii* was rather variable depending on fruit type, this was in line with parasitism rates reported during previous field surveys on native range of the pest (Daane et al., 2016, Girod et al., 2018b, Giorgini et al., 2019), offering a positive sign of re-establishment of the interaction between the two alien species in recently invaded areas, reverberating the biocontrol effect on cultivated fields.

Ability of *L. japonica* to find its host on this wide range of plant families, even in species probably absent in the native areas, is promising for an effective control of *D. suzukii*.

On the most ubiquitous hosts of the mid-late season, *L. japonica* seems to perform better on *R. ulmifolius* than *S. nigra*, with higher parasitism rate found during all the sampling period on the former species. Failure to detect parasitization on some collected samples of *S. nigra* could be mainly due to the intrinsic chemical composition of berries, thus not related to host finding defect.

Indeed, *S. nigra* contain compounds that are potentially toxic (i.e. cyanogenic glycosides, lectins), which not being optimal for its host *D. suzukii* as previously observed (Poyet et al 2015, Wolf et al., 2020), may have an adverse effect also on the larval parasitoid.

Beyond spontaneous plant species, *L. japonica* emerged also from cultivated crop hosts, such as blueberries, blackberries and raspberries, even though biological control by parasitoid wasps on crops could be marginal due to pesticide susceptibility.

Concerning molecular characterization of larval parasitoids, we observed phylogenetic relatedness of our specimens to *L. japonica* subspecies *japonica,* as both COI and ITS1 markers clustering with the previous identified GROUP III of Japan strains of *L. japonica* (Sapporo and Tokyo). Furthermore, during the analysis of Trento population of *L. japonica* we identified the presence of the peculiar COI pseudogene that seems evolved after the divergence of northern populations of subsp. *japonica* from the southern ones subsp. *formosana. (*Novković *et al., 2011*). It is still unknown if there is geographic differentiation in the strains of *L. japonica japonica* related to virulence variation against *D. suzukii* (Kimura, M.T. 2015a). We suggest this aspect should be further investigated, also in the light of recent reports both in Europe and in North America, to better understand the possible origins of the introduced strains.

Interaction among chemical application, cultural practices and the landscape configuration/composition need to be further investigated in order to gain new insights for integrated management of *D. suzukii*, including habitat manipulation techniques that could ameliorate the agroecological context in which natural enemies carry out their action against the insect pest (Thies et al., 1999, Jonsson et al., 2010).

With this study we explored the ecological interactions that arise between host and parasitoid in the new environment, observing that *L. japonica* is very active against *D. suzukii* and widely distributed on Trento province. Furthermore, this study allowed to improve the planning releases of the classical biological control agent *G. brasiliensis* (Lisi et al., 2021) and in the next few years it will be possible to follow the evolving dynamics between these two larval parasitoids and *D. suzukii*.

CHAPTER 3

Unravelling the reproductive behaviour of wild-caught *D. suzukii* as a basis for implementation of autocidal control tactics

Abstract

Remating frequency influences the reproductive trajectories of a population through sperm allocation and genetic variability. Multiple mating triggers the arena for sperm competition and cryptic female choice, conditioning patterns of sexual selection.

As post-copulatory mechanisms following female remating may also reduce effectiveness of sterile insect technique, investigations are required before the implementation of area-wide control programmes.

Here, we shed light on polyandry extent and sperm use in a natural population of *D. suzukii*, an invasive pest of stone and soft-fruit crops.

Paternity analysis by means of microsatellite loci genotyping of the progeny of wild-caught females, revealed high levels of multiple paternity with 83% of the broods having more than one sire.

Overall, we found no evidence of paternity skew, but an equal paternity share among fathers, indicating no bias towards a last-male sperm precedence.

We discuss the potential implications of these results on reproductive behaviour of *D. suzukii*, with regard to pest management practices using sterile male release.

Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), also known as Spotted Wing Drosophila (SWD) (Kanzawa et al. 1939), native to eastern Asia has rapidly spread throughout Europe and America in the last decades and steadily expanding its range on new geographic regions (EPPO Global Database, 2021).

Within the genus *Drosophila*, belongs to the 'melanogaster' group of the subgenus Sophopora (O'Grady et al., 2018). Nevertheless, unlike most of other closely related species that oviposit on rotting substrates, *D. suzukii* has evolved an egg-laying preference for ripening and ripe healthy fruits, peculiar behavioural traits reached through changes in foraging sensory processing (Keesey et a., 2015, Karageorgi et al., 2017) and a female serrated ovipositor (Atallah et al., 2014).

Furthermore, ability to breed on a wide range of soft-skinned fruits (Poyet et al. 2015, Kenis et al., 2016, Panel et., 2018), phenotypic plasticity to climatic conditions (Gutierrez et al., 2016) and the lack of an efficient biological control in the invaded regions (Lee et al., 2019), elevated *D. suzukii* to a serious pest status, which is threatening worldwide fruit cropping systems leading to recurrent pesticide use (Ioriatti et al., 2020).

Beyond above mentioned, invasiveness of a species may strictly linked with sexual behaviour, which influencing mate choice modifies the trajectories of reproductive success (Markow, 2002), reverberating the effects on population dynamics. Moreover, mating induces physiological changes in both sexes at different levels, factors that conditioning animal behaviour in the environmental context (Dickson, 2008, Avila et al., 2011).

To date a few studies on mating strategies of *D. suzukii* have investigated pre-copulation activity during courtship behaviour (Mazzoni et al., 2013 Revadi et., 2015, Roy et al., 2019), cuticular hydrocarbon chemical communication (Dekker et al., 2015, Snellings et al., 2018) and post-copulatory olfactory perception (Crava et al, 2019).

However, despite remarkable amount of literature on several aspects of *D. suzukii*'s life (Tait et al., 2021), little is known about mating systems and sexual selection of this species, in particular polyandry (Parker et al., 2013).

Polyandrous behaviour, that occurs when a female has multiple matings with different males, is found to be a widespread phenomenon in natural populations (Taylor et al., 2014) and has been proven in several Drosophila species with various ecological specializations (Markow, 2002).

Benefits of a polyandry has been associated with the gaining in reproductive output and offspring fitness, resulting by fertilization success, genetic compatibility and genetic diversity (Kvarnemo et al., 2013).

Sperm competition (Parker, 1970) and cryptic female choice (Eberhard, 1996) are the main intrinsic mechanisms of post-copulatory sexual selection, shaping the fate of the offspring paternity in promiscuous species.

Understanding sexual behaviour of *D. suzukii* may raise new insights to better explain the population genetic structure and its ecological plasticity or to managing the invasive species by applied sustainable biocontrol methods, such as sterile insect technique (SIT) (Lanouette et al., 2017). SIT has been proposed for controlling *D. suzukii* in confined environments such as greenhouses (Nikolouli et al., 2018), reaching an advanced developmental stage in recent years (Krüger et al., 2019, Sassù et al., 2019, Lanouette et al., 2020). However, next step would push towards an open field application of this technique, where the released sterile males would have to outcompete with potential high seasonal density of wild males. For these reasons, we need to shed light on and deepen the sexual behaviour of natural populations, in order to improve its area-wide integrated management (Vreysen et al., 2021). Remating in polyandrous females may influence the outcome of SIT, since sperm utilization for egg fertilization may be biased in favour of the ejaculates of certain males, which if fertile wild ones would make females still reproductive (Scolari et al., 2014).

In the present study, we used microsatellite markers to assess multiple paternity in an Italian population of *D. suzukii.* Moreover, we carried out further life-history studies to better understand reproductive behaviour of this species.

Materials and methods

Study site and population monitoring

The study site was a wide area located at 500 m asl on Valsugana of Trentino province in Northern Italy (46.051333, 11.220275; **Fig.1 a,b**) The area is very rich of unmanaged *Prunus avium* trees which are known breeding sites for *D. suzukii*. The fly sampling was performed in 2019, starting from the second half of May and lasted till the end of July, covering the entire ripening period of sweet cherries.

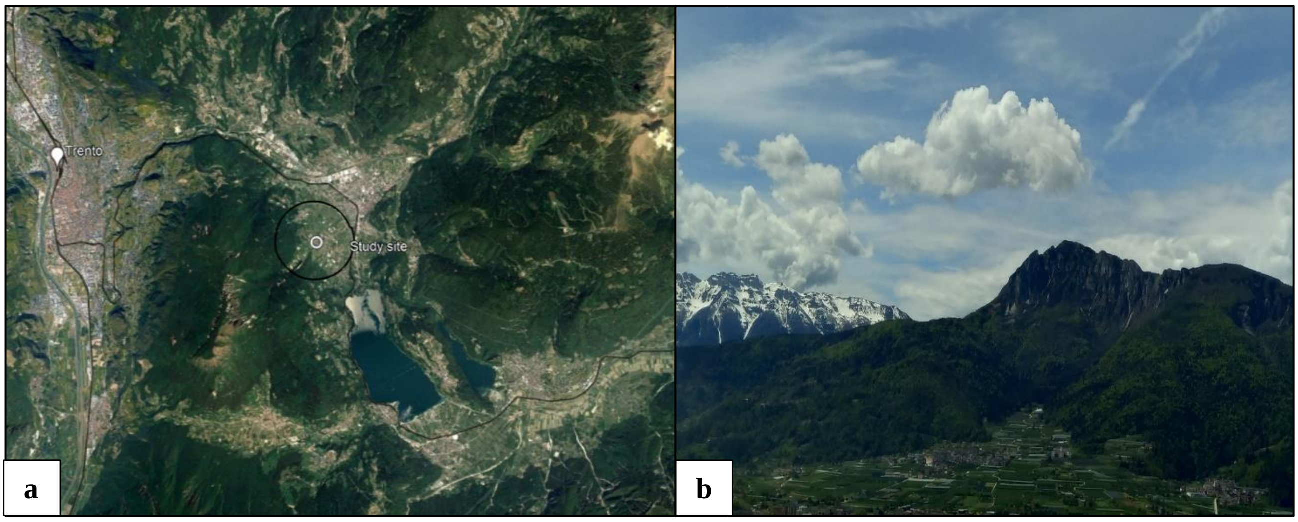


Figure 1. a) Map of the study site (black circle identifies the area subject to the wild females of *D. suzukii* sampling. **b)** A landscape view of the agricultural area.

This sampling approach allowed to derive information on the biological aspects of this population, such as flies density, fecundity, fertility, sex ratio and degree of polyandry which *D. suzukii* encounters in relation to the availability of its main host after the winter bottleneck.

Evaluation of *D. suzukii* population density has been performed by means of traps hung 1-1.5 m from the ground, in proximity (within 2.5 m) of unmanaged cherry trees. Each trap (*Droso-Traps*®,Biobest, Westerlo, BE) was filled with 200 ml of *Droskidrink* liquid bait (75% apple cider vinegar, 25% red wine, Prantil, Trento, IT), 4 grams of unrefined cane sugar and a drop of non-ionic surfactant Triton^{TM}X-100 (Sigma-Aldrich, Saint Louis, US). Traps were refreshed every seven days and content brought to the laboratory for inspection under magnification. Total catches of *D. suzukii* were recorded each week.

Infestation rate was evaluated by weekly collection of fruits from the study site as followed.

During the ripening period of *P. avium*, samples of 40-50 cherries were randomly collected from trees at each monitoring point. Samples were checked under stereomicroscope searching for typical white egg's breathing spiracles protruding out of the oviposition hole on the fruit's surface. Infestation rate was estimated as number of fruits with at least one egg on total fruits collected per sample.

Twelve monitoring points were set up over two plots 1.5 km apart from each other. Pest surveillance was conducted during 12 consecutive weeks (20-31) starting from May 16, 2019.

Wild flies sampling, laboratory fecundity and fertility phase for polyandry assessment

To estimate the presence and the degree of polyandry in this population, 31 females were collected within the two monitored plots by direct aspiration off ripe cherries from several unmanaged *P. avium* trees during the last week of June (26th week). This week was chosen taking into account the trap catches and fruits infestation, that suggested a build-up of *D. suzukii* summer morph population before the seasonal outbreaks. The collected females were immediately transferred into individual vials containing an uninfested fresh blueberry as oviposition substrate and a piece of cotton wool soaked of sugar solution to further nourishment and hydration. All females were kept in a climatic chamber set to 22 ± 1 °C and 70 ± 10% RH.

Blueberry and cotton wool were replaced with fresh ones every 48-72 hours until the death of the female.

Replaced fruits were inspected under magnification to assess egg-laid process by *D. suzukii*. Total number of eggs were recorded, then blueberries were incubated in new vials in order to raise eggs to adulthood. Offspring from each female were collected every 24 h and sexed.

D. suzukii females and their offspring were frozen and preserved in ethanol absolute (≥99.8%, Sigma-Aldrich, Saint Louis, US) till subsequent molecular analysis.

Microsatellite (SSRs) genotyping of female and progeny

Genomic DNA from females and their progeny were isolated from the whole body following protocol by Baruffi et al. (1995). Multilocus genotyping was carried out using four dinucleotides repeat microsatellite loci previously developed by Fraimount et. al. (2015) and recently employed in an extensive survey on Italian populations of *D. suzukii* (Tait et al., 2017).

Polymerase chain reactions (PCR) were performed following reference manual of Taq DNA polymerase recombinant (Thermo Fisher Scientific™) in a 20 µl reaction volume containing 1X PCR Buffer, 0.2 mM dNTP mix, 1.5 mM MgCl2, 0.5 µM of each primer, 0.5 U of Taq polymerase, 1–5 ng of genomic DNA. Amplification profile consisted of 3 min at 94 °C, followed by 30 cycles with 30 s at 94 °C, 30 s 57°C, 1 min at 72 °C and a final step of 10 min at 72°C. PCR products were visualized onto a 1.5% w/v agarose gel.

Diluted PCR products were genotyped at the Department of Biology and Biotechnology of the University of Pavia using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems®) with GeneScanTM 500 ROX (Thermo Fisher Scientific™) as internal lane size standard.

Alleles sizes were scored using GeneMapper software (Applied Biosystems®).

Parentage analysis

Allele binning was conducted with TANDEM v.1.08 (Matschiner et al., 2009), followed by visual inspection of integer allele values to verify robustness of the automated rounding process.

Mean number of alleles per locus, allele frequencies, observed and expected heterozygosity, polymorphic information content (PIC), null allele frequencies and combined exclusion probability (Excl) were computed with CERVUS 3.0 (Kalinowski et al., 2007).

Parentage analysis was performed using different approaches: a parsimony approach using GERUD 2.0 (Jones, 2005) and with a Bayesian computation method with SCARE software package (Jones et al., 2003).

GERUD uses an exhaustive algorithm to reconstruct parental genotypes from multilocus genotyping data of a progeny array. When the genotype of each brood’s mother is known, analysis is focused on estimation of the minimum number of sires per family. If multiple minimum-father solutions are possible, the software ranks the most likely solution based on Mendelian segregation and allele frequencies in the population. Reliability of our paternity analysis to detect remating was explored through GERUDsim 2.0, simulating set of five replicates with 1000 iterations each and using population allele frequencies based on all the four loci genotyped. Each set of simulations was performed keeping offspring number constant, two fathers and varying paternity skew from 0 to 0,9. SCARE is based on a Bayesian modelling method that describes the probability density of numbers of mates per female (α) in order to evaluate remating behaviour, and the proportion of offspring sired by the last-mating male (β), an estimation of sperm displacement. A Monte Carlo Markov chain (MCMC) is used to fit the parameters.

Results

Population dynamic and fruits infestation of the natural population of D. suzukii

A total of 15,049 *D. suzukii* adults were captured during the entire monitoring period with 12 liquid bait traps. There were 8,926 males and 6,123 females. Average catches on May 16th (week 20) revealed 0.3 (±0.1) males and 1.6 (±0.5) females, whilst at end of the monitoring periods on August 7th (week 31) there were 365.1 (±105.4) males and 185.6 (±53.6) females. At the begin of the ripening period mean flies per trap was 31.1 (±9.0) males and 31.1 (±9.0) females **(Fig.2a)**.

Cherries infestation was 1.7% (±1.1) on week 24, 46.9% (±6.8) on week 25, 91.0% (±4.6) on week 26 and reached 100% of infested fruits on week 27 **(Fig.2b)**.

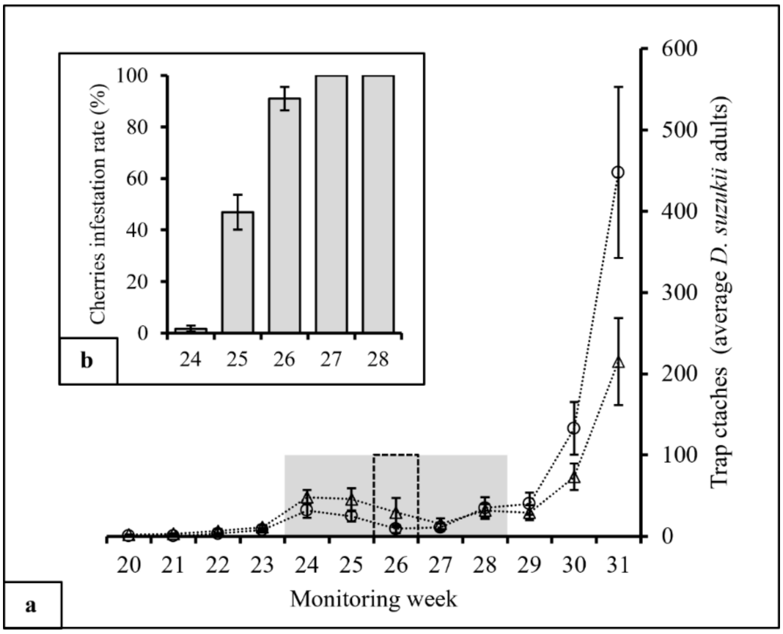


Figure 2. a) Population dynamics of *D.suzukii* in the study area. (empty circle=*D.suzukii* males; empty triangles=*D.suzukii* females) **b)** Fruits infestation by *D.suzukii* during the ripening period of *P.avium*. Gray area highlights the ripening periods (rectangle of broken lines indicate the week in which wild female of *D. suzukii* were collected).

Reproductive output of the wild-caught females collected

Out of the 31 females collected during the sampling bout, 26 females (83%) produced progenies. A total of 1267 eggs and 882 offsprings were obtained across all the oviposition days resulting in an average of 48.7 (± 3.2) eggs and 33.9 (± 2.4) offspring per female. Overall, sex-ratio of the progeny was 0.51 (± 0.02). Mean longevity of the captured females was 33.9 (± 1.3) days **(Fig. 3)**.

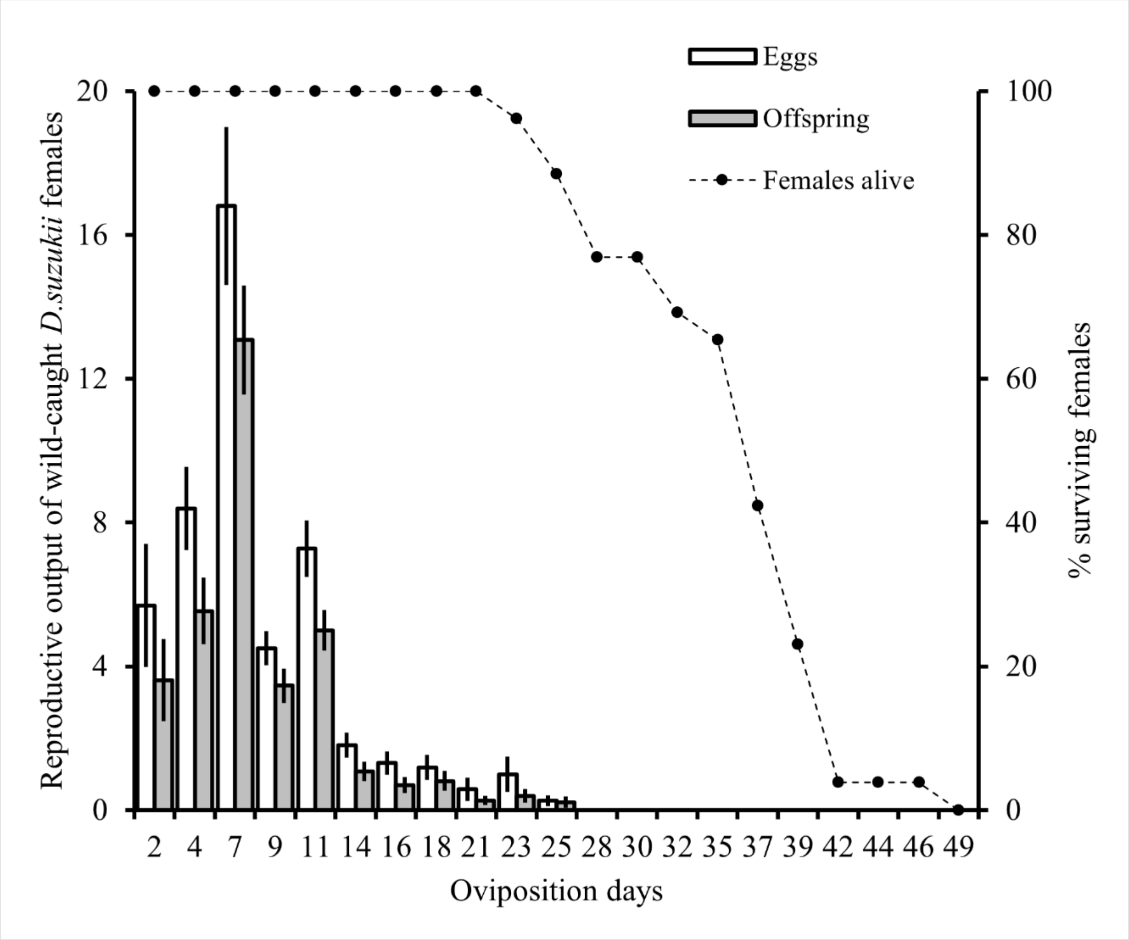


Figure 3. Lifetime summary of the reproductive dynamic of wild-caught females. White bars report the average egg number per oviposition days, grey bars the average offspring per oviposition days. Dotted line indicated the proportion of survival females across time.

Since total brood size of across families was variable **(Fig. 4)**, in order to increase paternity estimation we selected 12 families equally distributed among different brood sizes. From each of these families we genotyped about 50-60% of the progeny collected in each oviposition day, choosing randomly from both sexes. Furthermore, in order to increase accuracy in estimation of the number of sires, genotyping in five of these families was extended to 100%.

In total we genotyped 12 mothers and 328 offspring (mean per family=27.3 ± 4.4).

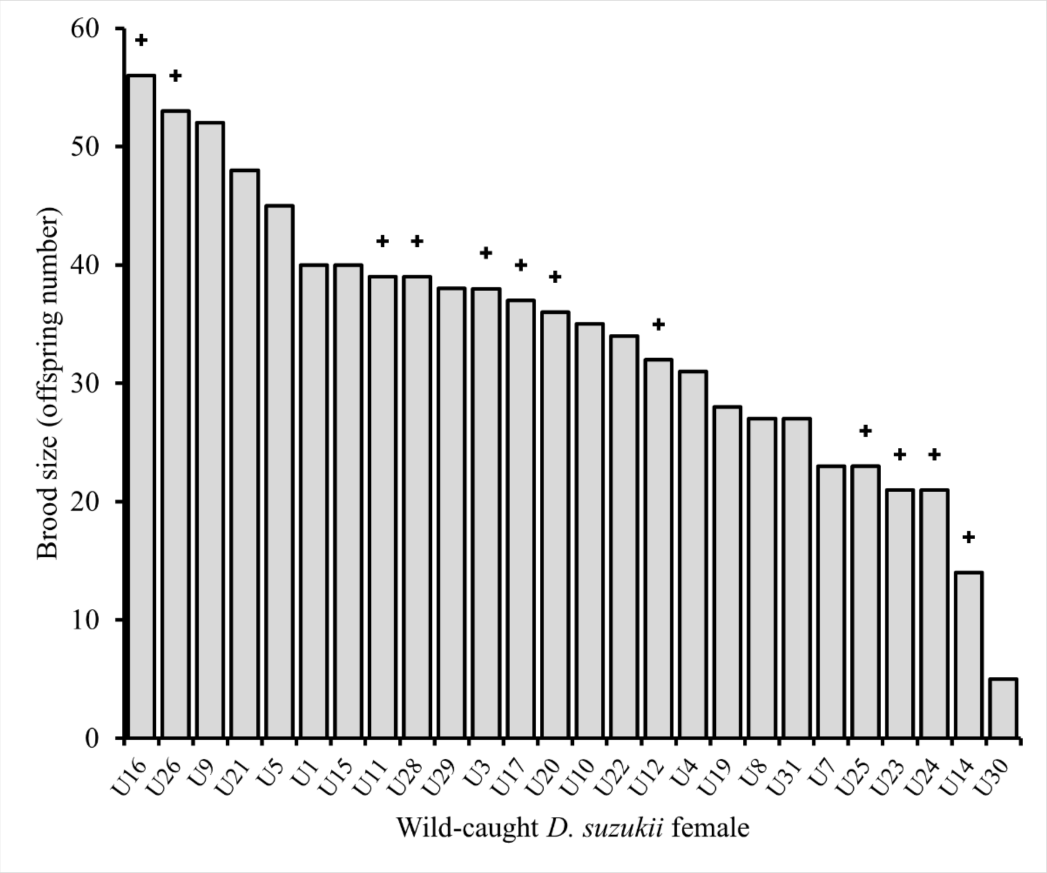


Figure 4. Brood size of the twenty-six wild-caught females. Cross over bars indicated the twelve selected families for paternity inference.

Sensitivity of SSRs for paternity analysis

Before starting the paternity analysis on *D. suzukii* mothers and their offspring, the sensitivity of microsatellites loci was assessed on an additional random sample of 25 wild *D. suzukii* that were collected in June 2019 at the same sampling area.

CERVUS analysis revealed a very high combined exclusion probability of 0.864 for the first parent and 0.966 for the second parent (when the first parent is known). Number of alleles per locus were six for loci Ds05 and Ds22, seven for loci Ds09 and Ds15. Mean expected heterozygosity was 0.789 and mean PIC was 0.739 for all loci genotyped. Since null alleles may cause a departure from Hardy-Weinberg equilibrium, compromising parental assignment, CERVUS calculates null allele frequencies. Our microsatellite loci obtained values much lower than the threshold set to 0.05 for reliable parentage analysis, according to Kalinowski et al. (2007), indicating reliability for parentage analysis at all loci.

Overall, microsatellites loci revealed an elevated sensitivity for the assessment of multiple paternity. Data are shown in **Tab.1**.

Table 1 Characteristics of four microsatellite loci used to parentage analysis of wild individuals of *Drosophila suzukii*

Locus	**k**	**H_O**	**H_E**	**PIC**	**Excl**	**F(Null)**
DS05	6	0.880	0.786	0.737	0.563	-0,0682
DS09	7	0.880	0.808	0.762	0.595	-0,0540
DS15	7	0.800	0.827	0.784	0.625	0.0086
DS22	6	0.720	0.736	0.677	0.489	-0.0065
					0.966*	

k, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content; Excl, exclusion probability for the second parent (*combined Excl); F(Null) null allele frequency estimate

Primer sequences in Fraimont *et al.* (2015)

Observed Remating Frequency

Estimation of reliability of our four polymorphic microsatellite loci to detect remating performed with GERUDsim revealed that multiple mating was detected in more than 99% of the simulations varying the paternity skew, thus it is highly unlikely that remating was misclassified as single paternity.

Values from the ten sets of simulations are reported below in **Tab.2**.

GERUD revealed that 10 out 12 of the families (83%) were sired by more than one father, ranging from 2 to a maximum of 5 fathers detected per brood with an average of 3.25 (±0.46) fathers (**Tab. 3**). In **Fig.4** are reported GERUD outputs, with the proportions of offspring sired by each father, deriving from the known maternal and progenies genotypes with the most probable paternal genotype.

Table 2. Gerudsim 2.0 simulations. The software analysis is reported as variation of paternity skew set during the simulations and the percentage of correct number of sires detected.

Paternity skew	0	0,1	0,2	0,3	0,4	0,5	0,6	0,7	0,8	0,9
% of simulations (±s.e.)	99,3 (±0,13)	99,2 (±0,07)	99,4 (±0,12)	99,3 (±0,02)	99,3 (±0,09)	99,1 (±0,09)	99,3 (±0,11)	99,2 (±0,16)	99,0 (±0,08)	98,7 (±0,07)

Table 3. Gerud 2.0 paternity analysis inferred on 12 broods of wild-caught *D.suzukii*. For each family the brood size, the number of genotyped offspring, the minimum father number, likelihood and exclusion probabilities are reported.

Family	**Brood size**	**Genotyped offspring**	**Minimum father number**	**Likelihood of most probable solution**	**Exclusion probabilities all loci**
U3	38	20	5	4,172E - 44	0,92
U11	39	20	2	7,918E - 14	0,67
U12	32	18	2	9,862E - 14	0,83
U14	14	8	3	3,604E - 19	0,86
U16	56	56	4	8,416E - 50	0,91
U17	37	36	4	1,985E - 44	0,88
U20	36	36	5	1,289E - 41	0,94
U23	21	21	1	*	0,82
U24	21	12	2	*	0,84
U25	23	22	1	*	0,92
U26	56	54	5	2,459E - 48	0,88
U28	39	25	5	1,812E - 43	0,93

*Unique solution reconstructed by Gerud 2.0

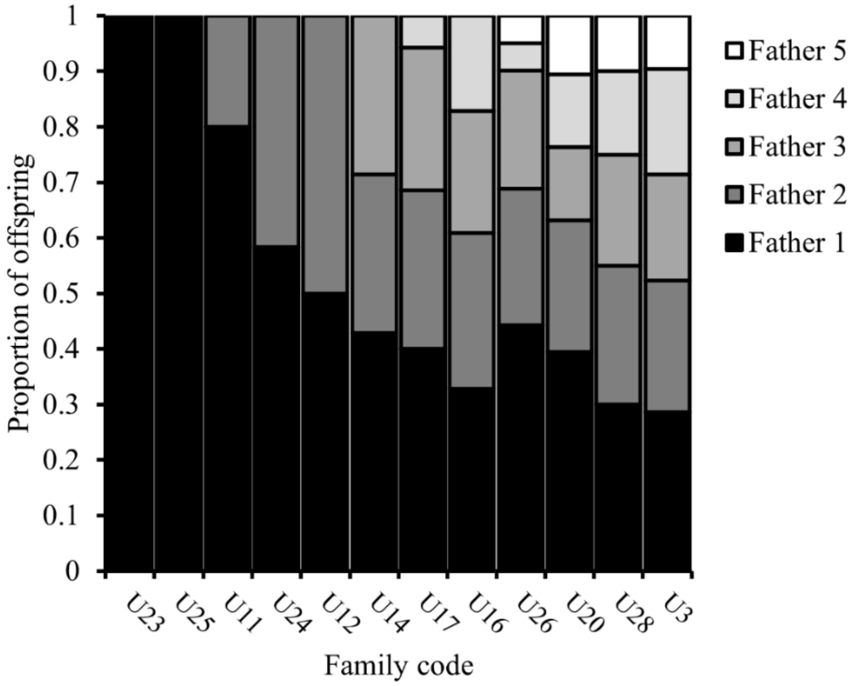


Figure 4. Patterns of multiple paternity in the broods of 12 wild-caught female *D. suzukii*.

SCARE computation revealed a high remating frequency among wild *D. suzukii* females. Paternity was estimated with a posterior mean value α= 5.03 (Confidence level 95% = 0.011) **(Fig.5a)**. Sperm displacement (β) was estimated to 0.504 (Confidence level 95% =8.502E-05) **(Fig.5b)**.

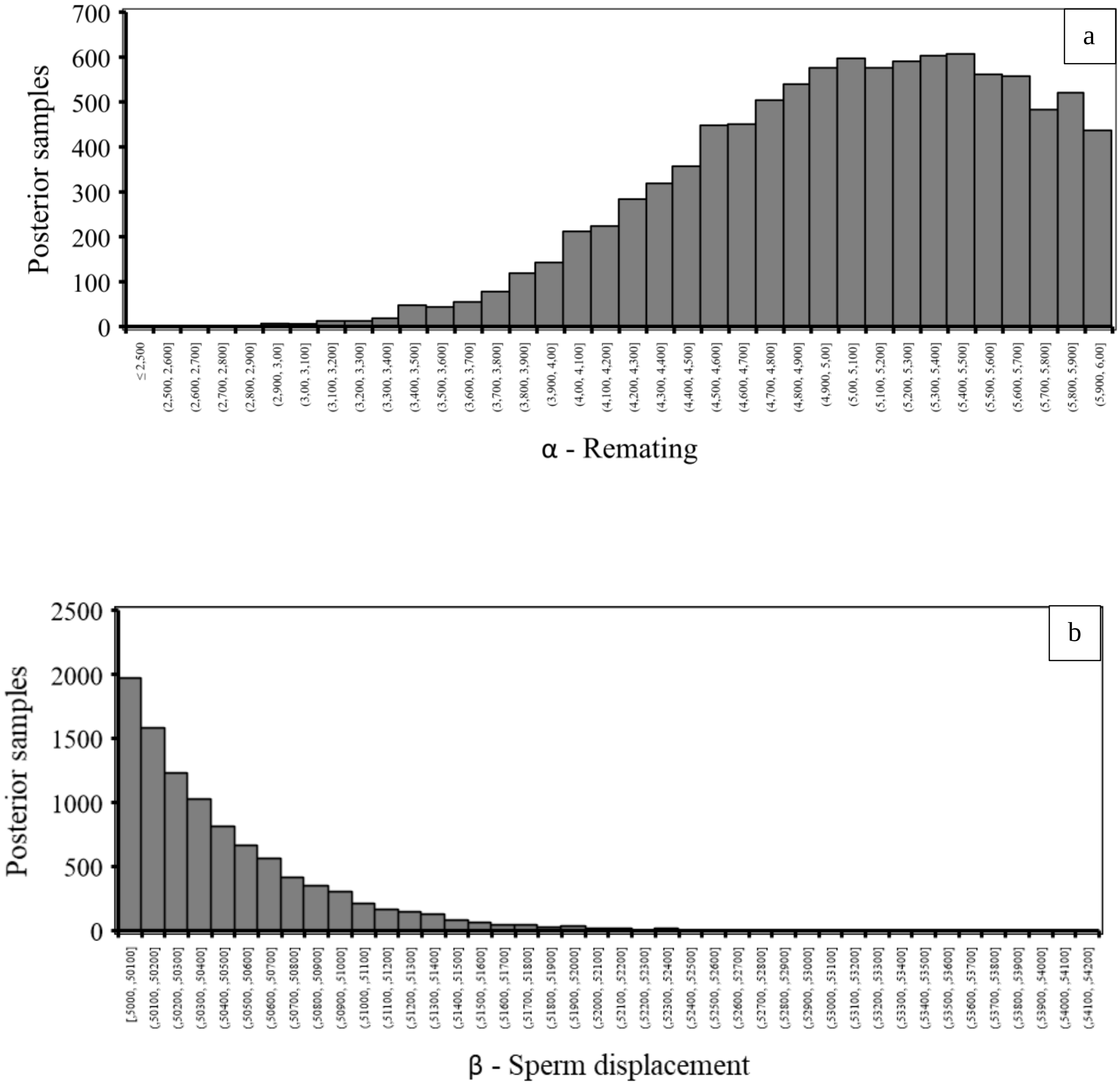


Figure 5. Histograms of the posterior mean distribution for a) α and b) β. Each histogram represents 10,000 samples computed for posterior mean.

Discussion

Paternity inference by genotyping microsatellite loci of offspring descending from wild-caught females of *D. suzukii* reveals extended multiple mating, giving evidence of the polyandrous behaviour of this species in nature. Using GERUD algorithm we found an average of three fathers per brood, with maximum values of five in some families. SCARE estimation of multiple paternity confirmed the polyandry of *D. suzukii*, with an estimated value (α) higher than GERUD.

Our choice of sampling natural population of *D. suzukii* at the beginning of summer is due to the knowledges on the ecology of this species became available in recent years.

After cold bottleneck period, *D. suzukii* can recover from overwintering diapause (Gutierrez, A.P et al. 2016) and from early spring onwards begin to breed on several spontaneous host fruits (Mitsui et al., 2010b, Kenis et., 2015, Panel et al., 2018), with a gradual transition from winter to summer morphotype (Grassi et al. 2018, Panel et al. 2018). In temperate regions during late spring, ripe *Prunus* species, particularly sweet and sour cherry trees, are the main host plants in which *D. suzukii* build up its new generations, leading to the first seasonal outbreak.

During our flies monitoring, infestation rate rapidly reached 100% within three weeks following the fruit colouring, suggesting the high reproductive output of this pest species (Wiman et al. 2014, Grassi et al., 2018). Moreover, the sampling area was surrounded by woodland, a natural habitat known to boost the pest population (Tonina et al., 2018b, Tait et al., 2020).

Another important thing to note is the lack of correlation between adult catches and infestation rate, stating the attractiveness competition between ripening fruits and liquid bait (Wong et al., 2018, Kirkpatrick et al., 2018, Clymans et al., 2019). Fruit sampling gives a better quantitative signal of insect pressure, indeed when cherries became overripe or fallen in a decaying state, adults catches shown a sharply increase.

Finally, it has been proven that period between spring and early-summer is a critical phase for *D. suzukii*, as adults survived overwintering period have a slow and weak reproductive recovery, therefore any pest management strategies implemented during this period, including SIT, may hinder or slow down late season population outbreaks, lowering crops chemical spraying with a direct benefit for agroecosystem. Furthermore, application of SIT later in the season could be compromised by the larger number of wild males in nature, requiring an overflooding sterile:fertile ratio that may be hardly achievable during inundative releases of sterilized mass-reared insects.

In this study, 83% of the wild-caught females produced offspring, confirming what previously reported by Grassi et al. (2018). Dissecting the specialized sperm-storage organs, seminal receptacle and the spermathecae, they found sperm presence in a high proportion of females captured from April onwards, also showing how males gradually gained sperm in their pair of testes. The gradual maturation trend of reproductive tract and oogenesis, followed by an increase in sperm presence in females, outline how this species may be sexually active early in season resulting in high levels of mated females when early host plants ripen. Furthermore, although first eggs can be laid in spontaneous berries, or other substrates, by overwintering females that have stored sperms from autumn matings (Panel et al., 2020), an increased proportion of captured females containing sperm in their storage organs throughout springtime means that: either a proportion of unmated winter morph females engage in mating (remating if their spermatozoa underwent fertility damage during cold period exposure or they are exhausted its stored sperm), or newly virgin summer morphs engage in mating, or both (Grassi et al., 2018, Panel et al., 2018). Recently, Panel et al. (2020) demonstrated that both the female morphotypes of *D. suzukii* were able to mate after being exposed to a cold period that mimicked a wintertime, producing offspring over spring-like condition with summer morphs having greater reproductive output than winter morphs, further confirmed by previous study of Leach et al. (2019b).

With our wild-caught females we found a gross reproductive output lower compared to more than 300 offspring per females obtained in life history experiments, in which males were given as continuous source for mating (Emiljanowicz et al., 2014). Despite variability in brood size among wild-caught females, the most numerous brood was less than 60 offspring. Eggs laid per day were in line with previous studies (Emiljanowicz et al., 2014, Tochen et al., 2014, Leach H., et al. 2019) and lifespan was on average a month, with some females reaching more extended period, as already reported for adult longevity of *D. suzukii* (Kanzawa 1939, Emiljanowicz et al., 2014).

Presumably, these differences imply either that some wild females had been alive for some time before our capture having already allocated a large part of their offspring in nature, or they have experienced sperm limitation at some point with the need of mating again to restore fertility success. Alternatively, this can be related to a different reproductive potential between wild-strain and lab-reared flies (Markow et al., 2002), thus requiring further investigations.

A wide range of ripening host plant from spring throughout the season creates abundant patches of breeding sites, boosting oviposition rate of the females (Kienzle et al., 2020) and the probabilities of sperm depletion, which would require remating in *D. suzukii*.

Furthermore, lack of niche competitors or native natural enemies is additional benefits to the invasive *D. suzukii* allowing rapid seasonal outbreaks that lead to high population size and likely increasing mating opportunity (Wiman et al., 2014).

Studies on *D. melanogaster* have shown that a mated female can use stored sperm to fertilize eggs for approximately two weeks, even though before entire sperm depletion the female usually remates within a few days after first mating (Lefevre at., 1962). During mating, male can transfer a few thousand of sperm to the female, but only a small fraction is stored, whilst the remaining part is discarded (Snook et al., 2004, Manier et al. 2010). Moreover, sperm load can be adjusted according to the female status (Lüpold et al., 2011) and in male-male competition (Garbaczewska et al., 2013). Restoration of female receptivity require partial sperm depletion after mating (Gromko et al., 1984) and it has been shown to be context dependent (Harshman et al., 1988).

Several molecular mechanisms are involved in the post-mating responses (Avila et al., 2011). Refractoriness in *D. melanogaster* is strongly influenced by the "sperm effect", that is the gradual release of the sperm-bound sex-peptide, a male seminal protein transferred to the female during copulation, which delay receptivity for several days after mating (Peng et al., 2005). A sex-peptide homologous has been found in *D. suzukii,* showing similar effect on refractoriness and oviposition of females (Schmidt et al. 1993). Physiological effect of these peptides may also have a key role in remating behavior of *D. suzukii* and the fecundity patterns may have been affected by these seminal male proteins which tends to increase ovulation soon after mating, decreasing receptivity in *D. suzukii* (Ohashi et al., 1991, Schmidt et al. 1993).

Revadi et al. (2015) firstly mentioned female remating in *D. suzukii* and the high propensity of males to remate within a short time window after previous mating. Lanouette et al. (2020) reported a similar male behavior, in which a single male was able to copulate with approximately six females in a 24-hour period, nevertheless they observed infrequent remating in females two and four days after mating.

Multiple mating in polyandrous species creates the arena for post-mating sexual selection, through sperm competition and cryptic female choice.

Sperm from different males compete for egg fertilization within the female storage organs to gain its own reproductive success against rival sperm. In *D. melanogaster*, during two-males mating experiment, offspring paternity was highly biased towards second male, a phenomenon called last-male sperm precedence, which involved physical sperm displacement (Manier et al., 2010). Nevertheless, laboratory studies typically tested only two successive males, so this may be quite different during recurrent and multiple mating by females (Laturney et al., 2018).

Indeed, beside this sperm competitive mechanism, in recent years the female choice has acquired considerable importance, resulting in a more complex dynamics between sexes for paternity fate (Mack et al., 2003, Lüpold et al., 2013, Manier et al., 2013).

Here, sperm displacement was estimated by SCARE with β value, giving no evidence of paternity skew with bias towards a particular male, a phenomenon that may be related to the female remating rate, favouring a more equally sperm allocation among fathers in a brood (Jones et al., 2003, Laturney et al., 2018). Of course, future studies will be necessary to establish how sperm use occurs over time, as well as clarify female refractoriness period after mating in natural population.

Since multiple mating may impact SIT programmes these aspects of the reproductive behaviour must be outlined to complete the framework in which this technique may be implemented.

Overall, our study offers a new perspective on reproductive behaviour of *D. suzukii*, giving first evidence of the polyandrous behaviour of this species, expanding its distinctive ecological characteristics and giving new insights concerning its management in global fruit production.

CHAPTER 4

Evaluation of vegetal species for integrating habitat manipulation practices against *D. suzukii* in cropping systems

Abstract

Habitat manipulation consist in agronomic tactics that modify cropping systems with the aims of reducing pest damage on cash crop, by exploiting and enhancing ecosystem services already present in the agroecosystem. Among manipulating practices, intercropping and trap plants are the most investigated and successful strategies implemented against crop pests, nevertheless little information about the use these practices to control *D. suzukii* are available.

Here, *Mentha x piperita, Origanum vulgare* L., *Thymus vulgaris* L., *Ocimum gratissimum* L. were selected as intercrops in blueberry orchards to examine their potential repellent effect on *D. suzukii*. Furthermore, we assessed *Prunus padus* L. as dead-end trap plant to limit infestation by *D. suzukii* on soft fruit crops production.

Overall, we found that intercropping with these species of Lamiaceae in blueberry crop was not effective to reduce the damage of *D. suzukii* on fruits, whilst a tree row of *P. padus* bordering the field edge was highly infested by *D. suzukii*, reducing the damage on a raspberry crop, as well as preventing immature stage of *D. suzukii* to develop to adulthood.

We further discuss pro and cons of managing *D. suzukii* through habitat manipulation practices on soft fruit cropping systems.

Introduction

Habitat manipulation resides on agronomic practices that aim to hinder the presence of the insect pest on crops and to favour a suitable environment in which the ecosystem service mediated by natural enemies is performed, thus preserving those resources necessary for their survival very often missing into the simplified landscape of the agricultural processes (Landis et al., 2000). Beyond the biocontrol purpose, other important practices related to the habitat manipulation attempt to enhance soil fertility or protect pollinators and endangered wild fauna (Tamburini et al., 2020).

Obviously, in-dept knowledges on the ecology of the target insects are essential and the road to success is very tortuous due to the real-world dynamics. However, in Europe, these strategies have been promoted by institutional bodies and implemented by growers through agri-environment schemes. Their effectiveness is currently monitored (Batáry et al., 2015).

Concerning *D. suzukii,* these techniques are still poorly investigated, nevertheless information about this species has grown enormously over the past ten years (Tait et al., 2021), opening new routes for its management at lower environmental impact.

Manipulation practices for *D. suzukii* may be carried out at different scales, from field to local or broader landscape level, as pest control is strictly interconnected among agroecosystem levels requiring a multi-step approach that consider this complexity (Thies C. and Tscharntke T., 1999).

For instance, sensitivity to abiotic stressor can be used to improve crop protection by manipulating some well-known cultural components, such as canopy pruning (Schöneberg et 2021), mulching (Rendon et al., 2020, McIntosh et al., 2022) and irrigation systems (Rendon et al., 2019b).

Focusing on sensory cues, recent studies have evaluated push-pull strategies for deterring *D. suzukii* from raspberry field (Wallingford et al., 2018, Cha et al., 2021), obtaining very promising results although further assessments on environmental and human health safety are needed (Stockton et al., 2021).

During the recent years laboratory screening on natural products have been actively pursued in order to find eco-friendly solutions for controlling *D. suzukii* and several compounds seem to have interesting properties (Dam et al., 2019). Nevertheless, as for essential oils, field trials have to be carried out to set up an optimized strategy enabling to guarantee the releases of an effective concentration for long periods (Lauren et al., 2015, Renkema et al., 2016, 2017, Gullickson et al., 2020, Wang et al., 2021). An alternative method could be using medicinal and aromatic plants by means of intercropping systems within the crop to protect (Beizhou et al., 2012).

D. suzukii is a highly polyphagous pest able to breed on a wide range of plant species (Poyet et al., 2015). However, the insect fitness and performance among these several host species can be highly variable, especially due to specific compounds that may compromise the proper physiological development because toxic or antinutritional (Fürstenberg-Hägg et al., 2013, Poyet et al., 2015).

Vegetal species that could be used as trap crops have been extensively investigated on horticultural and ornamental plant production, significant less on fruit crops (Sarkar et al., 2018).

New perspectives may derive by exploring the effect of trap plants on *D. suzukii*, such as the european bird cherry *Prunus padus* L. or firethorn *Pyracantha coccinea* M. Roem. planted nearby the crop field (Alhmedi et al., 2019, Ulmer et al., 2020).

Here, we evaluated the repellent/deterrent activity against *D. suzukii* of intercropping aromatic plants on blueberry crops. Plants choice fell on four common species belonging to the Lamiaceae family, *Mentha x piperita, Origanum vulgare, Thymus vulgaris, Ocimum gratissimum,* as the properties of their essential oil constituents on the behaviour of the insects are well characterized and suggested for field application.

Moreover, we carried out field trials in order to investigate the effectiveness of *Prunus padus* as dead-end trap plant nearby cash crops for reducing infestation severity by *D. suzukii*.

In Trentino region, the scarce availability of land for large plots of soft fruit crops has created a mosaic of small plots distributed in a heterogeneous landscape including urban areas and woodland, with cultural practices that need to be adapted to the diverse context. Given this irregular distribution of land property and the key role of semi-natural habitat for *D. suzukii*, we decided to evaluate two options for the use of dead-end-trap plants, either a tree row of bird cherry in pots adjacent to the crop tunnels or a hedgerow of *P. padus* along the woodland edge nearby crop.

Material and methods

- ***Evaluating repellence activity towards D. suzukii by intercropping aromatic plants on highbush blueberry crop***

Part 1 *(Intercropping with peppermint)*

Field trial with the aromatic plants *Mentha x piperita* L. (Peppermint, Lamiaceae) was carried out into a blueberry crop (*Vaccinium corymbosum* var. Duke) of 400 square meters located in Pergine Valsugana, Trento, Italy (46.047204, 11.261046) at 618 m asl (**Fig.1a**).

Crop field was subdivided in two equal and adjacent plots of four rows with six bushes per row.

Each highbush blueberry row in the "mint plot" was planted with *Mentha x piperita* stolons at both sides, whereas the other plot was used as "control plot".

Mint stolons were purchased from an organic plant nursery (Beber, Pergine Valsugana, Trento, Italy) and planted on soil on March 29, 2021 (**Fig.1b**).

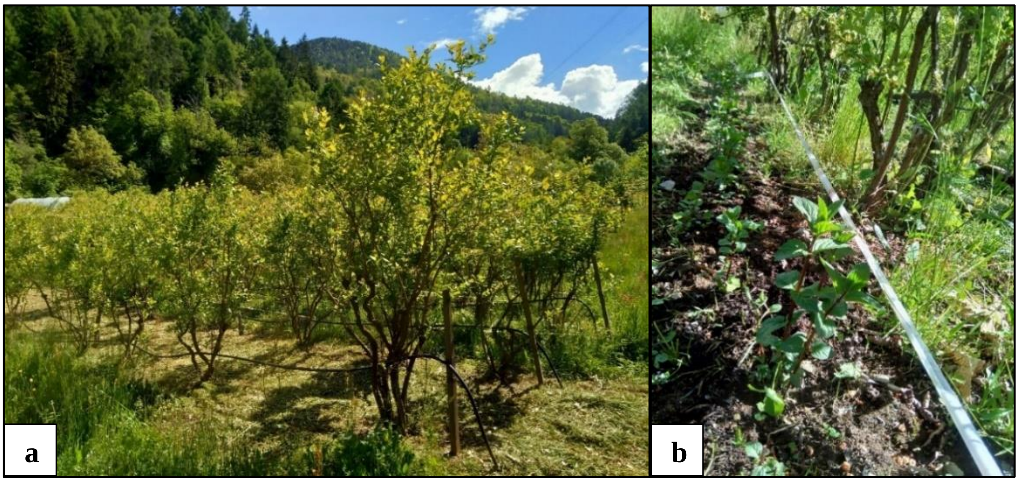


Figure 1. a) The highbush blueberry crop of the experimental trial**. b)** Peppermint stolons planted on soil during the preparative phase.

No insecticides were applied during the entire experimental phase and blueberries were regularly hand harvested every 2-3 days by growers.

Trial started according to fruit ripening on June 29 and was carried out during five consecutive weeks.

Infestation rate by *D. suzukii* was evaluated by sampling 30 blueberries per row once a week, equal to 120 total fruits/plot type/week.

Collected fruits were inspected under stereomicroscope searching for *D. suzukii* egg breathing spiracles. Infestation index was calculated as number of blueberries containing at least one egg on total fruits inspected.

Infestation data were compared using a repeated-measures Student t-test, comparing weekly infestation between different plots. Data were arcsine transformed to meet assumptions of normality.

All values are reported as mean±standard error.

Part 2 *(Intercropping with oregano, thyme and basil)*

A field trial with the aromatic plants *Origanum vulgare* L. (Oregano, Lamiaceae), *Thymus vulgaris* L. (Thyme, Lamiaceae), and *Ocimum gratissimum* L. (African basil, Lamiaceae) was conducted into a blueberry crop (*Vaccinium corymbosum* var. Brigitta) of 1300 square meters located in Fornace, Trento, Italy (46.123481, 11.211303) at 715 m asl (**Fig. 2a**)

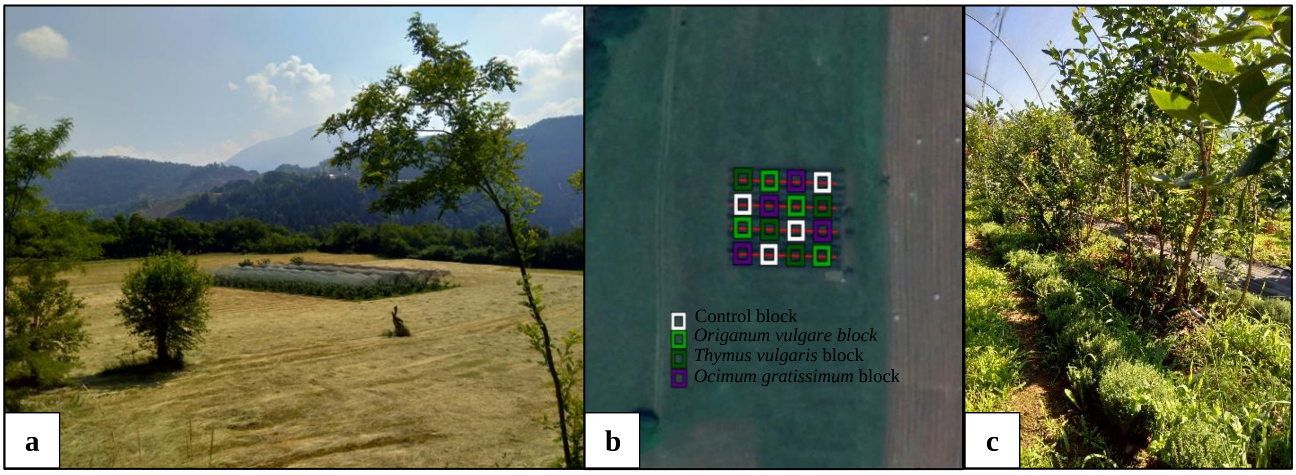


Figure 2. a) View of the blueberry crop field. **b)** Latin square arrangement of aromatic plant blocks, **c)** Thyme block

Crop field was organized on 12 rows of highbush blueberry, about 40 m long and spaced 2 m apart. Distance among bushes in a row was 2 m.

Experimental design was planned with a latin square arrangement of aromatic plant blocks and a control block along four blueberry rows (**Fig.2b**), in such a way having two rows between consecutive series of experimental block type. Each block was 6.7 m x 2.6 m and comprised four linear blueberry bushes with aromatic plants along both sides. For *O. vulgare* and *T. vulgaris* block, 25 plants per side 30 cm apart (total 50 plants/block), whereas for *O. gratissimum 37* plants per side, 15 cm apart (total 74 plants/block). Blocks on the same row were spaced by a buffer zone of about 4 m, which included a blueberry bush (**Fig.2c**).

Plants were purchased from a plant nursery (Tuttoverde, Trento, Italy) and transplanted on May 6, 2021. Following days after the transplant, each plot was fertilized once with a slow-release fertilizer (Biogard Plus, Lienz, Austria).

No pesticide spraying was applied during the entire experimental phase and blueberries were regularly hand harvested every 2-3 days by growers.

Trial started at fruit colouring on July 22 and was carried out during three consecutive weeks.

Infestation rate by *D. suzukii* was evaluated by sampling 15 blueberries per block once a week, corresponding to 60 total fruits/block type/week.

Collected fruits were inspected under stereomicroscope searching for *D. suzukii* egg breathing spiracles. Infestation index was calculated as number of blueberries containing at least one egg on the total fruits inspected.

Infestation data were compared using a repeated-measures ANOVA model, comparing weekly infestation among different blocks. Data were arcsine transformed to meet assumptions of normality.

All values are reported as mean±standard error.

- ***Assessment of Prunus padus as potential dead-end trap plant against D. suzukii***

Two field trials, on blueberry and on raspberry, were carried out at two selected crop sites 1 km apart.

At one site (*PpBlue*), located in Pergine Valsugana, Trento, Italy (46.078299, 11.270495) at 610 m asl., a blueberry crop (*Vaccinium corymbosum* var. Drapper) of 2200 square meters was selected and subdivided in four adjacent tunnels.

The other site (*PpRasp*), located in Sant'Orsola Terme, Trento, Italy (46.084814, 11.280378) at 708 m asl, comprised two crop tunnels of 900 and 1000 square meter of floricane raspberry (*Rubus idaeus*, var. Lagorai) 200 m apart

Figure 5. a) *P.padus* tree row adjacent to blueberry tunnel. **b)** Detail of the irrigation system adopted for *P. padus* in pot at the *PpBlue* site. **c)** Appearance of *P.padus* during its proper ripe stage.

No insecticide treatments were applied at both experimental sites and soft-fruit harvesting were regularly schedule by growers.

Concerning *PpBlue* site, we set a row 52 m long (30 plants, 2 m apart) of *P. padus* adjacent to two out four blueberry tunnels (DETP-plot) (**Fig.5a**). Trees were brought from the nursery to the experimental field on April 13 and were transplanted in pots of 40 cm diameter, connecting them to the drip irrigation system (**Fig.5b**). The control plot was constituted by the nearby two crop tunnels. *P. padus* bore ripe berries approximately from mid-June to mid-July. Infestation rate on blueberry was checked once a week starting at fruit colouring from June 26 until July 29.

We collected three samples of 30 blueberries each per plot once a week. Furthermore, along with crop fruits samples we sampled a total of 100-150 berries of *P. padus*, collecting fruits from all the linear distribution of trees row (**Fig.5c**).

In *PpRasp* site, we created a hedgerow 84 m long of *P. padus* trees close to the semi-natural area and 3-5 m far from raspberries tunnels (DETP-plot). Hedgerow was constituted by a total of 43 *P. padus* trees 2 m apart (**Fig.6 a,b**). Trees were transplanted in soil on March 8. Transplanted trees were fertilized once with a slow-release fertilizer (Biogard Plus, Lienz, Austria).

P. padus bore ripe berries from the beginning of July till the end of month. Infestation rate on raspberry was checked once a week starting at fruit colouring on July 1 for five consecutive weeks. Five samples of 10 fruits were collected on five different raspberry rows inside crop tunnel (**Fig.6c**), both for control plot and trap-plant plot. 100-150 of *P. padus* fruits were also weekly sampled.

After sampling, blueberries, raspberries and *P. padus* fruits were inspected under magnification.

The number of fruits containing eggs on total fruits per samples provided the infestation index. All the infested fruits were incubated in ventilated plastic container (21 x 21 x 8 cm; length, width and height) for two weeks in order to evaluate *D. suzukii* emergences. A week after field sampling fruits in the containers were checked every 48 h, thus *D. suzukii* pupae and adults were collected and counted.

Infestation data were compared using a repeated-measures Student t-test, comparing weekly infestation between different plots. Data were arcsine transformed to meet assumptions of normality.

All values are reported as mean±standard error.

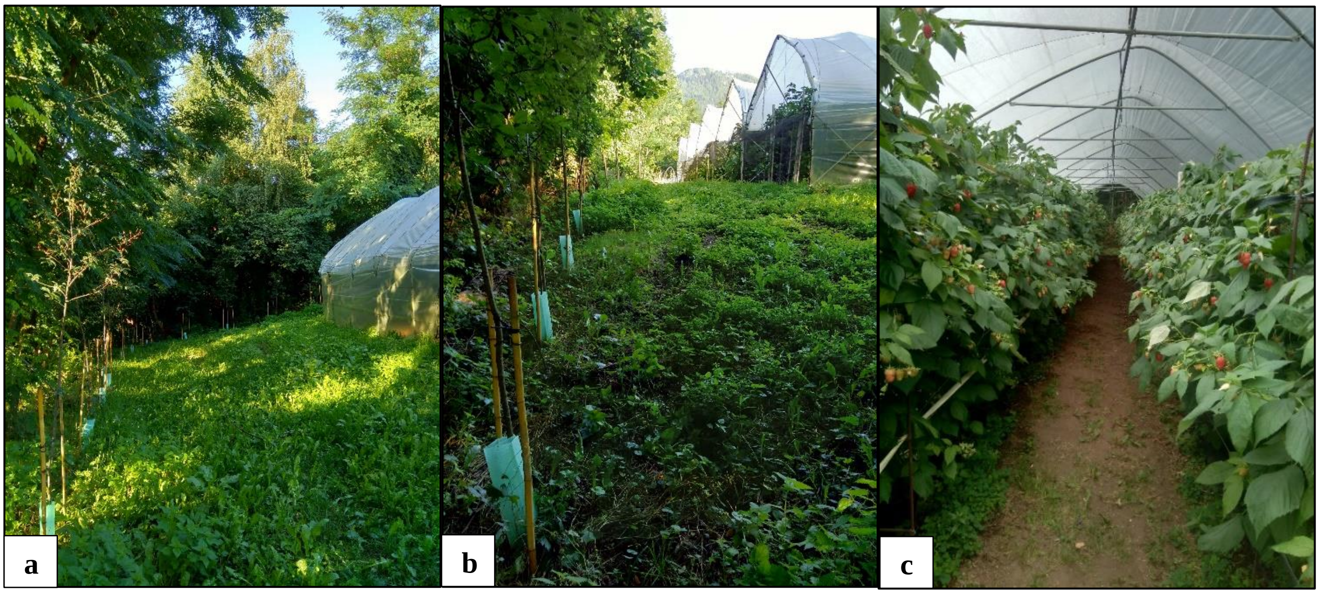


Figure 6. a, b) Edges of *P. padus* after transplantation. **c)** Raspberry tunnel in DETP-plot in *PpRasp* site.

Results

Blueberry intercropping with peppermint

Peppermint plot shown no difference on blueberry infestation due to *D. suzukii* when compared with the control plot over the entire sampling period (six sampling dates: June 29th: t= 0.88, d.f.=6, *p*=0.41 ; July 7th: t= 1.43, d.f.=6, *p*=0.20 ; July 14th: t= 0.40, d.f.=6, *p*=0.70 ; July 21th: t= 0.57, d.f.=6, *p*=0.59 ; July 29th: t= 0.59, d.f.=6, *p*=0.58). Data are reported in **Fig.3**

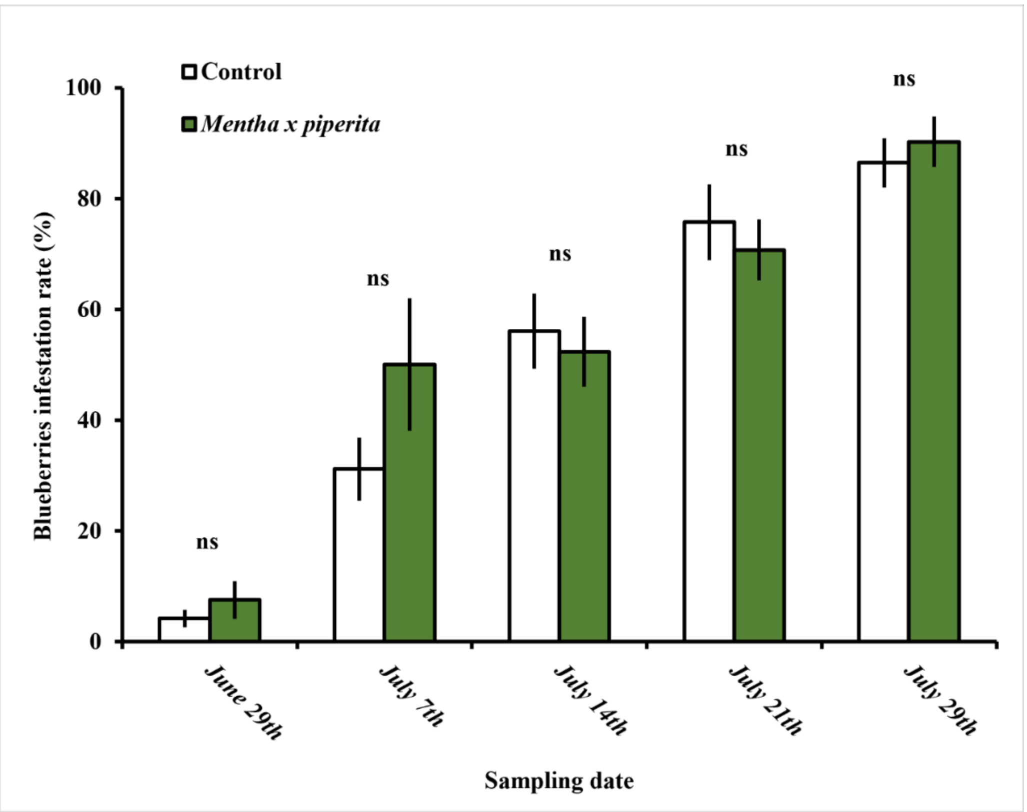


Figure 3. Infestation of *D. suzukii* on highbush blueberry crop when intercropped with peppermint and without intercrop. Bar charts for control and intercropped crop are reported in white and in green, respectively. Error bars report standard error. (ns: not significant).

Blueberry intercropping with oregano, thyme and basil

Intercropping blocks with oregano, thyme and basil did not decrease infestation rate by *D. suzukii* on blueberries compared with control block during the three consecutive weeks of fruit sampling; there were not significant differences among the diverse aromatic plant blocks (July 29th: $F_{3,12}=0.16$, $p=0.92$; August 5th $F_{3,12}= 2.81$, $p=0.08$; August 12th : $F_{3,12}=0.47$, $p=0.71$) (**Fig.4**).

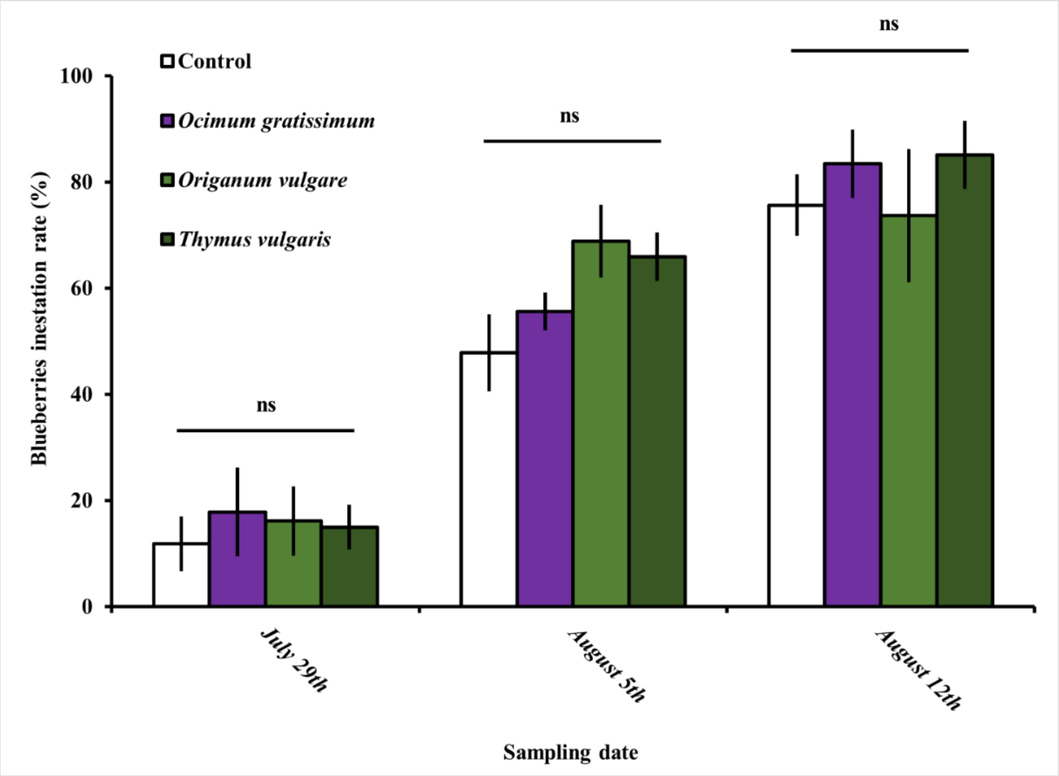


Figure 4. Effect of intercropping with aromatic plants on infestation rate by *D. suzukii* on the *Vaccinium corymbosum* crop. Bar charts are reported in white for the control plot, purple for basil plot, light green for oregano plot and dark green thyme plot. Error bars report standard error. (ns: not significant).

Assessment of P. padus as potential dead-end trap plant against D. suzukii

At *PpBlue* site fruit infestation on *P. padus* begun on June 24th with 6.4% (±1.8) of infested fruits, 35.1% (±1.01) on July 1st, 92.7% (±1.8) on July 8th and 93.1% (±0.8) on July 15th. During last two weeks of the field trial (on July 22nd and on July 29th) all the fruits of *P. padus* were lost due to natural senescence process.

Blueberries infestation did not differ between plots the consecutive sampling weeks (July 1st: t=1, $d.f.$=4, p=0.37; July 8th: t=2.15, $d.f.$=4, p=0.10; July 22th: t=0.51, $d.f.$=4, p=0.64; July 29th: t=2.16, $d.f.$=4, p=0.10), except for sampling date July 15th in which the plot adjacent to the *P. padus* row shown an higher infestation compared with control plot (t=4.25, $d.f.$=4, p=0.01). (**Fig.7**).

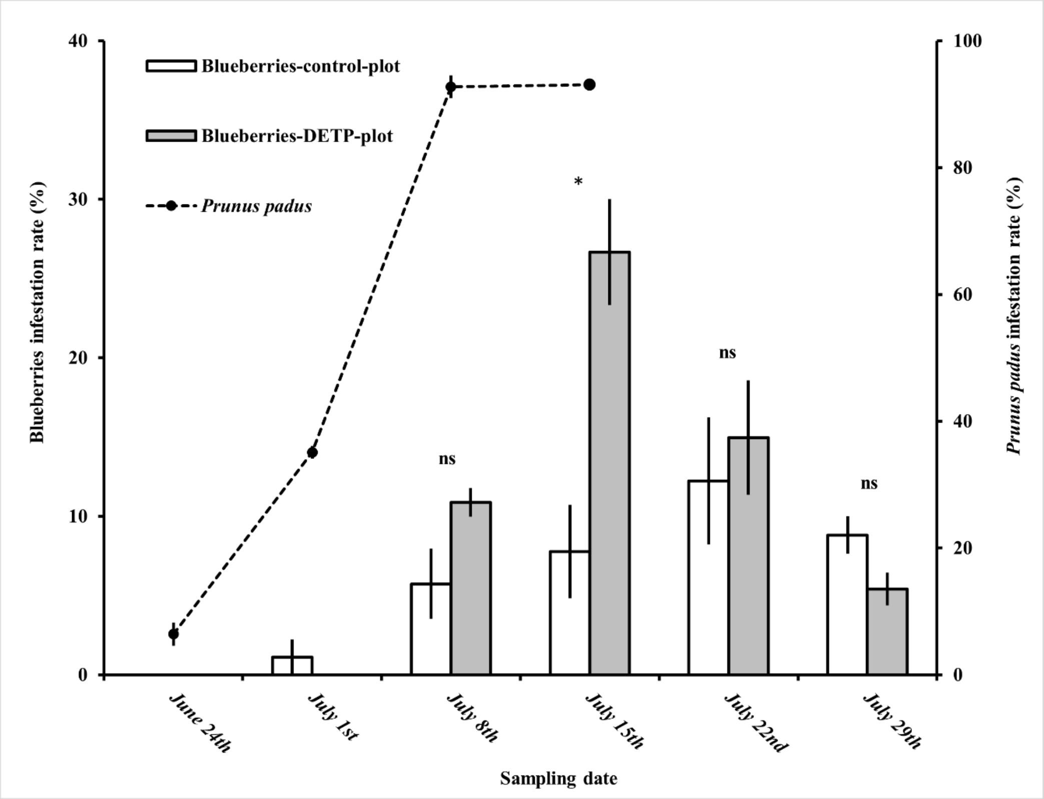


Figure 7. Average infestation rate of *D. suzukii* on *P. padus* and blueberries in plot with dead-end trap plantation (DETP) and in control plot during the six sampling weeks at *PpBlue* site. Bar charts for control and DETP crops are reported in white and in grey, respectively.
Error bars report standard error. (**p*<0.05, ns: not significant).

Overall, we collected 459 fruits of *P. padus*, of which 282 carried eggs by *D. suzukii*. Only seven adults of *D. suzukii* emerged from infested fruits, two on July 8th (102 infested fruits) and five on July 15th (135 infested fruits), equal to a *D. suzukii*/fruits ratio of 0.05 and 0.01, respectively.
At *PpRasp* site fruit infestation on *P. padus* begun on July 1st with 10.7% (±0.7) of infested fruits, 15.5% (±0.9) on July 8th, 37.9% (±1.3) on July 15th and 51.2% (±0.7) on July 22nd. Similarly, as for *PpBlue* site during last week of the field trial (July 29th) *P. padus* lost all the fruits due to natural senescence process.
Raspberries infestation did not differ between DETP- and control plot during the first and the last sampling week (July 1st: t=2.25, $d.f.$=8, p=0.054; July 29th: t=0.21, $d.f.$=8, p=0.8), however we found a significant lower infestation rate in DETP-plot than in the control plot during the sampling dates July 8th (t=3.06, $d.f.$=8, p=0.015), July 15th (t=8.76, $d.f.$=8, p<0.001) and July 22th (t=10.91, $d.f.$=8, p<0.001) (**Fig.8**).

Furthermore, we observed a better synchrony between trap plant and cash crop on *PpRasp* site than *PpBlue*. Moreover, there was a more similar and stricter trend on infestation rate both on *P. padus* and raspberries.

During the entire field trial we sampled a total of 603 *P. padus* fruits, of which 185 were infested by *D. suzukii*. No *D. suzukii* emerged from infested fruits.

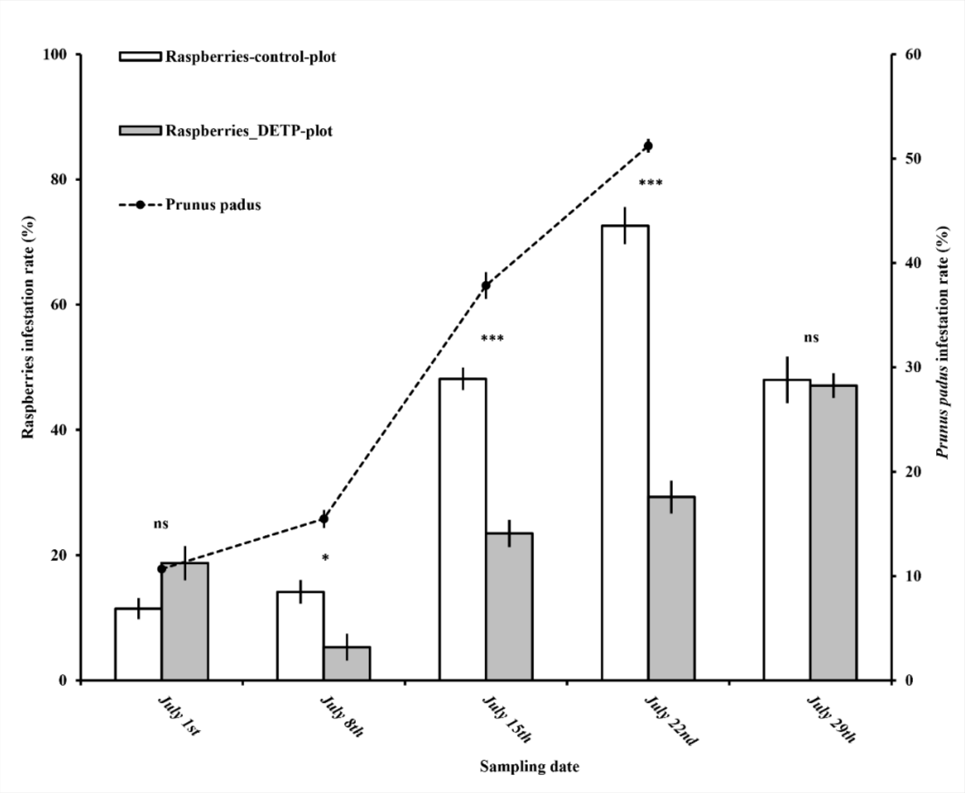


Figure 8. Average infestation rate of *D. suzukii* on *P. padus* and raspberries in plot with dead-end trap plantation (DETP) and in control plot during the six sampling weeks at *PpRasp* site.

Bar charts for control and DETP crops are reported in white and in grey, respectively.

Error bars report standard error. (*p<0.05, ***p<0.001, ns: not significant).

Discussion

Habitat manipulation techniques are quite varied, and their interaction may convey a synergistic effect to crops and environment, limiting the proliferation of insects harmful and benefiting from many ecosystem services.

Previous studies revealed contrasting results in peppermint intercropping, with a positive effect deriving from a reduction of *D. suzukii* in fruits (Gowton et al., 2021) or a negative effect, increasing *D. suzukii* infestation and the abundance of a Miridae pest on strawberries (Renkema et al., 2020b).

Our field trials with several plant species belonging to the Lamiaceae family, comprising also peppermint, did not find evidence in reducing *D. suzukii* infestations on blueberry crops, with an overall null effect on crops.

Furthermore, peppermint has been shown to increase pollinators and ground dwelling arthropods, but its essential oil seems to be noxious towards a pupal parasitoid of *D. suzukii (*Gowton et al., 2020, 2021).

These apparently irreconcilable results will require further studies in order to evaluate what could be the real benefit of this manipulation technique in the field, testing other plant species and detecting possible adverse effects on *D. suzukii*, but also on beneficial insects. Moreover, volatiles emission rates from aromatic and medicinal plants depend upon several genetic and environmental factors, as well as biotic stressor stimuli and physiological age (Dudareva et al., 2006). Latter may be related to the divergent outcomes during field trials, as to date no standardized field references are available to be confident that plants emitted the proper concentration necessary for the repellent activity against the pest in a specific growth phase after transplants. Future studies will have to take into account also these variables, carrying out trials with plants of different ages, as well as using germplasm accession with known emission profiles.

Interest towards plant species acting as trap crop for limiting population outbreaks of *D. suzukii* has grown considerably in recent times and laboratory/semi-field experiment confirmed the potential efficacy (Alhmedi et al., 2019, Ulmer et al., 2020), but no field trial confirmed their effectiveness yet.

Our field studies with European bird cherry highlighted some important aspects for a trap cropping system on soft fruits cultivation, which were not brought to light in the preliminary tests.

Temporal synchrony between cash and trap crop requires to be accurately schedule, indeed the ripening/ripe period of *P. padus* lasted three to four weeks between end of June and July, after that, fruits lost their turgidity starting a rapid senescence process that leads to fruits dropping. This feature may be essential since *D. suzukii* females have shown a greater preference for *P. padus* fruits during the ripening stage than the ripe stage (Alhmedi et al., 2019).

In the raspberry trial (var. Lagorai) trial we observed a better overlap compared with blueberry trial (var. Draper), with the ripening/ripe stage of *P. padus* lasted for most of the growers’ harvest time in the former, probably influencing the positive trial outcome that has been shown.

Soft fruits crop may also influence the effectiveness of the trap cropping system, as *D. suzukii* has shown different preference among fruit type/cultivars (Lee et al., 2011b, Burrack et al., 2013, Olazcuaga et al., 2019).

Spatial arrangement of trap planting on field border may be the optimal solution, albeit should be spaced from the cultivation tunnel (Aluja et al., 1997, Potting et al., 2005). Indeed, during this trial we stressed the role of distance, planting *P. padus* very close to the blueberry tunnels, noticing an increasing tendency of *D. suzukii* infestation on dead-end trap plant plot that became significant when infestation on *P. padus* reached its maximum level (>90%) the last two weeks of the ripening period. This issue may be similar to what has been reported in the case of mass trapping too close to the crop (Hampton et al., 2014). Based on our field evidence, when *P. padus* fruits were no more available on plant we did not notice an increase in infestation on blueberries adjacent to bird cherry row as consequence of a potential massive migration towards cash crop. These observations may be explained as the fruit dropping from trap plants occurred over time, triggering a slow and gradual loss of retention of the insect pest from *P. padus* trees, redistributing its population over the entire cultivated area with no significant infestation difference between control and treated plots. Speculations however need to be deepen and further explored for confirmation.

Planting *P. padus* on the entire perimeter surrounding cash crop, although it would be a very functional perspective, may not be feasible on soft fruits production mainly due to bird cherry tree growth habit and compatibility with handling in agronomic practices, also exceeding in shading of crops. The solution would be the implementation of hedgerows in those hotspots of the landscape interfacing the natural and the cultivated environment from which *D. suzukii* spreads (Tait et al., 2020). In this context an hedgerow of *P.padus* would probably also act as pesticide anti-drift barrier to protect beneficial macrofauna. *P. padus* might integrate the pull part in a push and pull strategies against *D. suzukii* where oviposition deterrents are applied (Khan et al., 2011, Stockton et al., 2021), Certainly, these preliminary studies bode well for the adoption of a dead-end trapping crop system for the management of *D. suzukii*, nevertheless it will be necessary to confirm the efficacy in different places and with different crop hosts. Furthermore, it is advisable to clarify which modalities are involved in the attraction, retention and mortality of the insect pest, as well as investigate the risks and benefits of adding these plants into agroecosystem in terms of food web dynamics and plant disease (i.e., crop pathogens and pests).

Moreover, to increase their effectiveness, trap plants need to be perfectly integrated with cultural practices that make more hostile the cultivated field to *D. suzukii* (Diepenbrock et al., 2017, Rendon et al., 2019b, 2020, Schöneberg et al., 2021), or by means of techniques focused on behavioural avoidance (Stockton D.G. et al., 2021), thus reducing the pest preference towards crops.

CHAPTER 5

Augmentorium*: a sustainable technique for conservation biological control of *D. suzukii

Abstract

Sanitation of crop field by means of removal and bagging infested fruits is one of most suggested management practice to reduce risk of infestation by *D. suzukii*.

However, infested fruits could be recycled into the *augmentorium*, a technique that aims to sequester adult flies while allowing the parasitoids to escape, thus facilitating biological control through conservation of natural enemies.

For this purpose, we designed a prototype of *augmentorium* that may be well adapted to *D. suzukii* and its parasitoids complex.

Performance and efficiency of the *augmentorium* were evaluated with some preliminary field trials, highlighting the suitability of the technique to sustain all the major indigenous pupal parasitoids.

Furthermore, our field analysis revealed that functioning of *augmentorium* may be extended by coupling this technique with releases of *Trichopria drosophilae.*

Augmentorium may become a practical tool adopted by growers for the conservation of *Drosophila* parasitoids with the purpose of an agroecological crop protection.

Introduction

Expanding the view from field to a larger scale, new insight can be reached by means of understanding how the management of the habitat surrounding fields could offer a contribute to the biocontrol of *D. suzukii* (Whitehouse et al., 2018, Schmidt et al., 2019, Trivellone et al., 2019, Wolf et al., 2020, Siffert et al., 2021), reducing pest population in natural and semi-natural habitats that may become huge pest reservoirs if not properly contained by natural enemies (Haro-Barchin et al., 2018, Tait et al., 2020).

During the last years several faunistic surveys have highlighted that outside its native area, few parasitoids are able to effectively parasitize *D. suzukii*, almost exclusively pupal parasitoids belonging to the Diapridae and Pteromalidae families with a wide host range (Miller et al., 2015, Knoll et al., 2017).

Among these, *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae) has been subjected to field experiments, suggesting its commercial use for augmentative releases (Rossi Stacconi 2018, 2019, Gonzalez-Cabrera et al., 2019).

Nonetheless, the combination of a complex of indigenous parasitoids with their peculiar life-history strategies may be further explored to improve their potential biocontrol of the pest (Wang et al., 2016, Wolf et al., 2021).

Furthermore, the management of cull fruits, especially those infested by *D. suzukii*, become an essential cultural practice for sanitation the cropping area in order to reduce pest recurrence (Leach et al., 2018). Beside fruit wastes deriving from crop fields, also wild flora fruits may be reused within an integrated pest management process, as it plays an important role for *D. suzukii*, particularly those found nearby the crops (Leach et al., 2019a, Grant et al., 2021).

Conservation of indigenous parasitoids for biocontrol purpose in open field may be pursued combining what previous mentioned using the *augmentorium*, an innovative and very practical technique developed for controlling tephritid fruit flies in Hawaii (Klungness et al., 2005, Jang et al., 2007), and here adapted to the peculiar characteristics of *D. suzukii* and of its parasitoids.

Materials and methods

Mesh size efficiency

Netting efficiency in sequester insect pest and letting pass parasitoids was evaluated by means of a preliminary laboratory experiment, testing the 25-mesh net (hole size: 0,97 x 0,83 mm, 3325BT BioRete Agrotextiles&Techtextiles Arrigoni, Uggiate Trevano, Italy), already used in crop protection as exclusion netting for the management of *D. suzukii* in Trento province, thus commercially available for growers.

To evaluate sequestration/escaping index of the gauge of mesh we cut rectangular pieces of 25-mesh net and substituted the original nylon netting on the lid of pint-size plastic cups (BugDorm, MegaView Science Co., Ltd., Taichung, Taiwan) with our net.

Plastic cup was placed inside insect cage (BugDorm-4F3030 Insect Rearing Cage, MegaView Science Co., Ltd., Taichung, Taiwan). Inside each cup a standard number of insects were housed. Ten individuals per cup (sex-ratio 50:50) were used and five replicates per species were done. Insect species tested were *D. suzukii*, *Pachycrepoideus vindemmiae* (Rondani, Hymenoptera: Pteromalidae), *Trichopria drosophilae* (Perkins, Hymenoptera: Diapridae).

The lab strain of *D. suzukii* was established by an initial population collected in S. Michele all'Adige (Trento, Italy). Strain was reared on artificial *Drosophila* diet slightly modified from Woltz et al. (2015).

T. drosophilae strain was kindly provided by Biobest Group (Westerlo, Belgium) (**Fig.9a**).

P. vindemmiae strain was established from individuals emerging from *D. suzukii* pupae collected by means of banana sentinel traps. Both the pupal parasitoids were reared on 1-day old *D. suzukii* pupae and fed with a 10% honey-water solution.

Insect colonies were kept in a climate-controlled chamber at 22 ± 1°C, 70±10% RH, and 16:8 L:D photoperiod.

In order to attract insects outside the plastic cups through the net, the cups were held horizontally and parallel to the cage floor, nearby to the cup lid was a Petri dish containing strawberry and a banana slice for *D. suzukii*, whereas a small Petri dish containing *Drosophila* diet with 3rd instar larvae/1-day old pupae of *D. suzukii* was used for pupal parasitoids. Laboratory assay lasted 12 h. Insects inside and outside the plastic cups were counted every 2h.

Design of the augmentorium prototype

Augmentorium was designed in order to be easily realized by growers and other interested users with common available materials (**Fig.9b**).

A plant pot (Ø = 30 cm) was drilled in four equally distant points. Furthermore, tiny holes were realized at the bottom for the outflow of water and fruits juice produced.

A metal mesh cylinder (h = 50 cm) was fixed to the plant pot with iron wire.

The structure was wrapped with a cut out of 120 x 120 cm of insect net, joining the net edges to create a thick border and a hermetic seal with stapples.

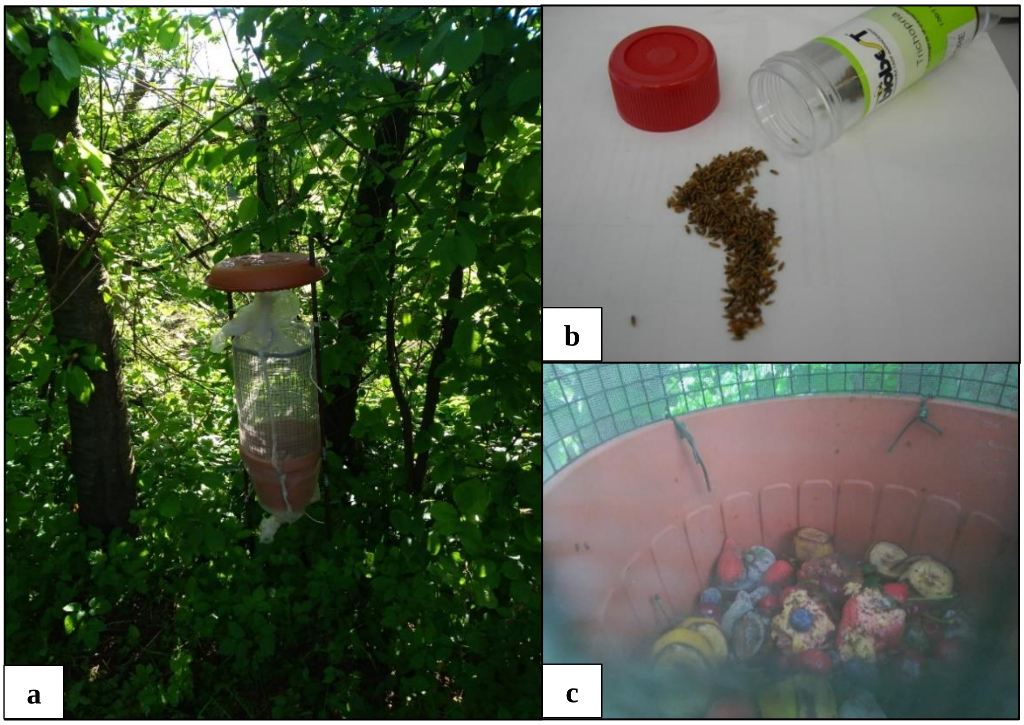


Figure 9. a) The *augmentorium* prototype inside a semi-natural area. **b)** *D. melanogaster* pupae parasitized by *T. drosophilae.* **c)** Internal fruit mix during the preliminary field trial.

Preliminary field trial

Field trials were carried out on two 3000 square meters plots of unmanaged mixed orchards on Valsugana. Sites were 1 km apart and they were selected based on similar conditions both of vegetation cover and *D. suzukii* pressure. At one plot we tested only the *augmentorium* (AUG) while in the other the *augmentorium* was combined with *T. drosophilae* addition (AUG+Td).

As for biological control with *T. drosophilae* it has been suggested to start early in the season to be effective against *D. suzukii* (Rossi et al., 2019), the field trial begun on April 16th (week 16) 2019. In each plot, two *augmentoria* were hung on iron rods placed inside spaces obtained on the hedgerows used as field borders on the experimental sites. *Augmentorium* pot was filled with universal potting soil. As naturally infested fruits by *D. suzukii* were poor available, each augmentorium was weekly managed in a specific way. During the first five weeks a “SWD starter” was loaded in each *augmentorium*. “SWD starter” was mainly composed of 10-20g of artificial diet infested by 3rd-instar larvae of *D. suzukii* and 1-2 kg of commercial fruits (banana and strawberry)

(**Fig.9c)**. From the sixth week onwards 1-2 kg of cherries or soft fruits infested by *D. suzukii* were collected from nearby crops substituting the “SWD starter”.

On week 16, 18 and 20, approximately 50-100 *T. drosophilae* (Biobest, mix of parasitized pupae/emerged adults, sex-ratio 50:50) per augmentorium/week were added at AUG-Td plot.

Parasitoids monitoring was carried out by means of sentinel trap composed of banana slice pre-infested by 10-20 3rd-instar of *D. suzukii* close to pupation, laid on a solid agar layer inside a white plastic cup. The cup was covered with a 20-mesh net and placed inside Delta trap.

We carried out two different methods for parasitoids monitoring during the field trials: an external sampling, in which we deployed three sentinel traps per *augmentorium*, about 10 meters away, two at both lateral side and a central one in front of the *augmentorium* and an internal sampling with a smaller version of the sentinel traps above-mentioned, using a single 100 mm x 15 mm Petri dish instead of the plastic cup that were lowered inside the *augmentoria*. Concerning, the external sampling all the sentinel traps were hung 30 cm from the ground level.

External monitoring was carried out during the two consecutive weeks (21-23) after the first catches of *D. suzukii* at experimental sites (by Drosotraps and apple-cider vinegar/red wine liquid bait, data not shown), then during the ripening period of cherries (early ripe stage on week 24/25 and late ripe stage on week 26/27) and during last two weeks of the field trials (30-32). Internal monitoring was carried out as above but without the earlier two samplings. During the sampling periods, traps were serviced weekly.

Following the period of exposure in the field the sentinel traps were incubated in climatic cells 22 ± 1°C, 70±10% RH, and 16:8 L:D photoperiod for 35 days.

Emerged insects were identified and conserved in absolute ethanol.

When field trial ended on August 7th, all the *augmentoria* were brought to the laboratory.

Internal content was removed and incubated for five weeks inside an insect rearing cage (BugDorm-6E, MegaView Science Co., Ltd., Taichung, Taiwan) at room temperature.

Twice a week cages were inspected, emerging Drosophilidae and parasitoid wasps were aspirated and identified.

Insects obtained from sentinel traps on each experimental plot were pooled and data used to a qualitative analysis of the entomofauna composition at specific time point of sampling.

Results

Mesh size efficiency

On five replicates, all individuals of both species, *T. drosophila* and *P. vindemmiae,* were able to exit from plastic cup passing throughout mesh gauze, resulting in 100% of escaping parasitoids.

Concerning *D. suzukii*, we further confirmed the total sequestering capacity of the mesh size by means of five replicates in which no adult was able to pass the netting, thus remaining sequestered inside the plastic cup.

Preliminary field trial: external monitoring

External parasitoid monitoring revealed that most abundant species were *T. drosophilae* and *P. vindemmiae* both at AUG and AUG+Td plots.

On week 24/25, 25% of parasitoids sample belonged to an unidentified *Spalangia* spp. (probably *S. erythromera*, to be confirmed by DNA barcoding).

At AUG+Td plot, on 4 out 6 monitored weeks *T. drosophilae* was collected by means of sentinel traps (ranging from 47-80% of total parasitoids) compared with AUG plot in which *T. drosophilae* was detected only on week 26/27 (13% of total parasitoids). During the cherries ripe period (week 24-27) *T. drosophilae* was no detected in sentinel traps at AUG+Td plot.

P. vindemmiae was the only species sampled on AUG plot (87-100% of total parasitoids), except for week 26/27. On AUG+Td, *P. vindemmiae* ranged from 20-100% of total parasitoids during the six sampling weeks (**Fig.10**).

Preliminary field trial: internal monitoring

During internal parasitoid monitoring, two species of Drosophila parasitoids emerged from sentinel traps, *T. drosophilae* and *P. vindemmiae*.

T. drosophilae was detected during cherries ripe period (80% on week 24/25 and 5% on week 25/26) inside augmentoria at AUG plot, whereas on 4 out 4 weeks in augmentoria at AUG+Td plot (range: 9-90%). No parasitoids were sampled on week 30/31 and only *P. vindemmiae* emerged on week 31/32 at AUG plot.

P. vindemmiae represented between 20-100% of total parasitoids at AUG plot and 10-91% at AUG+Td plot (**Fig.10**).

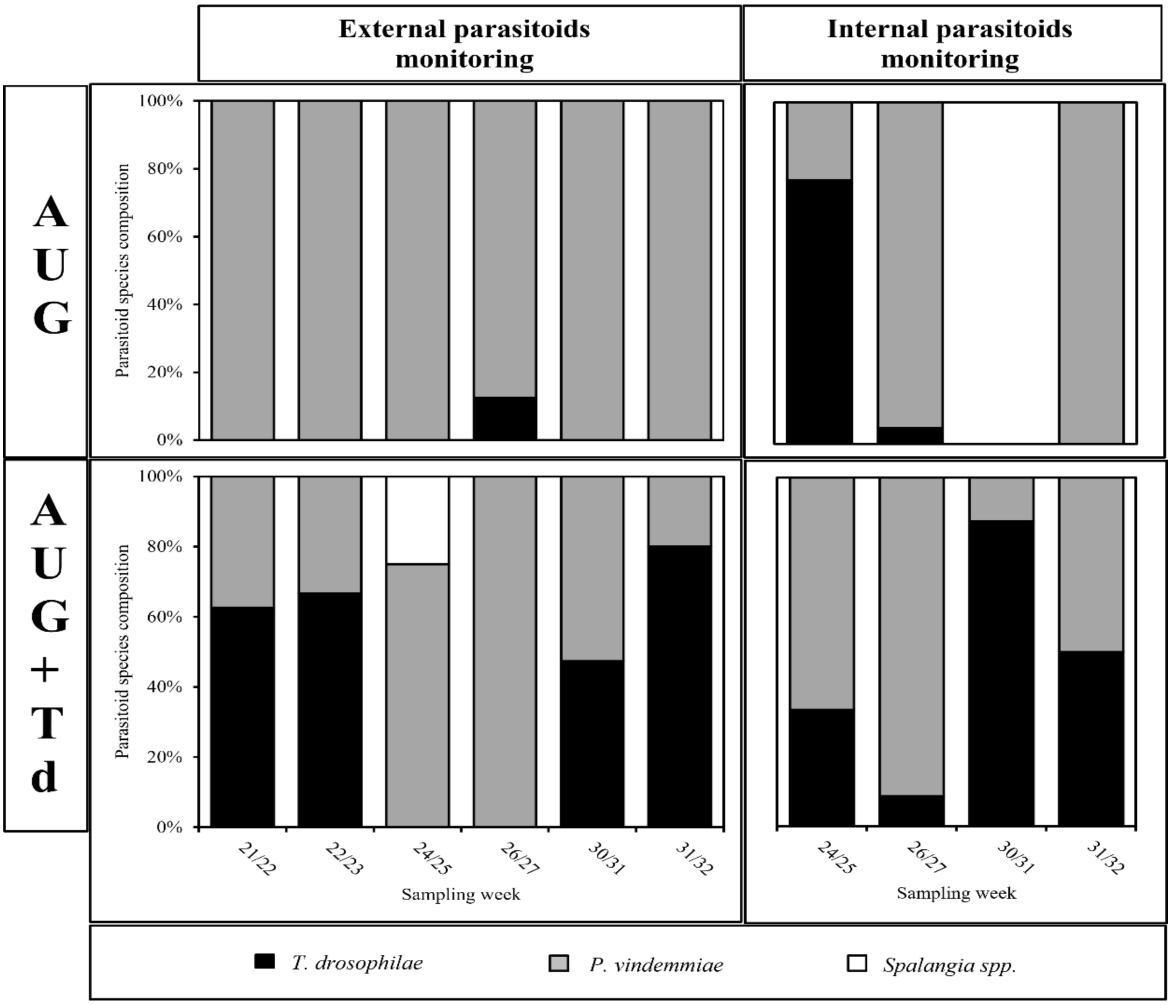


Figure 10. Parasitoid species sampled by means of sentinel traps during the field trial with *augmentoria*. (AUG=augmentorium without *T. drosophilae* releases, AUG+Td=augmentoria with *T. drosophilae* addition).

Augmentoria residual content analysis

After the laboratory incubation of residual fruit content inside each *augmentorium* the species composition was:

-AUG+Td I: parasitoid wasps = 56% of *T. drosophilae*, 3% of *Spalangia* spp., 41% of Drosophila larval parasitoids ; drosophilids = 9% D. suzukii and 91% *Drosophila* spp.

-AUG+Td II: parasitoid wasps = 43% of *T. drosophilae*, 3% of *P. vindemmiae,* 9% of *Spalangia* spp., 45% of Drosophila larval parasitoids ; drosophilids = 1% D. suzukii and 99% *Drosophila* spp.

-AUG I: parasitoid wasps = 100% of Drosophila larval parasitoids; drosophilids = 100% *Drosophila* spp.

-AUG II: parasitoid wasps = 2% of *P. vindemmiae*, 37% of *Spalangia* spp., 61% of Drosophila larval parasitoids ; drosophilids = 13% D. suzukii and 87% *Drosophila* spp. (**Fig.11**).

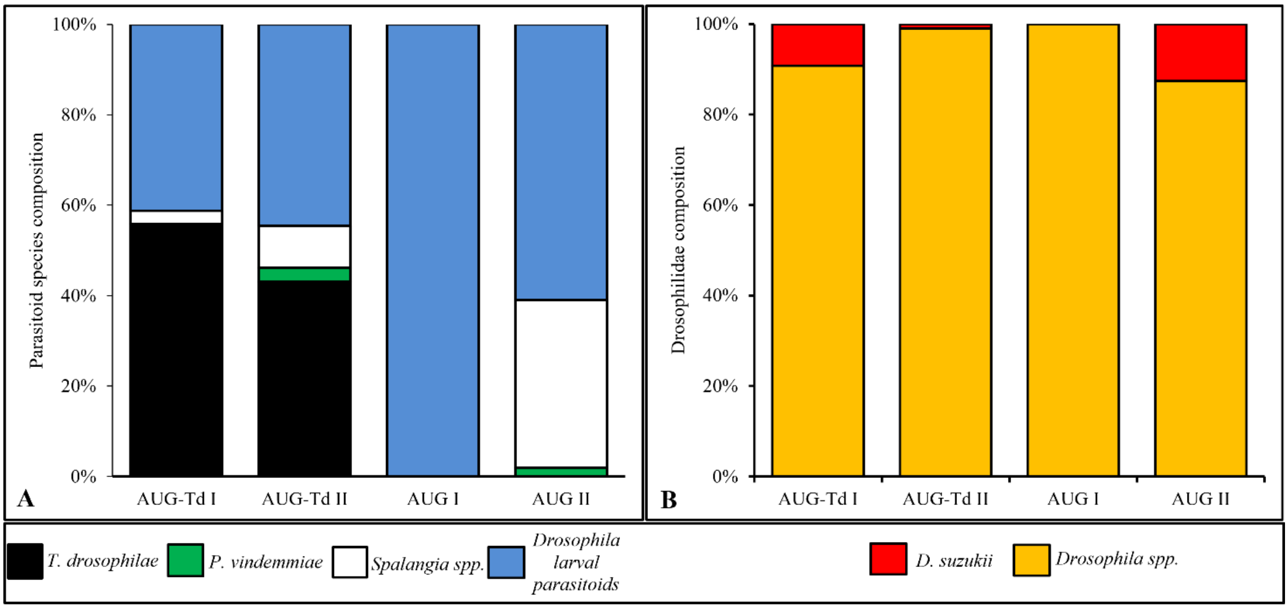


Figure 11. Parasitoid and drosophilids composition deriving from the residual fruit content inside the *augmentoria*. (AUG+Td I&II and AUG I&II are the *augmentoria* with or without *T. drosophilae* addition, respectively).

Discussion

Sanitation of the agricultural area from the infested fruit has been suggested to reduce *D. suzukii* emergences (Leach et al., 2018, Hooper et al., 2020).

Augmentorium would allow the fruit wastes recycling for a biocontrol purpose, thus potential also avoiding the killing of any recently introduced larval parasitoids (Puppato et al., 2020, Abram et al, 2020). Furthermore, in addition to cultivated infested fruit, it may also accept other kind of fruit material (Bal et al., 2017), as well as wild berries (Poyet et al., 2015, Grant et al., 2021) in order to have a constant supply along the season.

Preliminary laboratory and field tests with our *augmentorium* prototype allowed us to determine its capacity to attract and sustain the indigenous pupal parasitoids of *D. suzukii* (*T. drosophilae, P. vindemmiae* and *Spalangia*) and that its proper management could guarantee its functioning for a long-time during fruiting season.

Furthermore, carrying out few releases with a low number of *T. drosophilae* (0.1-0.2 *T. drosophila*/m^2) we observed its long-term presence both inside and outside the *augmentorium*, likely sustaining their activity by means of the infested fruits loaded weekly. This is a crucial factor since augmentative releases are time consuming and cost challenging, thus the facilitation of biocontrol practices by farmers is a highly sought-after prospect for a wider dissemination of the technique. Nevertheless, also without releases we observed the capacity of natural population of *T. drosophilae* to explore the *augmentoria*, even though its late arrival (during the ripe period of cherries) could not be enough to increase the parasitoids population, as that observed in the plot with *augmentoria* plus early releases of *T. drosophilae*, probably compromising an effective biocontrol activity against *D. suzukii*.

During residual fruit content analysis of *augmentoria* we observed a huge colonization by some Drosophila species and a very low number of *D. suzukii*, despite the 16 weeks of loading with *D. suzukii* infested fruit. Drosophilids' eggs or larvae, mainly belonging to *D. melanogaster* and *D. simulans*, were probably already present on the infested fruits and unintentionally inserted during the weekly loading. Nevertheless, we cannot rule out that adults are able to pass through the mesh of the net thanks to the smaller size compared with *D. suzukii*.

In addition to the mortality caused by the parasitoids, the low number of *D. suzukii* may be also explained by the presence of these drosophilids whose outcompeting would limit the development of *D. suzukii* (Dancau et al., 2017, Shaw et al., 2018), favoring a "natural sanitization" of the *augmentorium*.

Another interesting finding from internal content analysis derives from the presence of a large amount of Drosophila larval parasitoid, probably most specialized on other drosophilids than *D. suzukii*. However, although we need confirmation, *L. japonica* may also actively explore the *augmentorium* or being passively introduced with infested fruits, favoring build-up of the parasitoid population that could be used for inoculative biocontrol in areas in which *L. japonica* is less abundant.

Although the recent pandemic years have complicated a more extensive biocontrol evaluation on *D. suzukii*, the *augmentoria* implementation is still under investigation in Trento province, with the aim of establishing the impact of its use on a large scale together with the most recent classical biological control. Compared with *P. vindemmiae*, *T. drosophilae* seems to be a rarefied species (Miller et al., 2015, Knoll et al., 2017, this study) and *augmentorium* has demonstrated to enrich this species in the field, although interaction/competition among these parasitoids remains to be elucidated in open field (Wang et al., 2016, Wolf et al., 2021).

Augmentorium should be employed outside crops, as conservative biological control method offering a top-down effect against *D. suzukii* on those semi-natural areas that are intersperse in agroecosystem but enriched of non-crop host plants (Poyet et al., 2015, Tait et al., 2020, Grant et al., 2021).

CONCLUSION

Ten years after the first reports of *Drosophila suzukii* in Europe, many crucial aspects of the biology and ecology of this invasive pest have been elucidated, offering the opportunity to broaden the spectrum of solutions for its management.

Furthermore, it is increasingly evident that management of an alien species can no longer take place just at crop field level, but it must necessarily include a spatial scale as large as possible, which integrating the most promising strategies in a coordinated way.

Regard to *D. suzukii* area-wide integrated pest management could join the principles of agroecology, concentrating efforts on applied research that focusing on the complexity of the interactions present in the agroecosystem with the aim of stabilizing those dynamics that guide the balance in nature very often compromised by not selective interventions that tend to solve crop issues in a short-term without having a more far-sighted vision.

Time lag between the exit from the diapause of *D. suzukii* and the begin of the ripening period of early crop hosts, i.e. *P. avium* and *P. cerasus*, should become target of several techniques that merging together attempt to limiting the seasonal pest outbreaks of early summer. This strategy may condition the population dynamic reducing the pest pressure later in the season when a high number of *D. suzukii* is more difficult to control on crop fields.

The recent findings of *Leptopilina japonica* and the classical biological control by means of *Ganaspis brasiliensis* may facilitate the implementation of this new vision for a more sustainable management of this invasive pest.

L. japonica carried out its biological control service following the first pest infestations on *Prunus* species, continuing its activity on a wide range of host plants infested by *D. suzukii* along the season. *G. brasiliensis* releases may contribute with a synergistic effect on the biocontrol action against *D. suzukii*.

Furthermore, although the natural host-parasitoid asynchrony observed in late spring could allow a significant improvement of the new generations of *D. suzukii*, escaping from the biocontrol. This phenomenon may be reduced by recurring to the releases of sterile males on the areas before reaching the high pest density.

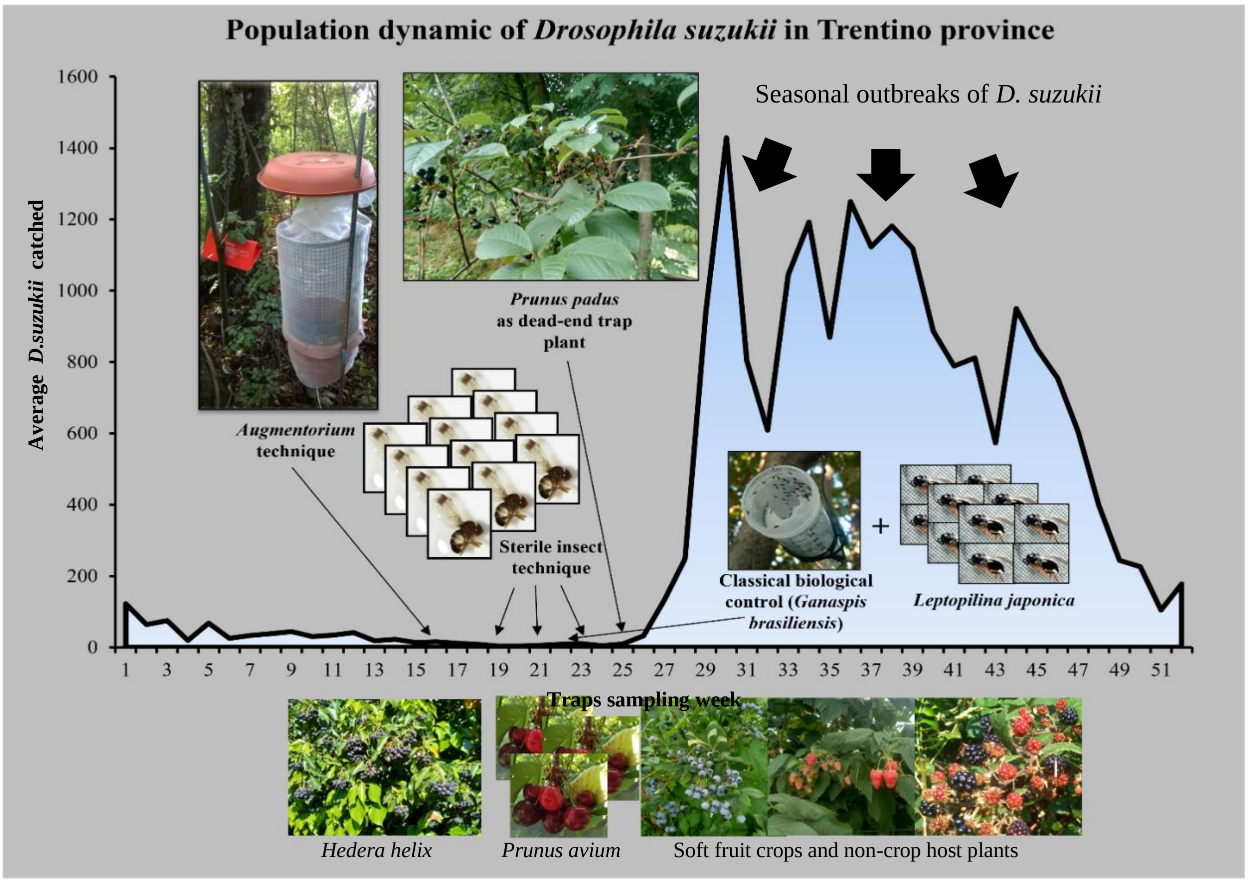
During crops production, the correct management through cultural practices could be accompanied by sanitization operations, placing the infested fruit inside the *augmentoria* arranged in the neighboring areas to the main crop, which aims to sequester adult flies emerging from infested fruits but let parasitoid wasps to escape.

Trichopria drosophila population may be increased by combining the *augmentorium* technique with some early releases of this parasitoid. In addition to *T. drosophilae*, the other main pupal parasitoids such as *P. vindemmiae* and *Spalangia spp.* could exploit the *augmentoria*, thus affecting the pupal stages of *D. suzukii* which develops on spontaneous plant hosts that grow at the edges of semi-natural habitats adjacent to crops.

Adding dead-end trap plants, such as *P. padus*, close to the crops to be protected, may further impact the spillover effect existing between the field edge and the crop, contributing to lower the pest pressure on crops.

Overall, even though preliminary studies requiring further confirmation, we hope to move towards an agroecological management of *D. suzukii*, integrating these practices with new methods that will be developed in the future such as push and pull strategies, more selective lure for monitoring and for attract and kill methods and other applied practices limiting the agrochemicals use.

Moreover, it will be precious all the knowledges that clarifying the influence of the landscape on the interactions of *D. suzukii* and its natural enemies, as well as will allow to re-design suitable agri-environment schemes that can be rigorously transferred to the farmers.



Summary of the agroecological approach. Strategies should be implemented in a coordinated way during late-spring/early-summer with the aim to limit late seasonal outbreaks.

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ACKNOWLEDGMENTS

Thanks:

To my parents, who have always supported me in doing what right for my life path, with joys and sorrows. You have guided by the naturalness and love that only parents by vocation can offer.

To Cristian, Erica and little Bernardo who show me every time how beautiful a young and cohesive family is.

To Alberto colleague and friend who beyond your indisputable experience and dedication to your job, you have confirmed me how magical and fundamental are those values in the bonds between people, humility, loyalty, kindness, sharing, perseverance for something in which you believe. For the beauty of understanding in a few words.

To Claudio, because despite the numerous commitments, you have always been there in times of need. You were able to guide me and make things happen, supporting me, quality of a tutor with considerable sensitivity of mind, professionalism and attention. Thank you so much for continuing to believe in me.

To Anna for welcoming me, educating me and for your inexhaustible energy in continuing to do what is part of your life. Thanks to your family from Pavia, Davide, Giulia, Giuliano and Ludvik.

To the family of Vigalzano, Angela, Evelyn, Franz, Gianpiero, Linda, Lorenzo, Luigi, Manuel, Mattia, Paolo, Roberta, Sandro, Sergio, Stefano and Tommaso for the strong spirit of mutual collaboration and for essential daily coffee breaks.

To Prof. Giuseppe Maiorano for coordinating the PhD course in the best possible way and Prof. Antonio De Cristofaro always available for clarifications and support.

To Sant'Orsola cooperative for believing in my work and allowing this training path. Many thanks to Dr. Gianluca Savini, Anna, Michele and all the growers for carrying out the field trials.

To the IAEA / FAO group for the wonderful week in Mendoza, among professionalism, laughs, cultural exchange and human enrichment.

To Federico for the countless PCRs and his essential help with biomolecular investigation.

To Lorenzo and Giulia for the precious scientific discussions and help in field trials.

To the Blueberry Breeding group, Lara, Matteo, Monica and to the Agricultural Entomology group, Gabriella, Gianfranco and Elisabetta.

To PAT Forest Service of plant nursery Casteller in San Rocco di Villazzano (Trento) for kindly providing us *Prunus padus* trees.

To all the friends and people who have shared experiences and words, enriching my life.