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Ganaspis kimorum as a classical biological control agent for *Drosophila suzukii*: from laboratory studies to field assessments.

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They say time flies when you are having fun, and these three years felt just like a moment!

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LIST OF SYMBOLS AND ABBREVIATIONS

AC	Control adults
AEM	Average Emersion Time
a.i	Active ingredient
AOW	Overwintering adults
AP-BB	Artificially Pierced Blueberries
APR	Apparent Parasitism Rate
a.s.l	Above sea level
BCA	Biological Control Agent
BL-C	Un-infested Blueberries (Control)
BL-I	Infested Blueberries
CAD	Cadenazzo (Switzerland)
CBC	Classical Biological Control
CLSA	Closed-Loop-Stripping-Analysis
$Ct_{<0}$	Cumulative time below freezing
CV	Coefficient of Variation
DAE	Days After Exposure
DD	Degree Day
DEL	Delémont (Switzerland)
DISAFA	Department of Agricultural, Forest and Food Science
Ds	*Drosophila suzukii*
$D_{T<0}$	Days where minimum temperature are below 0°C
$D_{T>30}$	Days where maximum temperature are above 30°C
EICA	Evolution of Increased Competitive Ability

ERH	Enemy Release Hypothesis
ES	Establishment Site
EPPO	European and Mediterranean Plant Protection Organization
FAE	Faedo (Italy)
FBL-C	Frozen Un-Infested Blueberries (Control)
FBL-I	Frozen Infested Blueberries
FE	First Emergence
FeCO	Femoral Chordotonal Organs
FEM	Edmund Mach Foundation
FET	First Emersion Time
FR	Field Rate
Gb	*Ganaspis brasiliensis*
GC-MS	Gas Chromatography-Mass Spectrometry
GEN	San Genesio (Italy)
Gk	*Ganaspis kimorum*
H-BB	Healthy (uninfested) Blueberries
HIPV	Herbicide-Induced Plant Volatile
IPM	Integrated Pest Management
IPSP	Institute for Sustainable Plant Protection
LAI	Laimburg (Italy)
LC	Lethal Concentration
L:D	Light:Dark ratio
LEP	*Leptopilina* spp.
LET	Last Emersion Time
Lj	*Leptopilina japonica*

LL	Local Larval parasitoids
LP	Local Pupal parasitoids
NICHE	Niche
NINT	Novel Interaction
NOV	Novale di Fié (Italy)
OD	Other *Drosophila* spp. (than *Drosophila suzukii*)
OH	Other Host flies
OW	Overwintering
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PE	Peak Emergence
PLAST	Phenotypic Plasticity
PRT	Patch Residence Time
RH	Relative Humidity
SINV	Species Invasiveness
SMA	San Michele all'Adige (Italy)
SR	Survival Rate
SS	Specificity Site
SWD	Spotted Wing Drosophila
SWD-BB	SWD-infested Blueberries
T_L	Lower Thermal threshold
TP	Total Puparia
T_U	Upper Thermal threshold
USA	United Stated of America

VOC Volatile Organic Compound

ABSTRACT

Agriculture forms the foundation of human society, providing one of the few essential resources we really need in life, food. However, agricultural production faces constant threats from emerging pests. While globalization facilitates access to agricultural products worldwide, it also opens the door to the spread of new invasive pests, leading to significant economic losses. One of the most emblematic examples of such event, is given by the spotted wing Drosophila, *Drosophila suzukii*. Native from East Asia, this fly colonized three continents in less than a decade. Its ability to oviposit in ripening fruit causes severe damage to berry and stone fruit crops. Current management relies heavily on pesticides, but growing interest in sustainable solutions has spurred research into biological control agents (BCAs).

Since native pupal parasitoids proved ineffective at controlling the pest below acceptable thresholds, research recently shifted toward larval parasitoids from its native range. With classical biological control gaining momentum, European countries and American states collaborated to identify the most suitable candidate for release, ultimately selecting the figitid wasp *Ganaspis kimorum* as a classical biological control agent. Prior to its release, extensive studies were conducted under quarantine conditions to evaluate its specificity, biology, and parasitization rate. However, key aspects regulating host-parasitoid communication and those requiring exposure to natural conditions in the introduction areas remained unexplored. The main objective of this thesis was to *assess the efficacy of G. kimorum as classical biological control agent in the newly introduced area* through comprehensive laboratory and field assessments.

The first part of this work focused to evaluating BCA establishment and ecological interactions. Pre and post-release monitoring assessed establishment, recapture rates, and interactions

with non-target species (Chapter 2). Results showed that *G. kimorum* successfully established in 50% of study sites, exhibited high specificity toward *D. suzukii* in ripening fruits and demonstrated the ability to survive the winter in some of the introduced habitats. Monitoring also revealed the abundant presence of the adventive larval parasitoid, *Leptopilina japonica*. Exposure in the field of *G. kimorum*-parasitized hosts showed that the BCA experiences significant competitive pressure, leading to an average 62% reduction in emergence, likely due to secondary infestation by *Drosophila* spp. and multiparasitism by *Leptopilina* spp (Chapter 3).

To investigate overwintering dynamics, *G. kimorum* was exposed to variable temperature regimes across seven sites in Switzerland and Northern Italy (Chapter 4). Consecutive dissections conducted throughout the winter revealed that *G. kimorum* was diapausing as first larval instar (L1) residing within early pupal stage of *D. suzukii*. A coefficient of variation (CV) and degree day (DD) analysis confirmed this hypothesis, suggesting a lower thermal threshold (T_L) for development of 9°C and an average post-diapause DD accumulation of 329±6. The parasitoids were able to overwinter in four out of the seven sites under study, and temperatures played a major role to regulate survival rates that ranged from 0.4 to 17.6%.

Host-searching mechanisms of *G. kimorum* were investigated in both medium and short range (Chapters 5-7). Olfactometry and volatile organic compound (VOC) analyses showed that parasitoids attraction toward the host was time-dependent (Chapter 5). While *G. kimorum* females were attracted by host's cuticular hydrocarbons present in fruits infested with young larvae, they were repelled by fermentation compounds found in older and deteriorated fruits. Vibrational assays revealed that *D. suzukii* larvae produce incidental vibrations in the form of a series of broad band pulses (frequency range of 0.1–2 kHz and amplitude range of 12.1- 946 µm/s) (Chapter 6). Behavioral assays suggested that *G. kimorum* uses these cues to detect larvae inside infested fruit (Chapter 7). When larvae were

killed inside berries and vibrational cues were absent, oviposition behavior was significantly diminished, with extrusion and insertion times reduced by 54% and 78% respectively.

The project also assessed *G. kimorum*'s integration into the agroecosystem. In particular we assessed its susceptibility to insecticides commonly used against *D. suzukii* (Chapters 8). Laboratory topical exposures showed that Spinosad and λ-cyhalothrin were the most toxic active ingredients, while Cyantraniliprole exhibited the least toxicity. Field residual exposures confirmed these findings, with Spinosad and Deltamethrin causing 92% mortality immediately after application compared to the 52% caused by Cyantraniliprole.

In conclusion, this thesis confirmed the efficacy of *G. kimorum* as biological control agent of *D. suzukii* in its newly introduced area (Trentino-Alto Adige). Post-release studies demonstrated its ability to establish and persist in natural environments, with successful overwintering observed in several sites despite the challenges posed by low temperatures. However, this thesis also identifies significant biotic and abiotic stressors limiting its establishment, in particular interspecific competition and winter survival. Overall, these findings provide valuable insights for the ongoing classical biological control program and help refine future risk assessments.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Biological invasions

Species naturally expand into new territories to enhance their fitness. Biological dispersal is a primary mechanism of gene flow and plays a central role in species evolution (Gibbs et al., 2010). For millennia, geographical and ecological barriers limited species movement through space (Caplat et al., 2016). However, the expansion of human trade has removed these dispersal barriers, promoting the spread of species through deliberate and accidental introductions (Hulme, 2009). Alongside transportation, climate change and socio-economic shifts are now actively reshaping species distributions and are considered main drivers of biological invasions (Essl et al., 2020). These invasions often have negative impacts on the invaded territories, typically quantified in terms of biodiversity and economic losses (Diagne et al., 2021; Galiana et al., 2014; Zenni et al., 2021). The annual mean cost associated with managing invasion reaches $ 26.8 billion worldwide, exhibiting a consistent threefold increase per decade (Diagne et al., 2021). In Europe alone, over the past sixty years, expenditures associated with invasive species reached $ 140 billion (Haubrock et al., 2021). Taxonomically, invertebrates, particularly insects, represent the costliest group, accounting for 90% of these expenses (Diagne et al., 2021). Not surprisingly, the agricultural sector is particularly susceptible to invasive species (Paini et al., 2016), as arthropod pests can significantly reduce crop yield by 18-20% (Sharma et al., 2017). Moreover, damage and yield losses are expected to increase due to climate change, posing additional concerns for food security (Skendžić et al., 2021; Subedi et al., 2023; Tonnang et al., 2022).

The invasion process is often categorized into three consecutive transitions that must be met for a successful biological invasion: 1) importation to introduction, 2) introduction to establishment, and

3) establishment to pest status. Importation refers to the physical movement of a non-native species into a new geographic area, establishment is defined as the species' ability to self-sustain in the new environment, and pest status indicates that the organism has a negative environmental and/or economic impact in the introduced area (Heimpel and Mills, 2017a). The probability of each transition occurring successfully varies depending on the circumstances. However, a well-known hypothesis, the "Tens Rule," suggests that each phase is successful in approximately 10% of cases (Williamson and Fitter, 1996).

A wide range of hypotheses have been proposed to explain the success or failure of biological invasion (Daly et al., 2023), many of which are non-exclusive. Some assumptions focus on the genetic traits of the invasive species (*e.g.*, SINV, PLAST), while others emphasize the abiotic conditions they encounter in the invaded territories (*e.g.*, NICHE, NINT) (Table 1). In contrast, the biotic resistance hypothesis highlights the pressure that native organisms can exert on incoming non-native species (Heimpel and Mills, 2017a). For example, the Enemy Release Hypothesis (ERH) suggests that non-native species can thrive and rapidly increase abundance and distribution when natural enemies are absent or provide insufficient control in the newly invaded areas (Keane and Crawley, 2002). This advantage can be further amplified through the Evolution of Increased Competitive Ability (EICA), where invasive species reallocate resources normally dedicated to defence toward growth, competitiveness, and reproduction (Blossey and Notzold, 1995).

Table 1.1: Summary of biological invasion hypothesis from Daly et al. (2023).

Acronym	Hypothesis description	Key references
SINV	*Species INVasiveness*, the likelihood of a non-native species becoming invasive is influenced by its trait performance, originality, and/or plasticity	(Van Kleunen et al., 2010)

PLAST	According to this theory, invasive species exhibit higher *phenotypic PLASTicity* in ecologically important traits than non-invasive species, enhancing their ability to establish and spread in invaded area	(Jonathan and Heger, 2018)
NICHE	In this scenario, non-native species colonize new territories by filling an empty *niche*. Contrary, establishment will be unsuccessful in a community with greater niche overlap, where functional similar species are coexisting	(Stachowicz and Tilman, 2005)
NINT	The combinations of native and non-native species result into a N*ovel INTeraction* that affect the establishment success of the non-native species	(Poyet et al., 2015)
ERH	The *Enemy Release Hypothesis* suggests that non-native species can thrive and rapidly increase abundance and distribution thanks to the absence of natural enemies	(Keane and Crawley, 2002)
EICA	If non-native species escape natural enemies in the invaded territories (ERH), they can go through *Evolution of Increased Competitive Ability*, by investing resources normally dedicated to defence in growth, competitiveness and reproduction, enhancing establishment success	(Blossey and Notzold, 1995)

1.2 Biological control, in theory

Regardless of the underlying theories explaining invasion success, the potential economic losses caused by invasive species justify the implementation of eradication and control strategies (El-Sayed et al., 2006; Hanley and Roberts, 2019). While biosecurity measures play a crucial role in preventing or delaying biological invasions (Cook et al., 2011), once the invasive species become a pest, most control measures (from short to long-term) rely on costly chemical pesticide applications, with harmful consequences for human health and the environment (Cimino et al., 2017; Heimpel et al., 2013; Kim et al., 2017).

An alternative approach is biological control, which Heimpel & Mills (2017) define as "the indirect positive effect of a biological control agent (BCA) on humans that is mediated by direct or indirect negative effects of that agent on populations of one or more target species". In simpler terms "The enemy of my enemy is my friend". Biological control is categorized into different models, distinguished by the extent of human influence on biocontrol agents (Heimpel and Mills, 2017a; Stenberg et al., 2021) (Figure 1.1). When humans have no direct influence on the biocontrol agent, the process is termed *natural biological control*, in which naturally occurring predators, parasitoids, or pathogens regulate pest populations. If humans introduce a biocontrol agent from another region to control a pest, this is known as *importation biological control* (also called *classical biological*

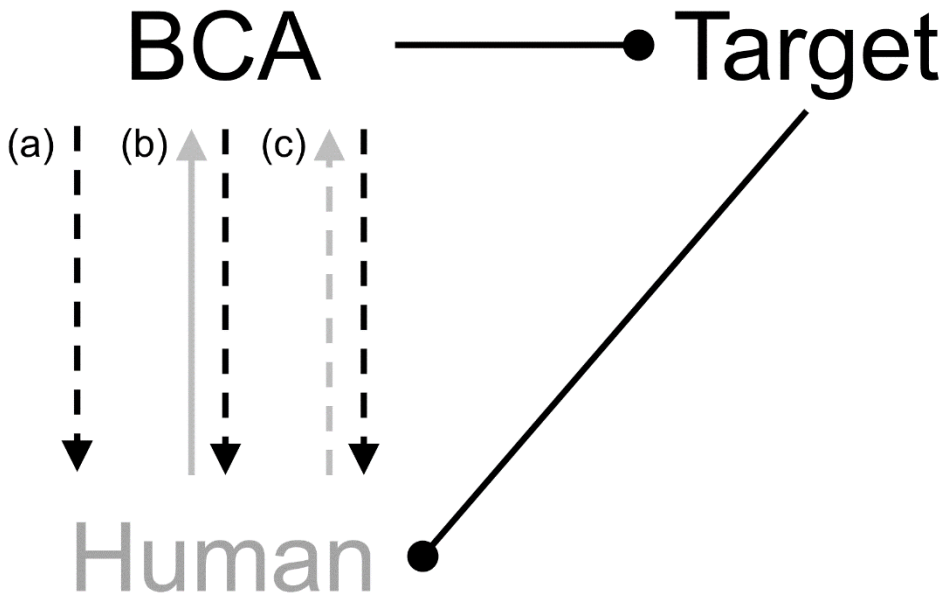

Figure 1.1: Interaction diagram modified from Heimpel & Mills (2017). Solid lines indicate direct effect, while dashed lines indicate indirect effects. Arrowheads indicate positive effect and round heads indicate a negative effect. Different classification of (a) natural biological control, (b) importation (classical) or augmentative biological control, and (c) conservation biological control.

control). Alternatively, when humans enhance biological control by releasing additional individuals of an already present biocontrol agent, it is referred to as *augmentative biological control*. Finally, when human intervention indirectly supports biocontrol agents by modifying the environment to improve their effectiveness, this approach is called *conservation biological control*.

Both classical and augmentative biological control involve the release of additional organisms. However, in classical biological control programmes the added organisms are intended to permanently establish in the new area, while in augmentative biological control they are intended to establish only temporarily (Stenberg et al., 2021).

Among the different forms of importation biological control, classical biological control is the most well-documented and widely recognized, largely due to its long history, which dates back to 1888. During the 20th century alone, it contributed to the partial or complete control of over 200 insect species, accounting for approximately 10% of all introduction cases (Cock et al., 2016; Heimpel and Cock, 2018). Heimpel and Cock (2018) identified a historical shift in the application of classical biological control. While the first hundred years were largely focused on its benefits (resulting in frequent introductions), the 1990s marked a growing emphasis on its potential risks, leading to a sharp decline in new introductions. This shift prompted the development of protocols and guidelines designed to assess both the benefits and risks of biological control, ensuring that species introductions are conducted with greater ecological safety. The primary tool used for this purpose is known as *risk assessment*.

1.3 Risk assessment: the intricate road to release

The aim of a risk assessment is to estimate the likelihood of negative effects following the intentional release of a biological control agent (Heimpel et al., 2024; Heimpel and Mills, 2017; Van Lenteren et al., 2003; Wright et al., 2005). The results of these evaluations determine whether a biocontrol agent (BCA) can be released in the first place. The primary factor typically assessed before release is *host specificity*, which examines the direct risks a BCA might pose to non-target species (Barratt et al., 2023; Heimpel and Mills, 2017). This is often tested in controlled choice tests conducted under quarantine conditions, but whether the results are truly representative of field conditions

remains uncertain. While host-specificity is central to decision-making, there are other important benefit-related factors (such as efficacy and successful establishment of the BCA) that are often difficult to be investigated due to the limitations of quarantine conditions. These factors, which are critical for understanding the long-term success of the BCA, typically remain unexamined until after the release.

1.4 The invasive pest: *Drosophila suzukii*

1.4.1 Facts and theory of Drosophila suzukii biological invasion

One of the most successful biological invaders of our modern history is the spotted wing *Drosophila*, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) (Zhao et al., 2023). Native to East Asia, this fly colonized three continents in less than a decade. First described in Japan in the early 20th century (Kanzawa, 1939), it was recorded in North America and Europe in 2008 (Cini et al., 2012; Asplen et al., 2015; Calabria et al., 2012; Hauser, 2011), in South America in 2013 (Andreazza et al., 2017) and in Africa starting from 2017 (Boughdad et al., 2021; Kwadha et al., 2021). According to Little et al. (2020), the key to its success is plasticity, which allowed *D. suzukii* to adapt behaviourally, physiologically and morphologically to new environments, fulfilling many of the invasion hypothesis mentioned earlier (See paragraph 1.1).

Unlike native *Drosophilidae*, which lay eggs in rotting fruit, *D. suzukii* lays eggs in ripening healthy fruit, thanks to its serrated ovipositor (Figure 1.2) (Atallah et al., 2014). This adaptation enabled the pest to rapidly exploit new ecological niches (NICHE hypothesis). *Drosophila suzukii* is highly polyphagous, with 139 reported plant species from which the fly can develop and emerge (Rossi Stacconi, 2022). In Europe alone, *D. suzukii* has been reported to emerge from more than 80 plant species, including both wild and crop plants (Kenis et al., 2016). The wide trophic niche that *D.*

suzukii discovered in the introduced areas has led to novel interactions with new host plant species, positively influencing its establishment (NINY hypothesis) (Poyet et al., 2015).

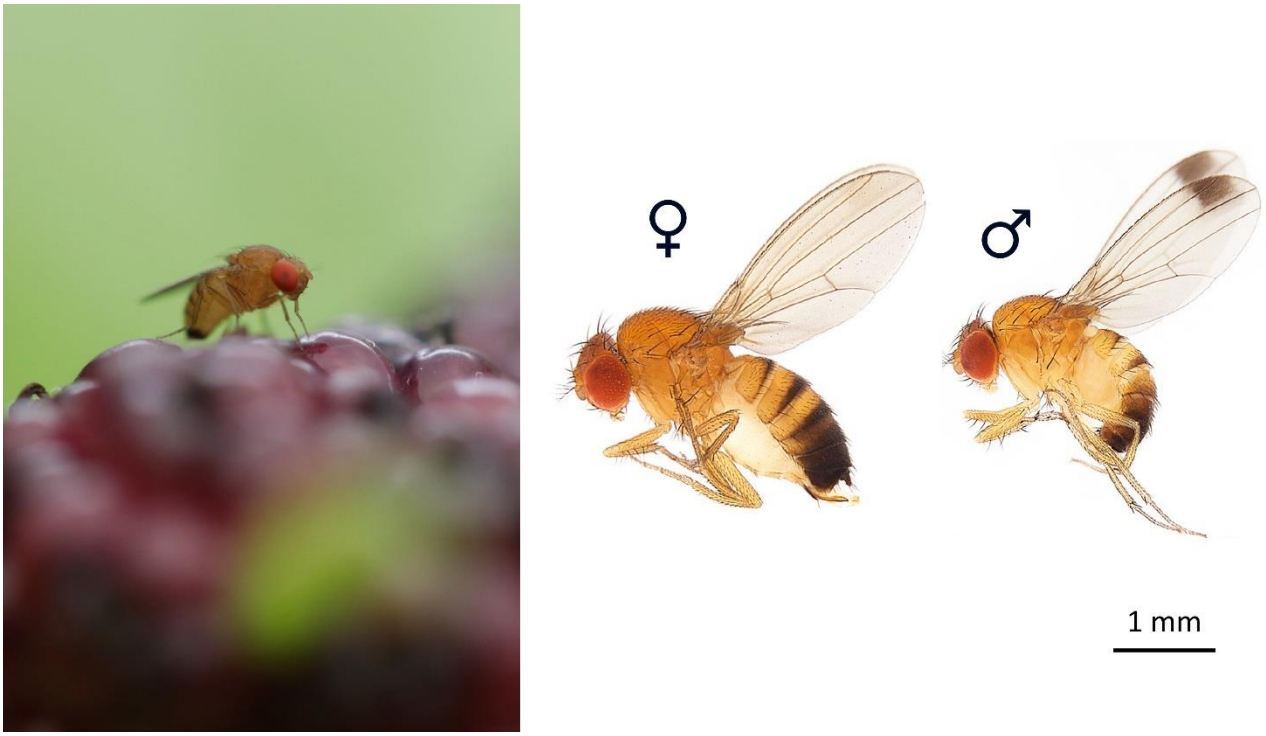

Figure 1.2: *Drosophila suzukii* adult female on a mulberry looking for an oviposition site (left). Lateral views of a female (♀) and male (♂) and male adult (right). Female is characterized by the serrated ovipositor, while males are easily distinguished by the black dots present on the wings. High resolution diagnostic images modified from Mc Evey, 2017.

Other relevant physiological and behavioural traits that contributed to its invasion success include a rapid development cycle and high dispersal ability (Tait et al., 2020). Although abiotic and biotic conditions can affect development parameters, lifetime fecundity can exceed 400 eggs under optimal conditions (Hamby et al., 2016). These traits have enabled *D. suzukii* to successfully invade new territories (SINV hypothesis).

Many authors have reported that the absence of effective natural enemies is a primary biological factor contributing to the successful invasion and severe agricultural damage caused by *D. suzukii* (Farnsworth et al., 2017; Lee et al., 2019; Lisi et al., 2022). In both North America and Europe, native pupal parasitoids such as *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) and

Trichopria drosophilae (Perkins) (Hymenoptera: Diapriidae) are capable to locate and parasitize *D. suzukii* pupae, but natural occurring parasitism is generally lower than 10% (Lee et al., 2019). These low parasitism rates are mainly due to the fact that these parasitoids are generalists and tend to prefer attacking other *Drosophila* species. The absence of effective natural biological control agents has likely facilitated the rapid spread and distribution of *D. suzukii*, enhancing its establishment by reallocating resources typically used for defense toward growth, competitiveness, and reproduction (ERH, EICA hypothesis).

Currently, in the province of Trento (Italy), the estimated economic impact of *D. suzukii* on producers of soft skin-fruit (cherries, strawberries, raspberries, blueberries and blackberries) amount to € 2.73 million, equivalent to 9% of the potential revenue (De Ros et al., 2021). In California (USA), raspberry farmers reported losses of $ 39.8 million due to *D. suzukii* infestation, accounting for 2.2% of their realized revenues (Farnsworth et al., 2017). In response to the significant yield losses caused by this invasive pest, farmers are implementing a variety of control methods.

1.4.2 Control methods

A wide range of methods and techniques are used worldwide to control *D. suzukii*, with their application depending on the stage of invasion, climatic conditions, and pest abundance. The most comprehensive approach is Integrated Pest Management (IPM), which combines multiple strategies, including chemical, cultural, and biological controls, habitat manipulation, and the use of resistant plant varieties (Haye et al., 2016; Tait et al., 2021).

1.4.2.1 Chemical control

Currently, both conventional and organic farmers rely on insecticides to control *D. suzukii* (Shaw et al., 2019; Shawer, 2021). However, the limited availability of effective active ingredients,

especially in organic cultivation (Tait et al., 2021), along with the increasing resistance to numerous products (Civolani et al., 2021; Disi and Sial, 2021; Ganjisaffar et al., 2022; Gress and Zalom, 2019) and the health and environmental concerns associated with pesticide use (Cimino et al., 2017; Heimpel et al., 2013; Kim et al., 2017), highlight the urgent need for more sustainable pest management practices.

1.4.2.2 Cultural control

Cultural practices have proven effective in reducing *D. suzukii* pressure at different crop production stages. Physical barriers, such as exclusion netting, can reduce infestation by 48% (Kuesel et al., 2023), and delay the first larval infestation by 10 days (Leach et al., 2016). Habitat manipulation can be adopted to make microclimate less favorable for the pest (Schöneberg et al., 2021). For instance, using drip irrigation instead of overhead sprinklers can lower humidity and increase temperature, ultimately reducing *D. suzukii* survival (Rendon and Walton, 2019). Similarly, pruning decreases canopy density, leading to a slight reduction in humidity (Schöneberg et al., 2020). Sanitation measures are another valuable cultural control strategy. The removal of dropped unharvested fruits and byproducts eliminates key breeding sites for *D. suzukii*, particularly during the off-season (Bal et al., 2017). While cultural practices have demonstrated effectiveness in reducing pest infestation, many require significant economic investment and/or are labor-intensive (Schöneberg et al., 2021; Tait et al., 2021).

1.4.2.3 Biological control

Multiple biological control agents have been tested against *D. suzukii*, through conservative, augmentative and classical biological control methods (See paragraph 1.2), such as predators, nematodes, entomopathogenic fungi and parasitoids. While several fungi and nematodes have

demonstrated effectiveness in laboratory assays, their applicability in field trials remains limited (Lee et al., 2019). Consequently, most attention is directed toward predators and parasitoids.

Predators. Common predators of *D. suzukii* include earwigs, spiders, ants, damsel and pirate bugs, which primarily target juvenile stages (Lee et al., 2019). Natural predation can significantly impact pest populations, reducing larval survival by 49%, and pupal presence by 91% (Woltz and Lee, 2017). An example of common predator in the invaded territories is the European earwig *Forficula auricularia* L. (Dermaptera: Forficulidae) which preys on *D. suzukii* juveniles and reduce its reproductive rate, but its role as a biocontrol agent is still limited (Bourne et al., 2019). Augmentative biological control using predators is generally discouraged, due to its high costs and low efficiency, with conservation practices to enhance naturally occurring predators being the preferred approach (Lee et al., 2019).

Parasitoids. Parasitoids play a crucial role in regulating herbivore insect populations (Rodríguez and Hawkins, 2000). Among them, hymenopteran wasps are the most abundant, with different groups specialized in attacking specific insect life stages (Polaszek and Vilhemsen, 2023). Parasitoids of *D. suzukii* are classified into two categories based on the host stage they parasitize: *larval parasitoids* and *pupal parasitoids*. Additionally, from a biological control perspective, *D. suzukii* parasitoids can be grouped into: 1) *resident parasitoids* (indigenous to the invaded regions) and 2) *native parasitoids* (exotic to the invaded regions and originating from *D. suzukii*'s native range). Three main resident larval parasitoids were recovered in the invaded range (Europe and Noth America), *Asobara tabida* (Nees) (Hymenoptera: Braconidae), *Leptopilina boulardi* (Barbotin) (Hymenoptera: Figitidae) and *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) (Chabert et al., 2012; Lue et al., 2016). Among them, only *L. heterotoma* successfully parasitizes and develops within *D. suzukii* (Rossi-Stacconi et al., 2015), while the others primarily attack other drosophilids, as *D. suzukii*'s immune

defences block their development (Kacsoh and Schlenke, 2012; Poyet et al., 2013). Two pupal parasitoids, *P. vindemiae* and *T. drosophilae* were mostly evaluated as biocontrol agents (Gabarra et al., 2015; Rossi Stacconi et al., 2013). These species are considered generalists due to their ability to parasitize multiple drosophilids, including *D. immigrans* and *D. melanogaster* (Esteban-Santiago et al., 2021; Rossi-Stacconi et al., 2015; Wang et al., 2016). In Italy, augmentative releases of *T. drosophilae* successfully reduced pest emergence and fruit infestation (Rossi-Stacconi et al., 2019, 2018), though the benefits were largely confined to areas near the release points and protected cropping systems. Since resident parasitoids did not provide satisfactory *D. suzukii* control, research efforts shifted toward native parasitoid species from the pest's original range in East Asia. Explorations conducted in China, Japan and South Korea identified at least 21 *D. suzukii* parasitoids species (Wang et al., 2020). Prevalent species included *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae) in South Korea (Daane et al., 2016), *Leptopilina japonica* Novković & Kimura (Hymenoptera: Figitidae) and *Ganaspis* cf. *brasiliensis* Ihering (Hymenoptera: Figitidae) in China and Japan (Giorgini et al., 2019; Girod et al., 2018a). Further studies suggested that *Ganaspis* cf. *brasiliensis* comprised multiple cryptic species, categorized into different lineages (G1-G5) (Nomano et al., 2017; Seehausen et al., 2020). Genetic analysis and morphological observations of the ovipositor recently confirmed this hypothesis, leading to a taxonomic revision (Sosa-Calvo et al., 2024). The lineage previously referred to as G1, which exhibited the highest specificity toward *D. suzukii* (Biondi et al., 2021; Daane et al., 2021) has been renamed *Ganapsis kimorum* Buffington. [As redescription occurred during the doctoral period, some of the following publications will still refer to the old nomenclature: *Ganaspis brasiliensis* lineage G1]. Due to its host specificity, *G. kimorum* was selected as a biocontrol agent for classical biological control programs in both Europe and the U.S. (Fellin et al., 2023; Gariepy et al., 2024; Lisi et al., 2022), with pioneering releases initiated in Italy in 2021 (Lisi et al., 2022).

1.5 The classical biological control agent: *Ganaspis kimorum*

1.5.1 Morphology

Ganaspis kimorum adults are black to dark brown, measuring on average 1.5 – 1.75 mm in length. Sexual dimorphism is evident in antennal morphology: male antennas are composed of 13 flagellomeres, while female antennas are shorter, composed of 11 flagellomeres, gently widened towards the apex and slightly clavate (Figure 1.3). The forewings feature a distinctive single triangularly-shaped enclosed cell, and the scutellum is dorsally surmounted by tear-drop shaped plate with a central pit. These morphological traits generally suffice to distinguish Figitidae from other *D. suzukii* parasitoids. However, identification at the species level within Figitidae requires further examination of key structures. In *G. kimorum*, the setal band at the base of the metasoma is "complete", and the scutellar plate is relatively large and convex (Abram et al., 2022b).

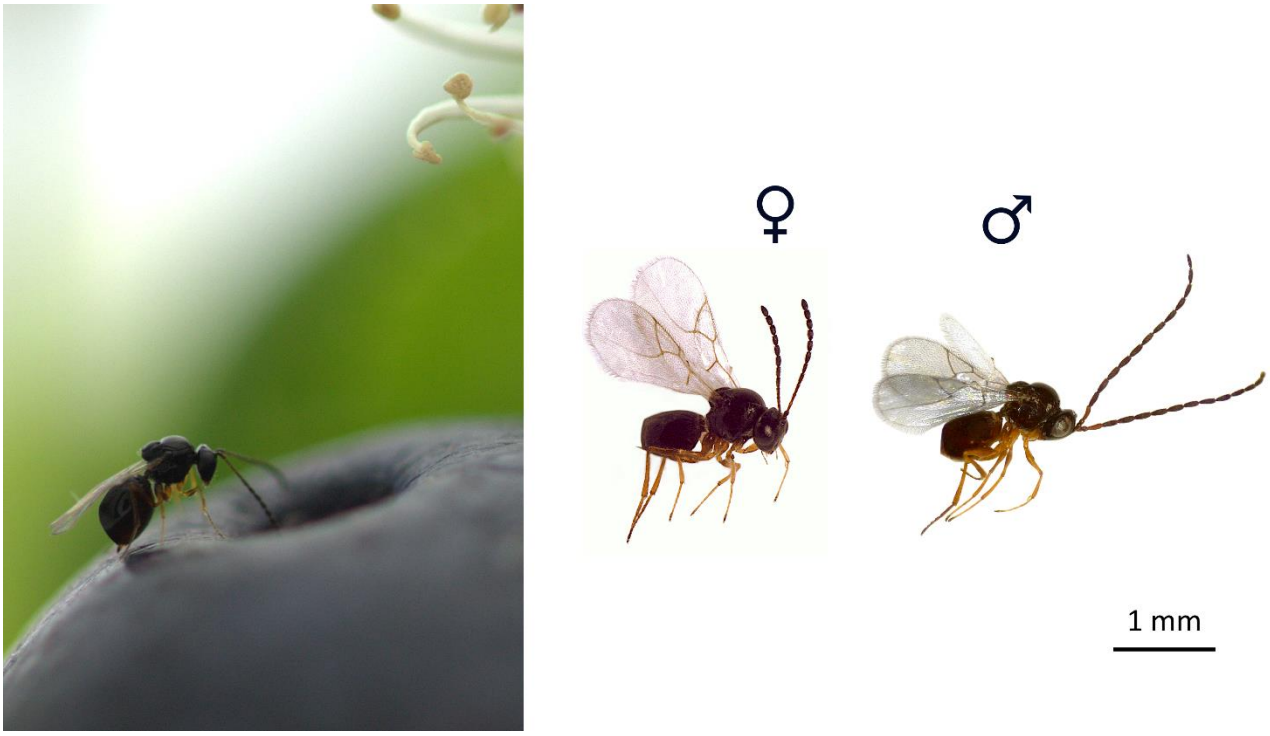

Figure 1.3: *Ganaspis kimorum* searching for *D. suzukii* larvae within an infested blueberry (left). Lateral views of female (♀) and male (♂) adults (right). Sexual dimorphism is evident in the number and shape of flagellomeres of antennae.

Morphologically, *G. kimorum* is nearly identical to *Ganaspis lupini* Buffington (Hymenoptera: Figitidae), and for this reason they were considered the same species until recently (Sosa-Calvo et al., 2024). They can be reliably distinguished only by examining the ovipositor clip, which is smaller and less developed in *G. kimorum* compared to *G. lupini*. While new identification keys are currently in preparation (Stahl et al., 2024), molecular tools such as polymerase chain reaction (PCR) with specific primers, combined with DNA sequence database comparisons for Drosophilidae parasitoids, offer a valuable diagnostic method (Gariepy et al., 2024; Lue et al., 2021).

1.5.2 Distribution

Within its native range, *G. kimorum* has been recorded in China, South Korea and Japan (Daane et al., 2016; Giorgini et al., 2019; Girod et al., 2018b; Matsuura et al., 2018; Wang et al., 2022) and based on available distribution data, the species is considered temperate (Stahl et al., 2024). Adventive populations have been established in the Northwest Pacific, including territories in the United States and in Canada (Abram et al., 2022a; Beers et al., 2022). Classical biological control releases have led to successful establishment in Northern Italy (Fellin et al., 2023) and multiple U.S. States such as Delaware, Georgia, Maryland, Oregon, Pennsylvania and Washington (Gariepy et al., 2024). Figure 1.4 displays current known insect distribution.

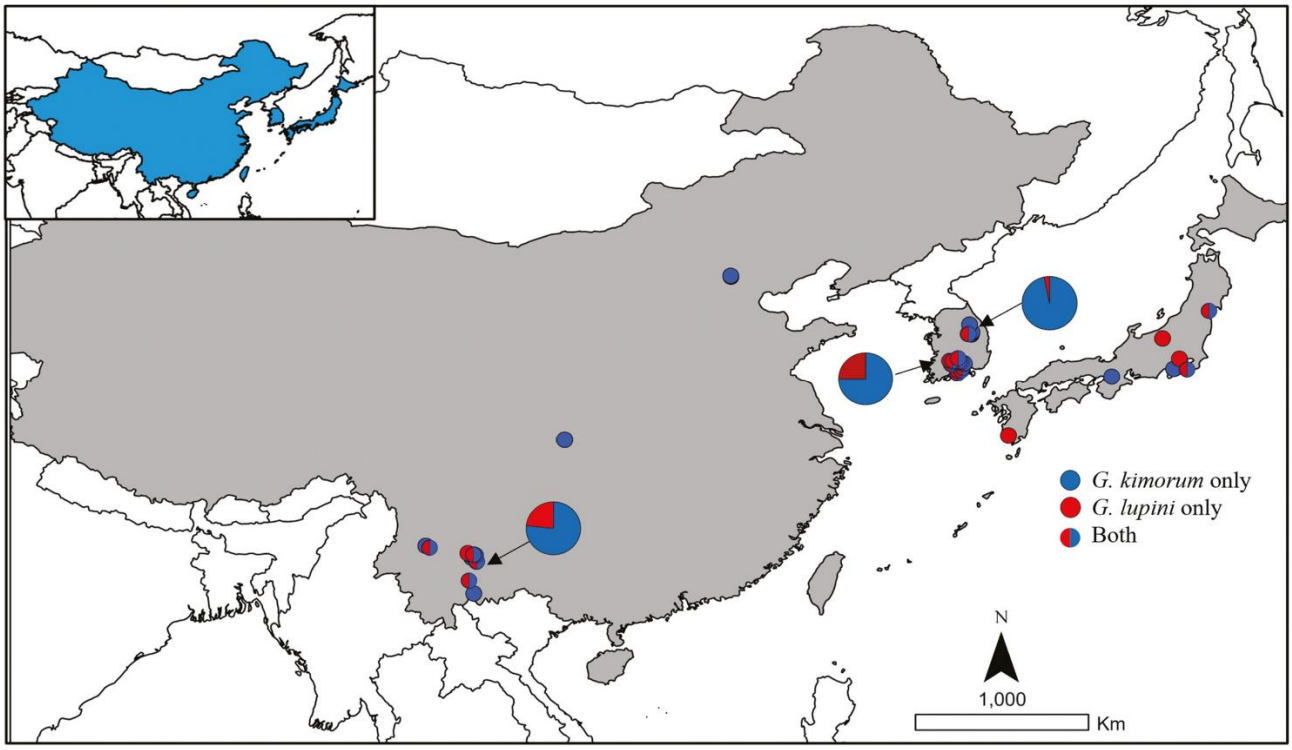

Figure 1.4: *Ganaspis* spp. distribution in the native environment (Stahl et al., 2024).

1.5.3 Life cycle

Ganaspis spp. are solitary, larval koinobiont endoparasitoids. Adult females locate fruit infested with host larvae, pierce the fruit surface with the ovipositor and deposit eggs inside the larval body. Bioassays have shown that *D. suzukii* first instars are preferred over later instar (Wang et al., 2018). Parasitoid eggs hatch approximately 72 hours after oviposition, with fully developed first instar emerging from the egg at around 96 hours. Transition to the second instar occurs after 140 hours (Wang et al., 2019). The parasitized host continues feeding and develops into a pupa, at which point *Ganaspis* spp. kills the host, feeds on the pupal body, and pupates inside the host puparium. Adult females emerge with an average of 40 mature eggs, increasing to 80 after 2-3 days. In general oviposition begins within two days after emergence. When continuously exposed to susceptible hosts,

Ganaspis spp. females survive approximately 18 days and produce an average of 98 offspring (Wang et al., 2018). Annual life cycle is shown in Figure 1.5.

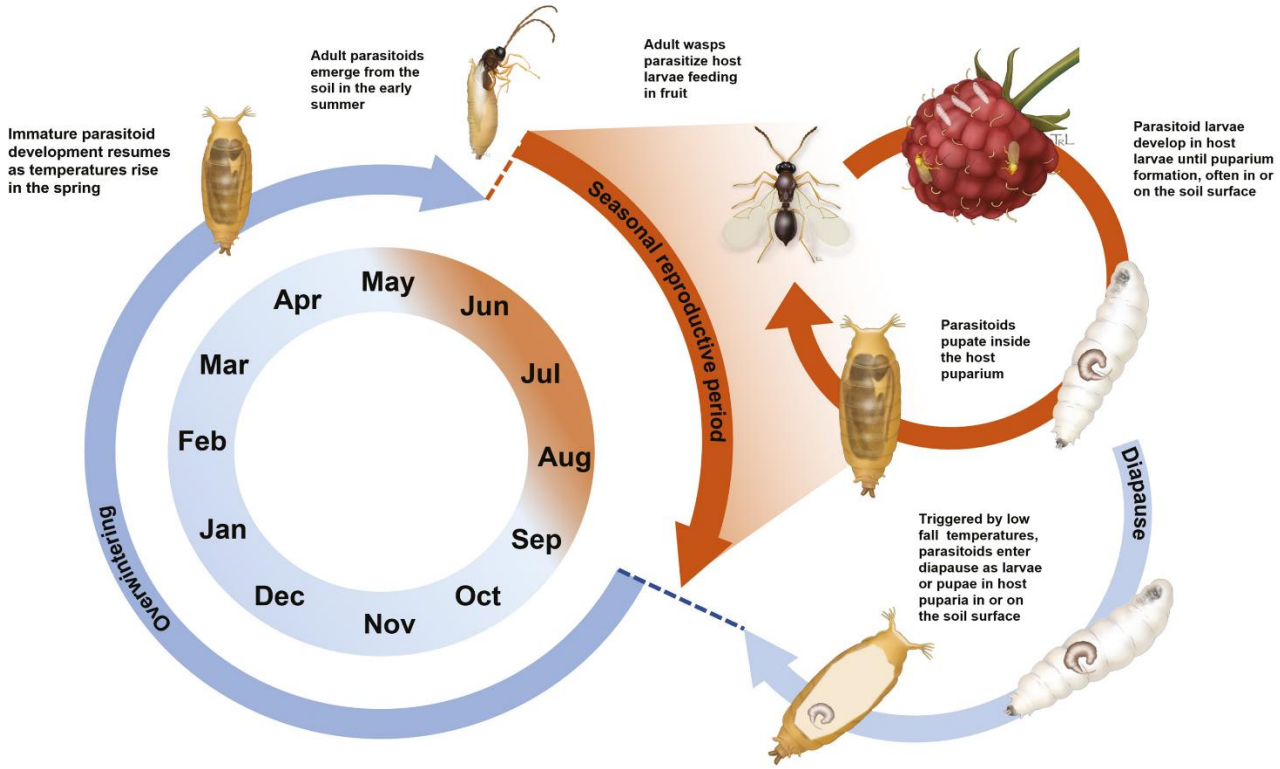

Figure 1.5*:* Graphical representation of *Ganaspis kimorum* life cycle (Stahl et al., 2024).

1.5.4 Host specificity

Host specificity is a central criterion in the selection of classical biological control agents and plays a major role in risk assessment (Barratt et al., 2023; Heimpel and Cock, 2018; Heimpel and Mills, 2017; Hoddle et al., 2021). While this aspect is one of the most extensively studied and well-documented, the recent redescription of *Ganaspis* species (Sosa-Calvo et al., 2024) has increased uncertainties in previous assessments, as early parasitoid trials conducted under the name *G. brasiliensis* included a mix of *G. kimorum* and *G. lupini*. Tests on a Japanese strain of *G. brasiliensis* (now considered *G. kimorum*) showed a strong preference for *D. suzukii*, whereas a Chinese

population (now considered *G. lupini*) was capable of parasitizing *D. melanogaster* and *D. subobscura* as well (Girod et al., 2018b). Seehausen et al. demonstrated *G. kimorum* specificity for *D. suzukii* compared to *D. melanogaster* in both laboratory choice tests (Seehausen et al., 2020) and in large-arena field cages (Seehausen et al., 2022). In fact, out of 957 emerging parasitoids, only one originated from the non-target *D. melanogaster* developing in decomposing fruit. These host specificity findings confirmed *G. kimorum* as a specialized parasitoid of *D. suzukii*, and supported its authorization for release in Italy (Lisi et al., 2022).

1.6 Thesis objectives

While the host specificity of *G. kimorum* was confirmed and its release authorized, several aspects of its biology, ecology and behaviour remained unknown, some of which are particularly relevant to the success of classical biological control programmes. The main objective of this thesis was to *assess the efficacy of G. kimorum as classical biological control agent in the newly introduced area*. To achieve this, the research was structured around four key research lines, each addressing one or more thematic hypotheses tested in specific studies.

1.6.1 Establishment and ecological interactions (CHAPTER 2-3)

Hypothesis: *Ganapsis kimorum* can successfully establish in the introduced area (CH 2).

A fundamental requirement for the success of a classical biological control program is the establishment of the introduced parasitoid. Following the first-ever propagative releases of *G. kimorum* in Northern Italy in 2021, pre- and post-release samplings were conducted to monitor its establishment, potential interactions with local non-target species, and overwintering capacity.

Hypothesis: Released *Ganaspis kimorum* is subjected to competitive pressure (CH 3).

One potential factor influencing the efficacy of *G. kimorum* as a biological control agent is interspecific competition. In particular, concerns were raised about its interaction with *Leptopilina japonica*, an adventive parasitoid species recently recorded in the release region (Puppato et al., 2020). Wang et al. (2019) conducted additive-series laboratory bioassays to evaluate interspecific competition among larval parasitoids of *D. suzukii*. Their results indicated that *Ganaspis* spp. could fully discriminate against *L. japonica*, avoiding already-parasitized hosts. However, *L. japonica*, due to its faster development, was capable of eliminating interspecific competitors, including *Ganaspis* spp. (Wang et al., 2019). Given the recent taxonomic revision of *G. kimorum* and the limitations of previous observations conducted in quarantine laboratories, this study focused on assessing interspecific competition under field conditions.

1.6.2 Overwintering (CHAPTER 4)

Hypothesis: *Ganapsis kimorum* is capable of overwintering in the introduced area (CH 4).

An important prerequisite for a successful establishment is that newly released BCA is able to overwinter in the introduced area. This aspect is particularly important when selecting release sites, as different climatic conditions may affect winter survival. Data on the thermal biology of *Ganaspis* spp. are limited to *G. lupini* (Hougardy et al., 2019; Stahl et al., 2024). Furthermore, these studies were conducted under laboratory conditions, which do not accurately reflect the fluctuating temperatures typical of natural environments. In this study, we investigated *G. kimorum* overwintering strategies by exposing the parasitoids to seven sites distributed at different altitudes in Switzerland and Northern Italy.

1.6.3 Host searching behaviour (CHAPTER 5, 6 and 7)

Hypothesis: *Ganapsis kimorum* host searching is regulated by chemical cues (CH 5).

Understanding how BCAs locate their host habitat and ultimately the target pest is critical for evaluating their specificity and efficacy. In this study, we conducted olfactometry and gas chromatography assays to assess the attractiveness of infested fruit and identify the volatile compounds most likely involved in *G. kimorum*'s response.

Hypothesis: *Ganapsis kimorum* host searching is regulated by vibrational cues (CH 5-6).

The role of vibrations in *Drosophila*'s parasitoids host detection has been investigated in the past, suggesting that some species may rely on vibrational cues (vibrotaxis) more than others (Vet and Bakker, 1985). However, there is currently no knowledge on the vibrations produced by the larval stage of *Drosophila* species, nor confirmation of their role as cues. We conducted two series of experiments: first, to identify and characterize the vibrations produced by *D. suzukii* larvae (Chapter 5), and second, to carry out quantitative behavioural observations to determine if *G. kimorum* host searching behaviour is influenced by them (Chapter 6).

1.6.4 Integration in the agroecosystem (CHAPTER 8)

Hypothesis: *Ganapsis kimorum* is susceptible to insecticides used to control *D. suzukii* (CH 8).

The ultimate beneficiaries of classical biological control programmes are those most impacted by pest damage, in this case, farmers. As *D. suzukii* continuously migrates between forest vegetation and orchards (Tait et al., 2020), parasitoids are expected to follow similar pathways to locate the host. Given that chemical applications remain the primary control method against *D. suzukii* (see paragraph 1.4.2.1) parasitoids are also likely to be exposed to insecticides and their detrimental effects. In this study, we evaluated *G. kimorum* susceptibly to agrochemicals by assessing the lethal and sublethal effects of both topical and residual insecticide applications in laboratory and field trials.

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CHAPTER 2. ESTABLISHMENT AND ECOLOGICAL INTERACTIONS

First report on classical biological control releases of the larval parasitoid *Ganaspis brasiliensis* against *Drosophila suzukii* in northern Italy

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Abstract

Current management strategy of the invasive fruit fly *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) exploits different tools but relies mainly on chemical control. In the invaded areas, the local natural enemy community mostly consists of generalist pupal parasitoids unable to control the pest efficiently. Conversely, in the pest native area, there are more specialized sympatric larval parasitoids attacking *D. suzukii*. Following foreign explorations and quarantine risk assessments, the larval endoparasitoid *Ganaspis brasiliensis* (Ihering) (Hymenoptera: Figitidae) was selected as the best candidate for classical biological control programs. In 2021, the first ever propagative biocontrol program using a Japanese G1 lineage of *G. brasiliensis* started in Italy. Here we report the results of the first year of releases in the province of Trento (Northeast Italy), wherein *G. brasiliensis* was released in 12 locations. Pre- and post-release samplings on fresh and fallen fruits were performed around the release points to assess the recapture rate, the impact of the exotic parasitoid on *D. suzukii* and its potential interactions with local non-target species. After releases, *G. brasiliensis* was

recovered at 50% of the locations. The exotic parasitoid only emerged from *D. suzukii*, mostly from fresh fruit still on the plant. Post-overwintering monitoring revealed the presence of a four *G. brasiliensis* individuals at two release locations.

Keywords:

Propagative biocontrol, Spotted wing *Drosophila*, Exotic parasitoid, Figitidae, Drosophilidae

2.1 Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), native to southeast Asia, has gradually invaded North America and Europe (2008), South America (2012) and Africa (2017) becoming a global economic threat to soft-skinned fruit crops (Cini et al., 2012; Asplen et al., 2015; Anfora, 2020; Hassani et al., 2020). The control of this highly polyphagous pest primarily relies on local area management, mostly on the use of insecticides (Tait et al., 2021). However, this approach is often not environmentally friendly and economically sustainable because of pest re-infestations occurring shortly after treatment from surrounding vegetation (Tait et al., 2020). On the other hand, an area-wide management approach would deal better with such mobile pest by reducing the total pest population within crop and non-crop areas (Haye et al., 2016).

In this sense, biological control strategies to suppress *D. suzukii* have been intensively studied, and include the use of predators, parasitoids, pathogens and competitors (Tait et al., 2021). In particular, it has been shown that parasitoids can regulate the population of drosophilids, either by directly causing high mortality rates (Janssen et al., 1988; Driessen & Hemerik, 1991) or by affecting competitive interactions between sibling host species (Boulétreau et al., 1991). In the invaded areas, two cosmopolitan pupal parasitoids have been identified to successfully parasitize *D. suzukii*: *Trichopria drosophilae* (Perkins) (Hymenoptera: Diapriidae) and *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) (Miller et al., 2015), with the first being released in Italy and Mexico and the latter in North America for augmentative biological control (Rossi Stacconi et al., 2019; Gonzalez-Cabrera et al., 2019; Hogg et al., 2022). However, natural parasitism by these generalist pupal parasitoids is below 10% (Lee et al., 2019) and the introduction specialist parasitoids from the native area of *D. suzukii* for classical biological control remains a preferable option (Lee et al., 2019).

Classical biological control can be an effective strategy to control invasive insect pests (Hajek et al., 2016) and can offer protection to natural ecosystem (van Driesche et al., 2010). Although its application and number of introductions gradually decreased since the 1970s, this method gained higher establishment and success rate in recent days, regaining momentum and trust (Cock et al., 2016). This positive trend is also the result of higher attention given from national and international regulatory bodies into providing appropriate tools for risk assessment, import and release of exotic biocontrol agents (BCA), all necessary steps to limit potential negative impacts on biodiversity in areas of introduction (Barratt et al., 2018).

In its native environment, *D. suzukii* is regulated by a multitude of natural enemies and, among these, at least 14 parasitoid species (Daane et al., 2016). However, field collections and laboratory studies have shown that two larval parasitoids of the Figitidae family are the most specialized towards *D. suzukii*: *Leptopilina japonica* Novković & Kimura and *Ganaspis brasiliensis* (Ihering) (Girod et al., 2018a, b; Giorgini et al., 2019; Daane et al., 2021). In particular, for *G. brasiliensis*, five clades (G1 to G5) where identified on a genetic basis (Nomano et al., 2017; Giorgini et al., 2019), with the G3 attacking few hosts in the *Drosophila melanogaster* Meigen species group and the G1 being considered a specialist on *D. suzukii*, thus rapidly gaining researchers' interest as a candidate for classical biological control. Adventive populations of *L. japonica* have been recently identified in northeastern Italy (Puppato et al., 2020) and in the northwestern North America (Abram et al., 2020a), whereas the G1 clade of *G. brasiliensis* was only recorded in British Columbia (Canada) and Washington (USA) (Abram et al., 2020a; Beers et al., 2022).

In 2021, after the evaluation of a comprehensive risk assessment submitted by Italian scientific institutions and phytosanitary services, the Italian Ministry of Ecological Transition authorized the release of *G. brasiliensis* G1 in seven regions and two autonomous provinces within the frame of a

national biological control program (Rossi Stacconi pers. comm.). Here we report the results of the monitoring program following the first-year releases of *G. brasiliensis* in the province of Trento (Northeastern Italy), where the exotic parasitoid was released at 12 locations.

2.2 Material and methods

2.2.1 Parasitoid rearing and releases

Parasitoids were reared according to the mass rearing protocol specified in Rossi Stacconi et al. (2022). For host, we used laboratory-reared *D. suzukii* that originated from multiple field collections of live adults at different locations of Trento Province (TN) Italy, during 2020 and 2021. The starting colony of *G. brasiliensis* derived from wild individuals collected during surveys in Naganuma park (Hachioji, Tokyo, Japan) from 2015 to 2017 (Girod et al., 2018a) and were provided by CABI's Swiss centre (Delémont, Switzerland). Parasitoid releases were performed at 12 locations in the Trento province, Italy (Table 2.1). Locations were selected according to presence of suitable unmanaged *D. suzukii* hosts, known presence of the pest and absence of ecological vulnerability constraints (protected areas). The sites were distributed along an altitudinal range (Table 1) and categorized as valley bottom sites (0–249 m a.s.l.), hillside sites (250–699 m a.s.l.) and mountain sites (700 + m a.s.l.). All sites were located at the margins of wooded areas, providing natural corridors for parasitoid movements. Four sites (#1, #2, #10 and #12) were in proximity (< 50 m) of crops considered non-host for *D. suzukii* (apple orchards and vineyards), four sites (#4, #5, #8 and #11) were in proximity of cherry orchards and four sites (#3, #6, #7 and #9) were in proximity of multiple small fruit orchards (blueberry, blackberry, raspberry and strawberry). At each site, a single release point was selected and parasitoid releases were performed once a week during three consecutive weeks. The dates of the releases were set according with local host plants' phenology (Table 2.1). Each release consisted of 200 three-day-old adults of *G. brasiliensis* (sex ratio 50:50 M:F).

Table 2.1: Site features, samplings dates and parasitoid releases. Longitudes and latitudes are provided as degrees, minutes, and seconds format and refers to North and East, respectively. All sites are located in Northeast Italy. Light and dark shading indicate pre- and post-release sampling events, respectively. SS = specificity site; ES = establishment site.

Site features					Week number and parasitoid releases									
#	Type	m a.s.l.	Latitude (N)	Longitude (E)	33	34	35	36	37	38	39	40	41	42
1	SS	227	46°2'6.684"	11°8'33.594"	1st	2nd	3rd							
2	SS	263	46°3'26.525"	11°8'21.703"	1st	2nd	3rd							
3	SS	353	46°2'58.128"	11°30'33.408"	1st	2nd	3rd							
4	SS	480	46°3'25.063"	11°12'49.514"	1st	2nd	3rd							
5	SS	544	46°4'33.816"	11°13'59.513"	1st	2nd	3rd							
6	SS	746	46°5'10.392"	11°16'40.033"		1st	2nd	3rd						
7	SS	747	46°4'50.952"	11°31'4.728"		1st	2nd	3rd						
8	SS	749	46°2'7.584"	11°13'2.525"		1st	2nd	3rd						
9	SS	1043	46°9'36.252"	11°17'27.672"		1st	2nd	3rd						
10	ES	179	45°55'33.492"	11°4'46.164"		1st	2nd	3rd						
11	ES	198	45°58'8.875"	11°5'33.63"		1st	2nd	3rd						
12	ES	211	46°11'19.932"	11°8'10.32"		1st	2nd	3rd						

2.2.2 Field surveys

The authorization to release *G. brasiliensis* was received on August 17th 2021 and parasitoid releases started within one week. Field surveys were performed before (1–2 pre-release samplings) and after (six post-release samplings) the first release event (Table 2.1). At each site, at least one pre-release sampling and five post-release samplings were conducted. Due to the lack of experimental evidence comparing the outcomes of different sampling methods (Abram et al., 2022a), the twelve release locations were divided into two subgroups, establishment sites (n = 3) and specificity sites (n = 9), each adopting a different sampling methodology. The goal of monitoring establishment sites (ESs) was to verify the ability of *G. brasiliensis* to disperse and reproduce in the new environments after releasing; to this aim, large quantities of fruits were collected from a wide area surrounding the release sites (see below). On the other hand, the goal of monitoring in the specificity sites (SSs) was to assess the host specificity of *G. brasiliensis* towards *D. suzukii* and other non-target species, and its

interactions with other parasitoids. To this aim, at each site a fruit was collected from few selected host patches and all fly puparia deriving from it were sorted prior to eclosion. In 2020 and 2021, monitoring activity was also conducted in multiple control sites (Supplementary Table S1) where no release of *G. brasiliensis* was performed. Monitoring in control sites was carried out according to the sampling protocol described for ESs, with the aim to assess the presence of adventive populations of the parasitoid in the area of the study (Trento province, Italy) and supporting results from the pre-release monitoring in ESs and SSs. In the ESs, post-overwintering surveys were conducted on May 27th and June 21th 2022 to check the survival of *G. brasiliensis* after the cold season.

2.2.3 Fruit collections

In the ESs, multiple fruit collections were conducted on host plants located in an area within 200 m from the release point. To account for spatial variation in host densities and parasitism levels, all plant patches included in the monitoring area and carrying fruit susceptible to *D. suzukii* attack were sampled and kept individually. In the SSs, fruit collection was carried out from only four pre-selected host plant patches located within 100 m from the release point. For both sampling methodologies, all sampling points were georeferenced using SMASH Digital Field Mapping (HydroloGIS S.r.l, Bolzano, Italy) to calculate their distance from the release site. At each sampling point and fruit quantity permitting, sub-samplings were carried out both from the plants (fresh fruit), randomly picking over the whole vertical height of the canopy of each selected host plant patch, and from the underlying ground (overripe and rotten fruit). Each sub-sample aimed to fill a 280 ml plastic container (Unipak 5011–21, Berry Superfos, Bologna, Italy) corresponding to 50–80 g of fruit, depending on the size and the water content of individual fruit. All sub-samples were incubated for three weeks in separate containers under optimal condition ranges (21–24 °C; 65–75% RH; L:d 16:8 photoperiod). Incubation was carried out in modified plastic containers similar to those described by

Abram et al. (2022a). Within each container, fruit was placed over blotting paper covering a layer of sand (1 cm). Such method allowed for a better control over humidity, as sand worked as moisture retainer and prevented formation of liquid pools.

2.2.4 Laboratory handling of collected fruit

Two methodologies were adopted to assess emergence of the different fly and parasitoid species depending on the collection site (ESs or SSs). For fruit collected in the ESs, emerged individuals (flies and parasitoids) from each sub-sample were regularly removed from the container three times per week and stored in 70% ethanol until taxonomic identification. For samples collected in the SSs, *Drosophila* puparia were collected from containers as they formed. Based on size, color and the unique morphology of the spiracles of *D. suzukii* puparium (EPPO, 2013; Abram et al., 2022a), puparia were divided in *D. suzukii* and non-*D. suzukii* and kept in separate containers over moist filter paper until emergence of flies or parasitoids. This method allowed for an accurate estimation of parasitization rate on target and non-target by *G. brasiliensis* and by other larval and pupal parasitoid species. For both methodologies, at the end of the incubation period, containers were carefully inspected and all remaining uneclosed puparia were collected, re-constituted in water for 24 h and dissected to check for presence of dead hosts or parasitoids.

2.2.5 Data analysis

The number of *G. brasiliensis* and other parasitoids as well as that of hosts emerged from the samples was recorded. Identification was conducted on a morphological basis using taxonomic keys (Markow and O'Grady, 2005; Lue et al, 2016; Buffington and Forshage, 2016; Forshage & Nordlander, 2008). After identification, samples of the released and recovered *G. brasiliensis* and samples of non-target drosophilids were deposited in the permanent collection of the Trento's Science Museum (Trento,

Italy) and made available for further verifications and analysis. Descriptive analyses were performed on the occurrence of *G. brasiliensis* with other species. For data collected in the SSs, the total percentage parasitism and the percentage of aborted parasitism were assessed based on the protocol described in Abram et al (2022a).

2.3 Results

2.3.1 Occurrence of G. brasiliensis

No *G. brasiliensis* emerged from fruit collected in the control sites (Supplementary Table S2.1) or at the release sites during pre-release sampling (Tables. 2.2, 2.3). During post-release sampling, a total of 87 *G. brasiliensis* individuals was obtained from fruit collected at six release locations (#1, #4, #8, #9, #10 and #12). (Figure 2.1). Among these, 42 emerged from six fruit samples collected in the ESs and 45 from 11 fruit samples collected in the SSs (Tables 2.2, 2.3). In the ESs, all *G. brasiliensis* individuals derived from plant-sampled fruit, whereas in the SSs 31.1% *G. brasiliensis* eclosed from ground-sampled fruit (Tables 2.2, 2.3). Overall, eclosions spanned from samples collected during week 34 (August 23rd–29th) to week 39 (September 27th–October 3rd), with all sites but #12 scoring occurrence of *G. brasiliensis* from multiple weeks. In the SSs, eclosions of *G. brasiliensis* occurred at all the altitudinal ranges, with individuals being recorded from one valley bottom, one hillside and two mountain sites. Most of the fruit samples from which *G. brasiliensis* was recovered were wild blackberry (*Rubus ulmifolius* Schott—nine samples), the remaining samples consisted in elderberry (Sambucus nigra (L.)—three samples), cropped blackberry (*Rubus fruticosus* (L.)—two samples), alder buckthorn (*Frangula alnus* Mill.—two samples) and cropped raspberry (*Rubus idaeus* (L.)—one sample) (Figures 2.2, 2.3, 2.4). In the SSs, all fruit samples containing *G. brasiliensis* were collected in the proximity of the release point (0–10 m), representing 18% of all collected fruit samples. In the ESs, 33.3% of *G. brasiliensis* individuals were recorded at 0–10 m

(26.6% of the fruit samples), 47.6% at 20–50 m (27.5% of the fruit samples) and 19.1% at 100 m far from the release point on a single fruit sample. During the post-overwintering surveys, four *G. brasiliensis* individuals were recorded at two locations: a single female eclosed from St. Lucie cherry (*Prunus mahaleb* L.) sampled from plant on May 27th 2022 at the site #10, while two males and one female eclosed from two common mulberry (*Morus alba* L.) samples collected from ground and from plant on June 21st 2022 at site #12.

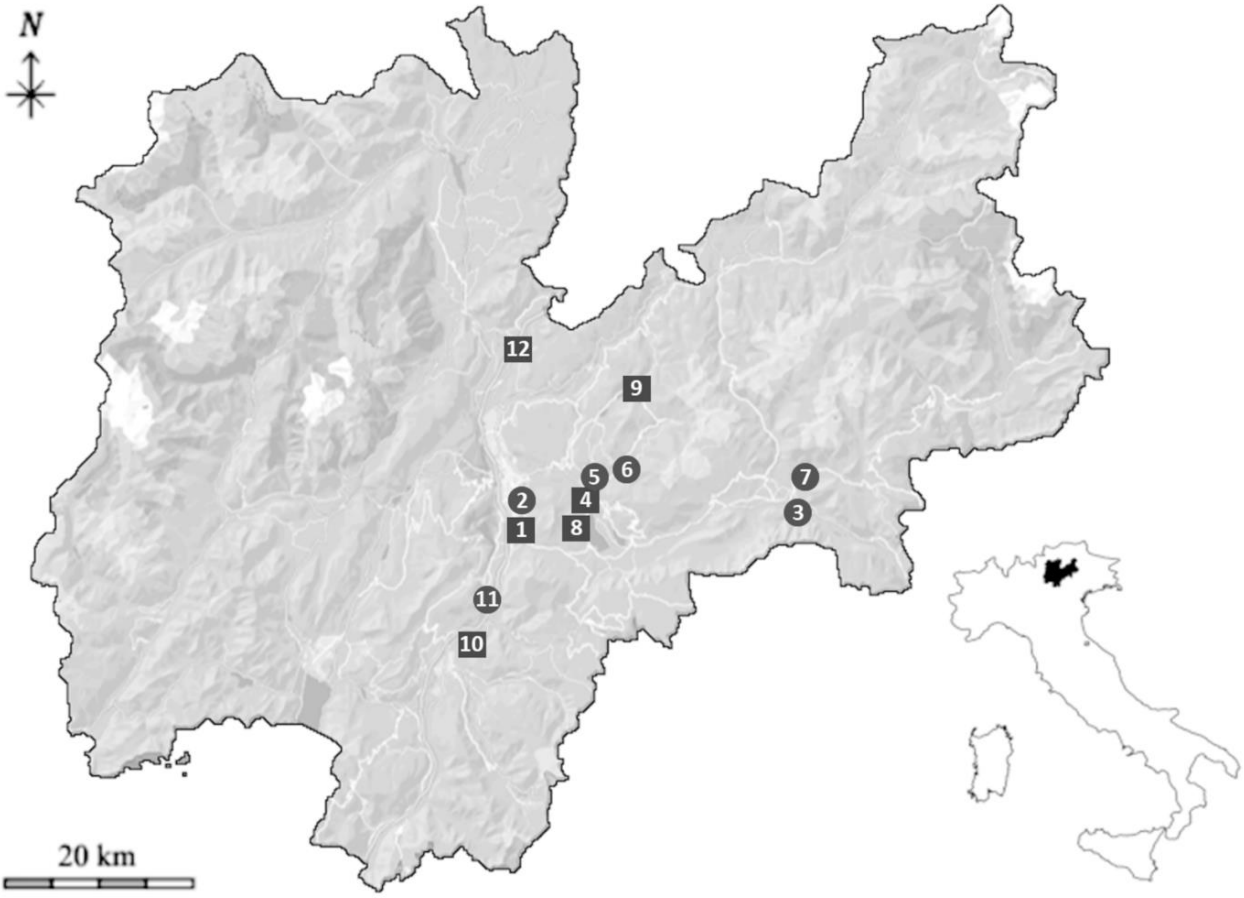

Figure 2.1: Distribution map of the release sites within the Province of Trento (Italy). Squares and circles indicate locations with and without *G. brasiliensis* occurrence during the monitoring, respectively. Location numbers refer to those listed in table 2.1.

Table 2.2: Number of *D. suzukii* (Ds), other host flies (OH), *G. brasiliensis* (Gb), *L. japonica* (Lj), local pupal parasitoids (LP), and local larval parasitoids (LL) eclosed from fruit samples collected in the establishment sites. The number of fruit samples for each category is shown in brackets. Partial sums for pre- and post-release samplings (bold) and the grand total (shading) are showed.

Establishment sites	***Ds***	**OH**	***Gb***	***Lj***	**LP**	**LL**
Plant (45)	3374	49	0	138	0	0
Ground (13)	193	800	0	49	84	113
Pre-release (58)	**3567**	**849**	**0**	**187**	**84**	**113**
Plant (116)	9086	150	42	549	12	0
Ground (55)	1070	2985	0	124	113	105
Post-release (171)	**10156**	**3135**	**42**	**673**	**125**	**105**
Total (229)	**13723**	**3984**	**42**	**860**	**209**	**218**

Table 2.3: Total number of puparia (TP) of *D. suzukii* and other host flies isolated from the fruit samples collected in the specificity sites and number of *G. brasiliensis* (Gb), *L. japonica* (Lj), local pupal parasitoids (LP), and local larval parasitoids (LL) eclosed from the puparia. The number of fruit samples for each category is shown in brackets. Partial sums for pre- and post-release samplings (bold) and grand totals (shading) are showed.

Specificity sites		**TP**	***Gb***	***Lj***	**LP**	**LL**
D. suzukii	Plant (17)	1631	0	98	0	0
	Ground (9)	816	0	293	27	0
	Pre-release (26)	**2447**	**0**	**391**	**27**	**0**
	Plant (126)	14511	31	964	9	0
	Ground (32)	986	14	168	15	0
	Post-release (158)	**15497**	**45**	**1132**	**24**	**0**
	Total	**17944**	**45**	**1523**	**51**	**0**
Other drosophilids	Plant (17)	30	0	1	0	0
	Ground (9)	65	0	8	0	0
	Pre-release (26)	**95**	**0**	**9**	**0**	**0**
	Plant (126)	202	0	2	0	0
	Ground (32)	60	0	0	1	0
	Post-release (158)	**262**	**0**	**2**	**1**	**0**
	Total	**357**	**0**	**11**	**1**	**0**

2.3.2 Interactions of G. brasiliensis with other species

Drosophila suzukii was the main host eclosing from fruit samples representing 98.5% and 43.9% of all the flies obtained from plant-sampled and ground-sampled fruit, respectively (Tables 2.2, 2.3). Other host species consisted in *D. melanogaster* (0.98% and 36.8% from plant-sampled and ground-sampled fruit, respectively), *Drosophila simulans* Sturtevant (0.37% and 11.6%), *Drosophila immigrans* Sturtevant (0.15% and 4.1%), *Drosophila subobscura* Collin (1.5% from ground-sampled fruit), *Drosophila hydei* Sturtevant (1.1% from ground-sampled fruit), *Drosophila busckii* Coquillett (0.9% from ground-sampled fruit) and *Drosophila funebris* (Fabricius) (0.1% from ground-sampled fruit). In the SSs, the occurrence of *G. brasiliensis* was recorded only from *D. suzukii* pupae collected in post-release samplings (Table 2.3). Likewise, in the ESs all *G. brasiliensis* individuals eclosed from fruit samples in which the recorded host eclosion was 100% *D. suzukii*. *Ganaspis brasiliensis* individuals accounted for 4.44% and 3.74% of all the parasitoids recorded in the ESs and SSs, respectively. Several other parasitoid species were recorded from both ESs and SSs during post-release monitoring. The most abundant was the exotic larval parasitoid *L. japonica* representing 71.22% and 94.18% of all parasitoid individuals eclosed in ESs and SSs, respectively. Among the local species, pupal parasitoids were *P. vindemiae* (9.50% in ESs and 1.49% in SSs), *T. drosophilae* (3.69% in ESs and 0.58% in SSs) and *Vrestovia fidenas* (Walker) (0.04% in ESs), while larval parasitoids were *Leptopilina heterotoma* (Thomson) (6.04% in ESs), *Leptopilina boulardi* (Barbotin) (4.89% in ESs) and *Asobara tabida* (Nees) (0.17% in ESs). In berry samples from the SSs in which *G. brasiliensis* was present, apparent parasitism was on average 2.3% (0.2–13.1), accounting for 17.9% (span: 1.8–67.8%) of the total apparent parasitism recorded in those samples (*G. brasiliensis* + other parasitoids). When *G. brasiliensis* parasitism occurred, *L. japonica* was always present and more abundant than *G. brasiliensis*. Local larval parasitoid species never eclosed from

plant-sampled fruit and were absent in the two ground-sampled fruit samples attacked by *G. brasiliensis* (Tables 2.2, 2.3). When dissecting D. *suzukii* uneclosed puparia (< 0.7% of the total puparia), no sign of aborted parasitism was recorded.

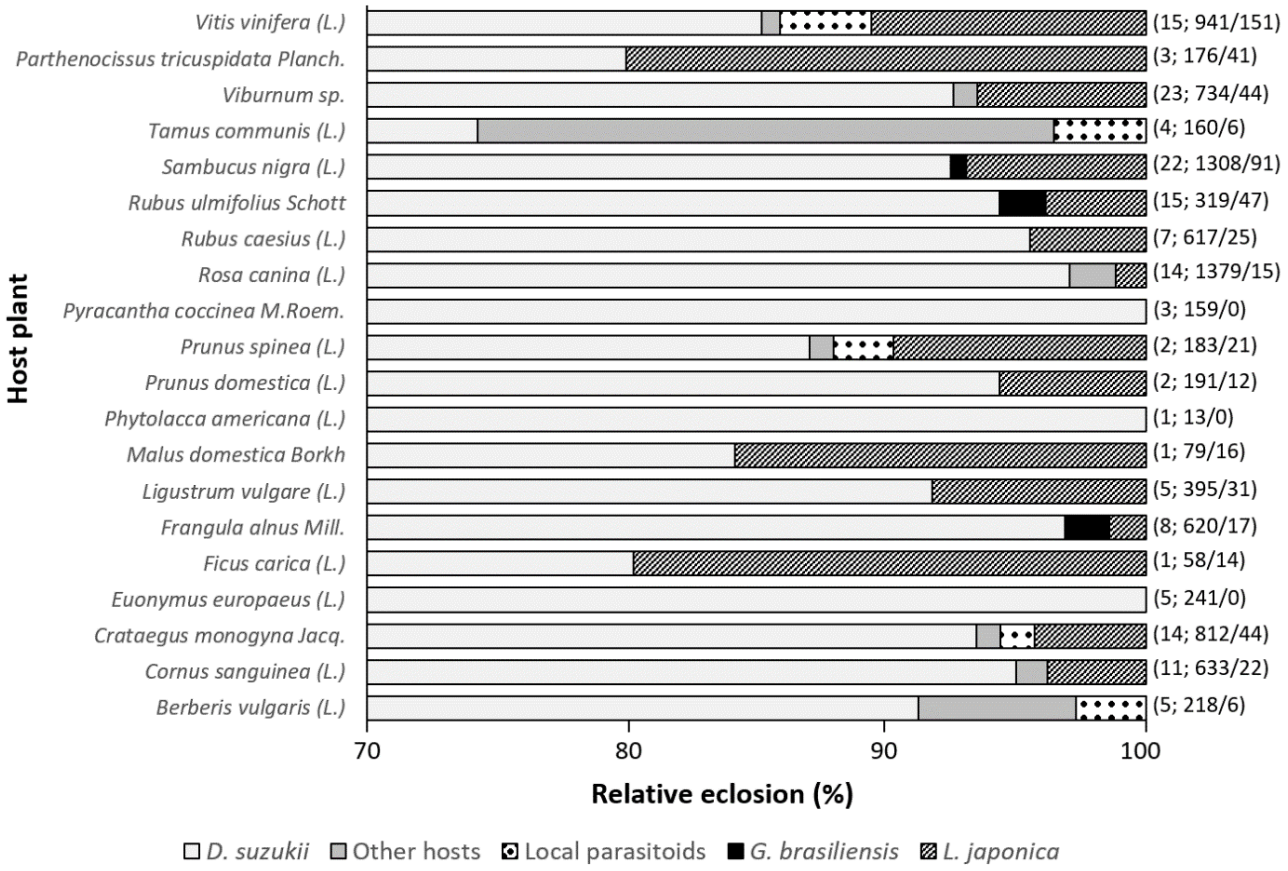

Figure 2.2: Relative eclosion percentage of flies and parasitoids in fruit collected from different host plants during post-release monitoring in the establishment sites. For each host plant species, the number of samples collected followed by the number of fly and parasitoid individuals eclosed are indicated in brackets.

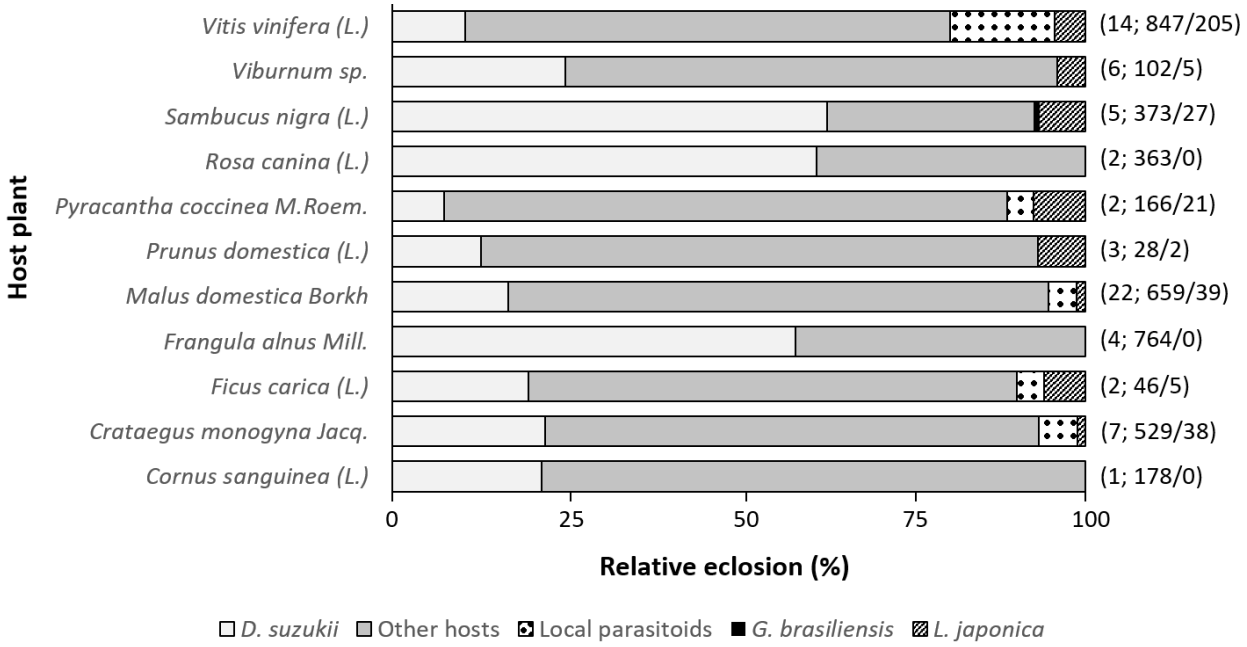

Figure 2.3: Relative eclosion percentage of flies and parasitoids in ground-sampled fruit collected during post-release monitoring in the establishment sites. For each host plant species, the number of samples collected followed by the number of fly and parasitoid individuals eclosed are indicated in brackets.

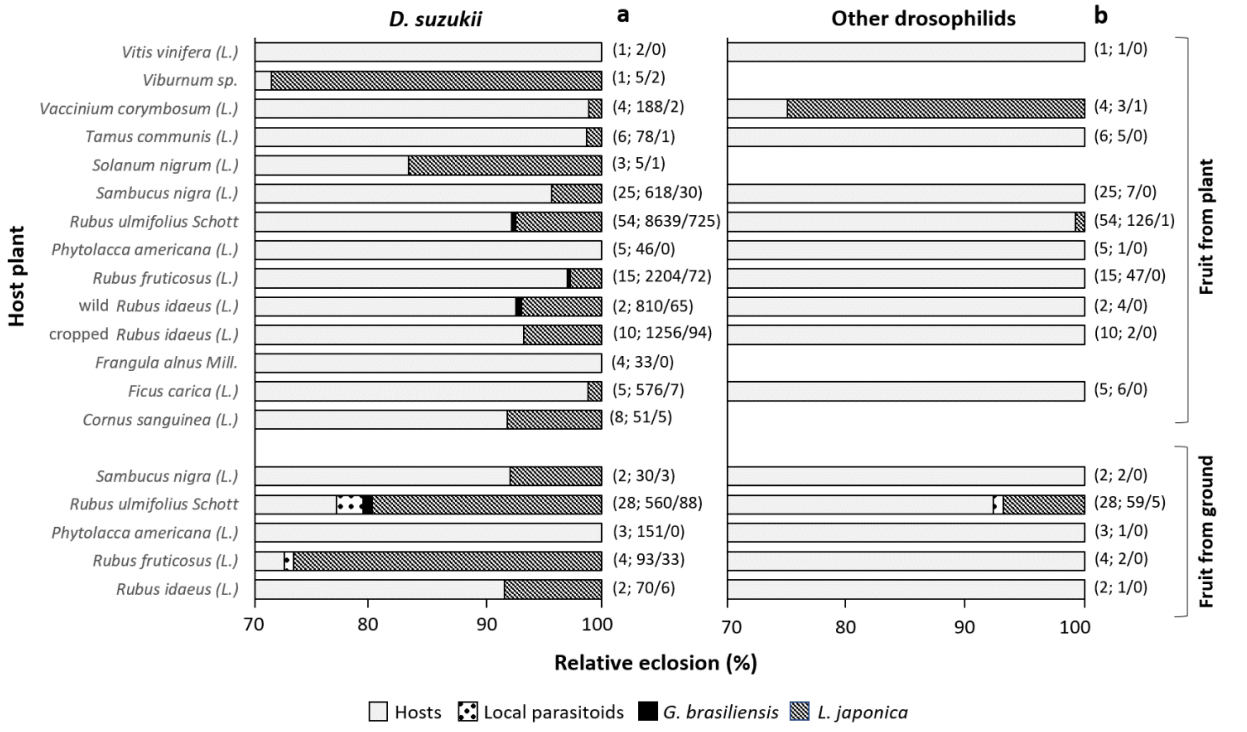

Figure 2.4: Relative eclosion percentage of flies and parasitoids from the puparia of *D. suzukii* (**a**) and of other host flies (**b**) collected from plant- and ground-sampled fruit during post-release monitoring in the specificity sites. For each host plant species, the number of samples collected followed by the number of fly and parasitoid individuals eclosed are indicated in brackets.

2.4 Discussion

Implementing a propagative (classical) biocontrol program involves efforts that are equally distributed between the pre- and post-release phases. Pre-release work seeks to draft a comprehensive risk assessment for the candidate exotic BCA, considering all positive and negative consequences of its potential introduction in a new environment (Barratt et al., 2010). Post-release work mainly aims to verify (1) the initial survival and potential establishment of the released BCA and (2) the interactions of the BCA with the target species and with other local organisms (non-targets) (van Driesche et al., 2010). In our post-release study, the results of the first-year monitoring showed that *G. brasiliensis* can reproduce and overwinter in the release area. In particular, the sites at which the parasitoid was recovered during the post-overwintering surveys are both located at the valley bottom and characterized by a temperate climate with hot summer and no dry season (Beck et al., 2018). This is consistent with the CLIMEX model prediction from Wang et al. (2020), indicating the suitability of most of the temperate European areas for the establishment of the exotic parasitoid. Despite the low number of parasitoids released per site (300 females and 300 males), *G. brasiliensis* was recovered in 50% of the sites during the six weeks following the first release event (weeks 34–39) and regardless the altitudinal range. Moreover, post-overwintering monitoring showed that, at least at two locations, *G. brasiliensis* was able to survive the cold season and to start new generations in spring. These findings strongly suggest a climatic suitability of the Province of Trento for the parasitoid. However, further data need to be collected in the next years, particularly in relation to the ability of the exotic BCA to overwinter in release area (Hokkanen and Sailer, 1985). In fact, winter 2021–2022 has been relatively mild compared with the local climatic historical series and this unusual temperature trend may have favored the survival of *G. brasiliensis*. Preliminary laboratory trials assessing survival of different *G. brasiliensis* stages at low temperature suggest that the parasitoid overwinters as pharate

adult within the host puparium (Rossi Stacconi pers. comm.), but further experiments are needed for confirmation. The few records of *G. brasiliensis* adults scored during the post-overwintering monitoring activity may appear as a rather disappointing result. However, recent examples of successful classical biological control shed a positive light on this data. For example, the parasitoid *Torymus sinensis* Kamijo (Hymenoptera: Torymidae) was released for the first time in 2005 and 2006 at three sites located in Piedmont (Italy) to control the invasive Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae). In 2007, out of 36.000 galls sampled at the same release sites, a total of only 30 adults of *T. sinensis* were collected (Quacchia et al., 2008). By 2009, the parasitoid emerged from 4.08% of the collected galls and after seven years from its first release (in 2012) *T. sinensis* parasitization rate was steadily above 80% (Ferraccini et al., 2019).

Two different monitoring approaches were adopted for establishment and specificity sites, allowing for different evaluations on the impact of *G. brasiliensis* in the release area. Monitoring in the ESs aimed to sample large quantity of fruit from a wide sampling area in order to maximize the probability to recover *G. brasiliensis*, to assess its spread capacity from the release point and to provide an overview of the host plants exploited by the parasitoid during host search. On the other hand, most of the sites were monitored as SSs sampling a reduced amount of fruit samples in order to evaluate in detail parasitism parameters and host specificity of *G. brasiliensis*, As expected, the occurrence rate of *G. brasiliensis* was higher in ESs (66.6% of the sites) than in SSs (44.4% of the sites), although in the SSs were found more fruit samples attacked by the BCA (n. 11/184) than in the ESs (n. 6/229). However, looking at the distribution of the attacked fruit samples, the ESs monitoring approach seems to provide a more accurate representation of the parasitoid movements within the sampled area compared to the SSs approach, showing some parasitoid activity away from the release point. Despite the practical differences, data from the two monitoring approaches were consistent with

the fact that *G. brasiliensis* G1 behaves as a specialist on *D. suzukii*, never eclosing from puparia of other species (SSs) or from fruit samples infested with other flies than *D. suzukii* (ESs). This aspect of the biology of *G. brasiliensis* G1 was already observed in its native range (Daane et al., 2016; Girod et al., 2018a), in quarantine facility studies (Girod et al., 2018b; Daane et al., 2021) and in field cage releases (Seehausen et al., 2022), but this is the first study reporting the parasitoid's strict association with *D. suzukii* in natural conditions outside its native range. A second aspect that has been further confirmed by our study, is that *G. brasiliensis* tends to avoid competition by attacking its hosts mostly within fresh fruit still on the plant (Giorgini et al., 2019), whereas local parasitoids of drosophilids parasitize their hosts on decaying fruit. Although chemical ecology studies have not yet been conducted on olfactory preference of *G. brasiliensis* G1 towards infested and uninfested fruit, such avoidance behavior was already suggested in a recent study considering the competitive parasitization outcomes of three solitary larval parasitoids of drosophilids; *G. brasiliensis* G3, *L. japonica* and *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae) (Wang et al., 2019). The study showed that *G. brasiliensis* G3 is outcompeted by both the other parasitoids and, in response to that, it discriminates against parasitized hosts. Also, we observed that the ovipositor of the released strain of *G. brasiliensis* is significantly shorter compared to that of other larval parasitoids recovered from the monitoring (Supplementary Figure S2.1). Such morphological feature would allow *G. brasiliensis* to only parasitize host larvae located just below the fruit skin of fresh fruits (*i.e.*, 1st and 2nd instars) and making it extremely difficult for the exotic parasitoid to attack hosts buried in a rotten substrate. In terms of impact on biodiversity, these functional traits (preference towards fresh fruit and ovipositor morphology) exhibited by *G. brasiliensis* creates a specific trophic niche for the BCA (Cohen, 1977) and reduces the risk of negative impacts on non-target organisms, such as local larval parasitoids.

Overall, the result of our monitoring showed that *L. japonica* was the dominant parasitoid species in all locations where *G. brasiliensis* was released. This adventive exotic species was reported in Italy (Province of Trento) for the first time in 2019 at one location (Puppato et al., 2020) and, since then, increasing catches have been reported. In its native range, *L. japonica* is frequently found on *D. suzukii*, which is considered a preferred host (Girod et al., 2018b; Giorgini et al., 2019), and recent studies reported that this parasitoid reproduces on a relatively narrow host range, although not narrow enough to be considered as a candidate for classical biocontrol programs (Girod et al., 2018a; Daane et al., 2021). Likely, the presence of *D. suzukii* as one of the most abundant species feeding on cultivated and wild fruit have been a key factor contributing to the rapid dispersal and population growth of *L. japonica* in the Alpine region and a similar trend can be expected for *G. brasiliensis*. However, population changes induced by biological control programs often require years to reach stable endpoints and the outcomes are function of many dependent and independent variables, including the intrinsic characteristics of the pest, the BCA and the target ecosystem (Hokkanen and Sailer 1985). Abram et al. (2022b) recently reported that in Canada (British Columbia) the two adventive parasitoids, *G. brasiliensis* and *L. japonica*, have re-built the same close association with *D. suzukii* observed in the pest's native range, with the latter being the dominant species. Extensive fruit samples carried out in 2020 showed *D. suzukii* parasitization by the two parasitoids to be time-structured and widespread across a variety of habitats and host plants. Such observations have been made a short time after the first reports of *G. brasiliensis* and L. *japonica* in the area (one and four years, respectively), and most likely the parasitoids' dispersal is still ongoing (Abram et al., 2022b). In the area of the present study, *D. suzukii* is considered a major pest of soft fruit and, more than a decade after the introduction, its impact still accounts for 9% of the potential revenues, mostly due to costly control measures (De Ros et al., 2020). Realistic expectations for impact of classical biological control in Italy might be that *G. brasiliensis* will contribute in restoring part of the top-down pest

suppression existing in the *D. suzukii* native area. However, such contribution will likely be difficult to quantify in terms of success or failure, as it is expected to be regionally variable and to add to that provided by other local and adventive biocontrol organisms (Abram et al., 2020b). In this sense, the establishment of stable populations of *G. brasiliensis* and their relative contribution to *D. suzukii* larval parasitism would be a first step towards success of the classical biological control program. Once this is accomplished, an evaluation should be done in the long run to assess whether the increased parasitism has resulted in a reduction of management costs.

2.5 Supplementary material

Table S2.1: Monitoring activities undertaken in 2020 and 2021 in locations where *G. brasiliensis* was not released. Samplings were conducted with the same modality described for the establishment sites and according with the phenology of the fruit at each location. The 2020 control sites included all 12 sites in which *G. brasiliensis* was released in 2021. The overall numbers of *G. brasiliensis*, larval parasitoids and pupal parasitoids eclosed from the sampled fruit is reported.

n° of monitored sites	Sampling period	*G. brasiliensis*	Larval parasitoids	Pupal parasitoids
27	May 18 - July 8 2020	0	372	185
18	July 7 - September 9 2020	0	425	34
7	July 30 - September 30 2020	0	827	230
18	May 31 - July 20 2021	0	185	4
4	July 12 - July 26 2021	0	33	0

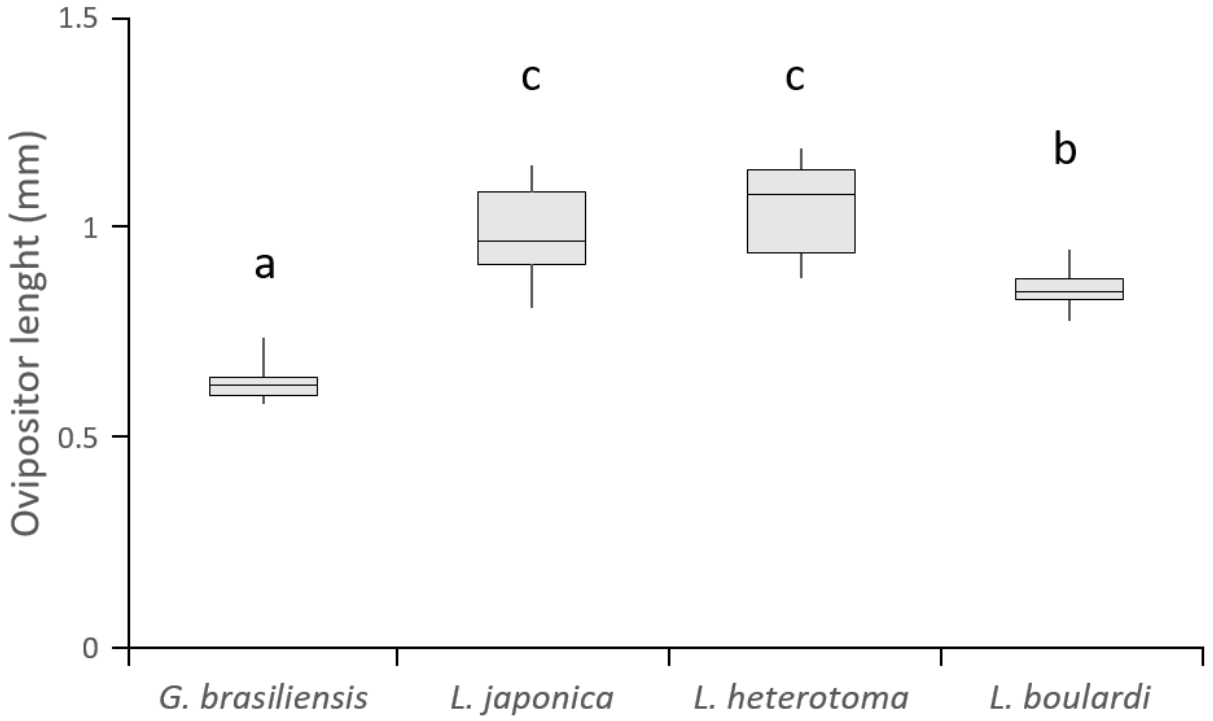

Figure S2.1: Boxplots showing the distribution of the ovipositor length measurement (mm) in *G. brasiliensis* (n = 20), *L. japonica* (n = 20), *L. heterotoma* (n = 17) and *L. boulardi* (n = 19). Each boxplot shows the minimum and maximum values (whiskers), the range of values between the 25th and the 75th percentile (box) and the median (central line). Different letters indicate significant differences ($p < 0.001$) after the Kruskal-Wallis test followed by Dunn's post hoc test. Parasitoids were immersed in 70% alcohol and carefully dissected under a stereomicroscope (Nikon SMZ800, Nikon, Japan) to remove the coiled ovipositors from the abdomen and from the ovipositor sheath. Each ovipositor was projected onto paper and digitally measured from the tip to the valvifers using an imaging setup (Nikon Digital Sight DS-Fi1, Nikon, Japan).

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CHAPTER 3. INTERSPECIFIC COMPETITION

Ecological challenges for a newly introduced biological control agent. Assessing interspecific competition affecting *Ganaspis kimorum*.

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Abstract

Ganaspis kimorum has been selected as a classical biological control agent (BCA) against *Drosophila suzukii* and has recently been released in Italy and North America. Although initial establishment has been reported, concerns persist regarding its competitive interactions with other parasitoids, particularly *Leptopilina japonica*. This study assessed the competitive dynamics of *G. kimorum* under natural conditions. Field experiments involved three treatments: (i) *D. suzukii*-infested blueberries protected by fine mesh to exclude external parasitoids (Control), (ii) *D. suzukii*-infested blueberries parasitized by *G. kimorum* and exposed to local parasitoids and drosophilids (Treatment), and (iii) uninfested blueberries exposed to the environment (Negative control). Principal component analysis revealed that treatment type was the main factor influencing insect emergence patterns. Emergence of *G. kimorum* in Treatment samples decreased by 62% relative to the Control. *Leptopilina* spp. consistently emerged earlier than *G. kimorum*, suggesting a temporal competitive advantage. Overall, our study demonstrated that *G. kimorum* residing in *D. suzukii*-infested fruits faces significant competitive pressure, most likely driven by secondary infestation of *Drosophila* spp. and by parasitoids of the genus *Leptopilina*. These results highlight the importance of considering biotic interactions when evaluating the long-term success of classical biological control programs.

3.1 Introduction

Ganaspis kimorum Buffington (Hymenoptera: Figitidae), has recently been selected as classical biological control agent (BCA) against the spotted wing Drosophila, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), and has been released in both Italy and North America (Fellin et al., 2023; Stahl et al., 2024). Initial reports suggest that *G. kimorum* is successfully establishing in the introduced areas (Fellin et al., 2023). However, concerns remain regarding its efficacy, particularly due to its interactions with native and adventive parasitoids, including *Leptopilina japonica* Novković and Kimura (Hymenoptera: Figitidae), another figitid parasitoid of *D. suzukii* that has recently been recorded in both Europe and North America (Beers et al., 2022; Puppato et al., 2020).

Wang et al. (2019) conducted additive-series laboratory bioassays to evaluate interspecific competition among larval parasitoids of *D. suzukii*. Their results indicated that *Ganaspis lupini* (previously referred to as *Ganaspis brasiliensis* G3) could fully discriminate against *L. japonica*, avoiding already parasitized hosts. However, due to its tendency to multi-parasitize the host and its faster development, *L. japonica* was capable of eliminating interspecific competitors, including *Ganaspis* spp. (Wang et al., 2019).

Given the recent taxonomic revision of *G. kimorum* (Sosa-Calvo et al., 2024) and the limitations of previous studies conducted in quarantine laboratories, this study aims to assess competitive pressure experienced by the BCA under field conditions.

3.2 Material and method

3.2.1 Insect colonies

Ganaspis kimorum specimens originated from a population collected in 2016 near Tokyo, Japan (Girod et al., 2018). Parasitoids were reared at Edmund Mach Foundation (FEM) in S. Michele all'Adige (Italy). Rearing followed the protocol described by Rossi-Stacconi et al. (2022), under controlled conditions (16:8 light:dark (L:D) photoperiod, 22 ± 2°C and 70 ± 10% relative humidity (RH)).

Drosophila suzukii colonies were established from locally collected adults and maintained in climatic chambers with a photoperiod of 16:8 L:D at 22-24 ± 2°C and 50-70 ± 10% RH.

3.2.2 Experimental set up

The experiment was conducted between August and September 2023 in three independent sites (>5 km apart) located in the Trentino-Alto Adige region (Italy): San Michele all'Adige (SMA), Faedo (FAE) and Laimburg (LAI). Each site featured similar landscape, consisting of a natural forest bordering an agricultural field (vineyard). At each site, ten metal cages (20 × 20 × 5 cm, 5 mm mesh) were positioned near *D. suzukii* host plants to prevent interference from birds, rodents, or other macro-organisms. Five cages were attached to plant branches of the canopy at a height of 1–2 m (CAN), while the remaining five were positioned on the ground beneath the plants (GRO). Each cage contained six small absorbent paper containers (5 × 5 × 4 cm) holding blueberries assigned to different treatments. Before field exposures, blueberries from the same batch and variety were rinsed three times with water, dried at room temperature, and placed on sterile Petri dishes (9 cm Ø). They were then exposed to *D. suzukii* in rearing cages for one hour. Post-infestation, blueberries were inspected under a stereomicroscope (M80, Leica Microsystem, Germany), and only those containing between 10 and 20 eggs were selected. The selected blueberries were transferred to *G. kimorum* rearing cages for 48 hours to allow parasitization. Following parasitization, blueberries were randomly distributed

into absorbent paper containers, each holding 8 blueberries (15 ± 0.5 g), and assigned to one of the following treatments:

- Control (C): *D. suzukii*-infested blueberries covered with a fine mesh to prevent any external infestation and parasitization.
- Treatment (T): *D. suzukii*-infested blueberries parasitized by *G. kimorum*, left uncovered to allow secondary infestations and parasitization.
- Negative Control (N): Uninfested blueberries left uncovered to assess the distribution of local insect species.

Each treatment was replicated twice for collection at 5 and 9 days after-exposure (DAT) (C5, C9, T5, T9, N5, N9) resulting in a total of 180 samples (3 Sites x 5 Cages x 2 Positions x 2 DAT x 3 Treatments). After 5 and 9 days, the field containers were collected and transferred individually into ventilated plastic cylindrical containers. These were maintained under controlled conditions (16:8 L:D photoperiod, 22 ± 2°C and 70 ± 10% RH) and supplied with 2 mL of distilled water weekly. The emergence of drosophilids and parasitoids was recorded every two days, and all emerged adults were preserved in 70% ethanol for taxonomic classification. Identification was conducted under stereomicroscope based on morphological characteristics using taxonomic keys (Buffington and Forshage, 2016; Forshage and Nordlander, 2008; Lue et al., 2016; Markow and O'Grady, 2005; Sosa-Calvo et al., 2024). For analysis, emerging insects were categorized into four groups: *Drosophila suzukii* (Ds), *Ganaspis kimorum* (Gk), Other *Drosophila* spp. (OD), and *Leptopilina* spp. (LEP). Within the LEP group, were further classified three species: *Leptopilina boulardi* (Barbotin) (Hymenoptera: Figitidae), *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) and *Leptopilina japonica* Novković and Kimura (Hymenoptera: Figitidae).

3.2.3 Statistical analysis

Principal Component Analysis (PCA) was performed to examine the patterns and relationships within the insect emergence groups (Ds, Gk, OD and LEP) across multiple treatments. Prior to PCA, the numerical variables were standardized (centered and scaled) to ensure equal variance. The first five principal components (PCs) were extracted, and the analysis focused on identifying key variables explaining the highest variance in the dataset. The analysis was carried out in software R (4.3.0) using the "FactoMineR" package (Husson et al., 2024; R Core Team, 2020). As data were not normally distributed (assessed via Shapiro-Wilk test), a non-parametric Kruskal-Wallis rank sum test was performed to evaluate whether categorical variables (Treatment, Site, Cage, Position and DAT) significantly influenced the two main Principal Components (PC1 and PC2).

To further analyze differences in insect emergence among treatments, a Kruskal-Wallis was conducted separately for each insect group. Post-hoc comparisons were performed using pairwise Mann-Whitney U tests with Bonferroni corrections. All statistical analyses and visualizations were conducted in R using "dplyr", "rstatix" and "ggplot2" packages (Kassambara, 2019; R Core Team, 2020; Wickham, 2011; Wickham et al., 2023).

3.3 Results

The principal component analysis (PCA) revealed that the first two principal components explained 57.3% of the total variance, with PC1 accounting for 31.0% and PC2 for 26.3%. The PCA results indicated a clear clustering based on Treatment (Figure 3.1). The Kruskal-Wallis analysis confirmed that Treatment was the only variable highly significant influencing both PC1 (χ^2= 92, df = 2, $P < 0.001$) and PC2 (χ^2 = 37.5, df = 2, $P < 0.001$), while none of the other tested variables were

significant for both components. Therefore, subsequent analyses focused on how treatments (C, N, T) influenced insect emergence.

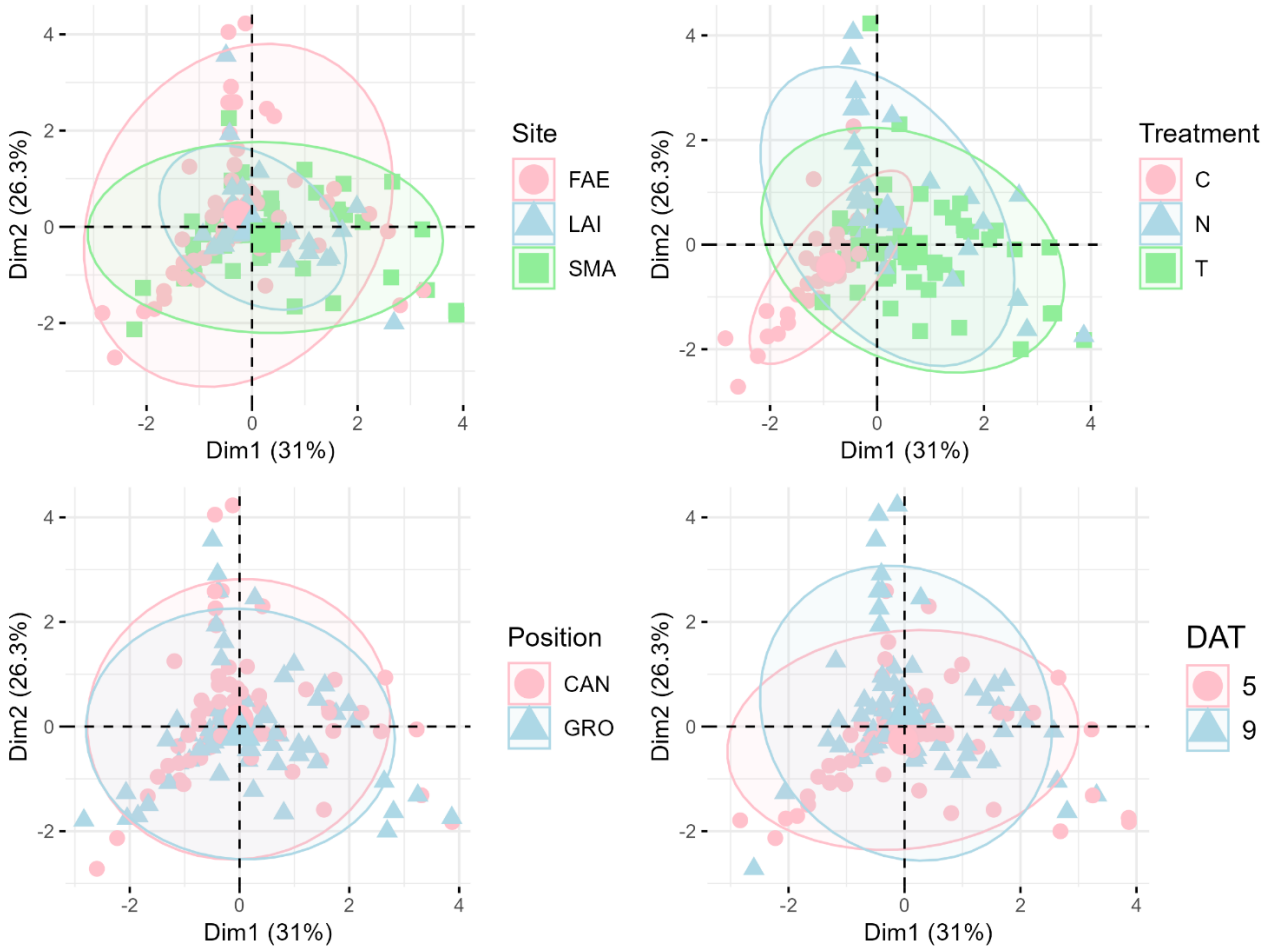

Figure 3.1: Biplot resulting from the Principal Component Analysis (PCA) of insect emergence data. Data are plotted on the two principal components (Dim1 and Dim2). All the factors recorded are shown, from top left (Site), top right (Treatment), bottom left (Position) and bottom right (Days After Treatment: DAT). Different colors and shapes identify the different factor levels, while ovals represent data clusters.

All insect groups were influenced by treatments, except for *D. suzukii*, which did not show significant differences among treatments (χ^2 = 5.68, df = 2, P > 0.05). Drosophilids (OD) exhibited 122% higher average emergence in the T group compared to N (U = 1095, P < 0.001, Figure 3.2a). Similarly, *Leptopilina* spp. (LEP) emergence was significantly higher in T compared to N group, with an increase of 104% (U = 1450, P < 0.05, Figure 3.2a). In contrast, *G. kimorum* (Gk) showed a 62%

lower average emergence in the T group compared to the control (C) (U = 2420, P < 0.01, Figure 3.2a).

The first emergence (FE) times were generally consistent within insect groups. However, in the T group, parasitoid emergence followed a distinct pattern: *Leptopilina* spp. emerged first at 21 days post-exposure, followed by *G. kimorum* at 23 days. The peak emergence (PE) of *D. suzukii* varied among treatments, occurring on Day 12 in the control (C) group, but delayed to Day 19 in the N and T groups (Table 3.1, Figure 3.2b).

Table 3.1: Days when First Emersion (FE) and Peak Emersion (PE) occurred. Data are shown for insect groups (Ds: *Drosophila suzukii*, OD: Other *Drosophila* spp., Gk: *Ganaspis kimorum*, LEP: *Leptopilina* spp.) among different treatments (C: Control, N, Negative control, T: Treatment). Data in parenthesis are the total number of emergences for the specific category.

	Ds		OD		Gk		LEP	
	FE	PE	FE	PE	FE	PE	FE	PE
C	12 (7)	14 (36)	-	-	21 (1)	26 (68)	-	-
N	12 (3)	19 (132)	12 (15)	19 (99)	-	-	21 (2)	26 (11)
T	12 (5)	19 (69)	12 (10)	19 (199)	23 (5)	26 (25)	21 (4)	26 (22)

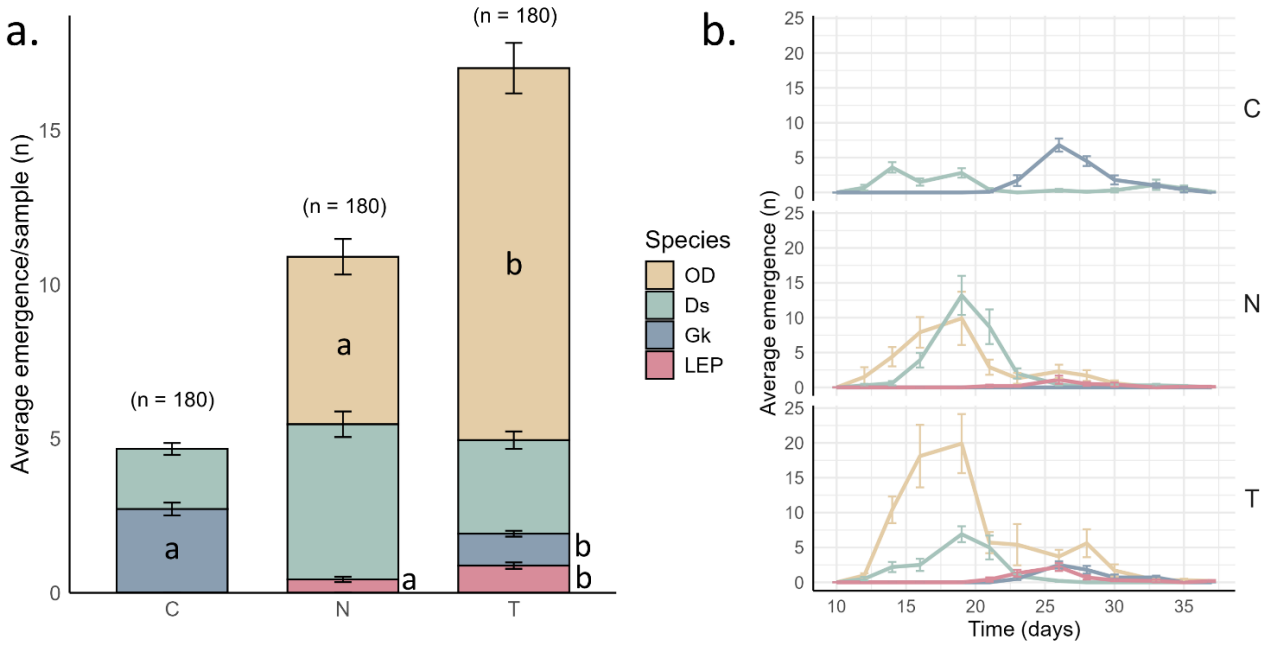

Figure 3.2: (**a**) Average emergence recorded for the insect groups (Ds: *Drosophila suzukii*, OD: Other *Drosophila* spp., Gk: *Ganaspis kimorum*, LEP: *Leptopilina* spp.) among different treatments (C: Control, N, Negative control, T: Treatment). Stacked bars show mean values, error bars Standard Error Mean (SEM), while different letters for the same insect group indicate significant differences according to the pair-wise Mann-Whitney U-test with Bonferroni corrections. (**b**) Trendline of insect emergence according to treatments. Error bars on the lines indicate SEM recorded on that specific day and insect group.

A deeper analysis of the LEP group revealed that out of the 79 emerging *Leptopilina* spp., the majority were *L. boulardi* (n = 33, 41.8%), followed by *L. japonica* (n = 25, 31.6%) and *L. heterotoma* (n = 21, 26.6%). The emergence of *L. boulardi* was significantly higher in the T group compared to N (U = 1510, P < 0.01, Figure 3.3a). Meanwhile *L. heterotoma* was found exclusively in samples collected from the ground (GRO) (U = 1530, P < 0.01, Figure 3.3b).

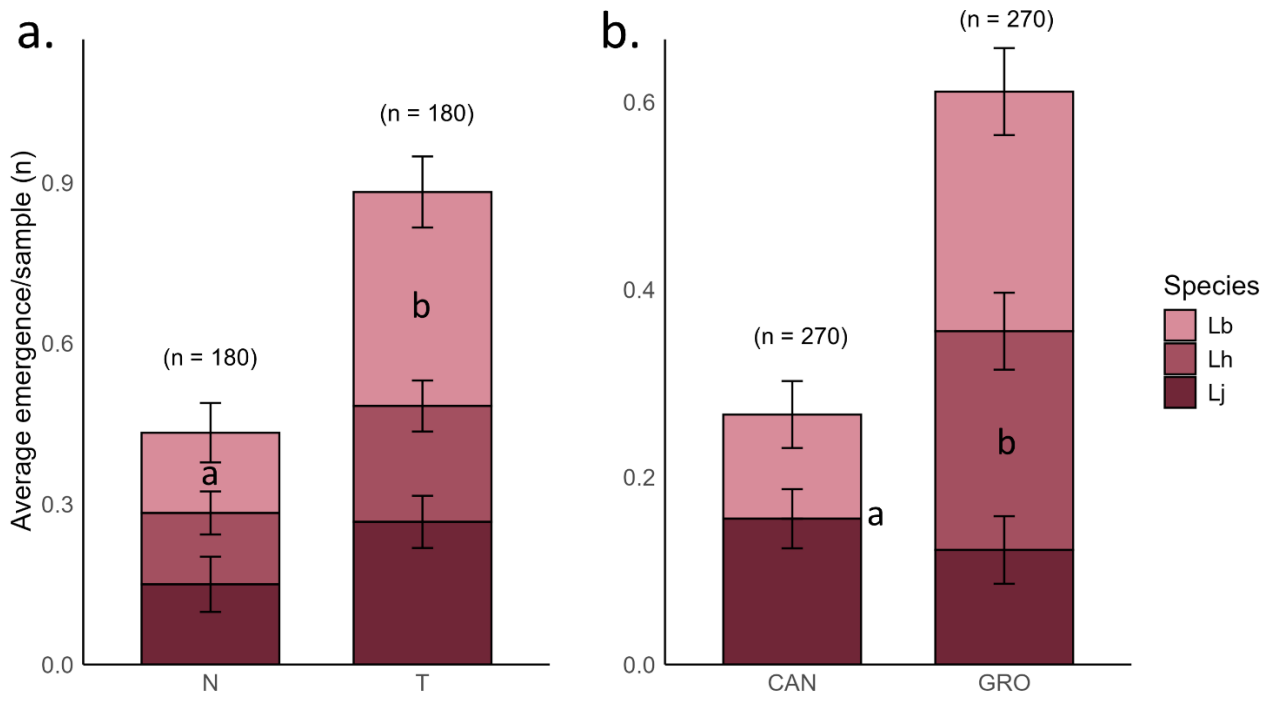

Figure 3.3: Average emergence of the *Leptopilina* spp. group (Lb: *Leptopilina boulardi*, Lh: *Leptopilina heterotoma*, Lj: *Leptopilina japonica*) among (**a**) Treatments (N: Negative control, T: Treatment) and (**b**) Position (CAN: Canopy, GRO: Ground). Stacked bars show mean values, error bars Standard Error Mean (SEM), while different letters for the same insect group indicate significant differences according to the pair-wise Mann-Whitney U-test with Bonferroni corrections.

3.4 Discussion

From parasitization to emergence, the immature stages of parasitoids encounter numerous challenges to successfully complete their development. In addition to abiotic stressors such as climatic conditions that regulate their growth, constant competition occurs at various levels (Harvey et al., 2013). In fact, inter- and intraspecific competition in natural ecosystems simultaneously affects both the host and the parasitoid residing within, making ecological interactions particularly complex.

The observed reduction of *Ganaspis kimorum* in the T group confirms that the biological control agent (BCA) is subjected to significant competitive pressure in the introduced area. However, the high overall emergence rates recorded in both the T and N groups suggest that resources

(blueberries) were not a limiting factor for *Drosophila* spp., excluding resource exploitation competition as the primary cause (Jones et al., 2009). Instead, the most likely explanation lies in inter and intraspecific competition occurring between host species and/or other parasitoids.

Drosophila suzukii exhibits cannibalistic behaviour at high population densities, with late larval instars capable of feeding on conspecific pupae (Bezerra Da Silva et al., 2019), a behaviour also observed in other *Drosophila* spp. (Vijendravarma et al., 2013). Although no significant differences in *D. suzukii* emergence were recorded between treatments, the higher trend observed in the N and T groups is most likely due to secondary infestations occurring in the field. This is supported by emergence timing. In fact, peak emergence (PE) in N and T groups occurred one week later than in the control group (19 days vs 12 days) (Table 3.1), suggesting that a substantial portion of emerging *D. suzukii* in these groups originated from wild populations. These secondary infestations could have intensified *D. suzukii* intraspecific competition, ultimately contributing to the reduction of *G. kimorum*.

Similarly, the numerous *Drosophila* spp. (OD) recorded in the T group could have fed on *D. suzukii* parasitized by *G. kimorum*. Cases of interspecific competitions within drosophilids are well documented, particularly between *D. suzukii* and *D. melanogaster*. (Dancau et al., 2017; Shaw et al., 2018). Under laboratory conditions with unlimited resources, *D. melanogaster* can outcompete *D. suzukii*, regardless of the oviposition timing (Dancau et al., 2017). Such negative interactions could have contributed to the reduced emergence of *G. kimorum*.

Multiparasitism, the acceptance of a host already parasitized by a heterospecific, is a common phenomenon. Parasitoids can generally discriminate against hosts parasitized by conspecifics but are less effective at detecting those parasitized by other species (Ardeh et al., 2005; Van Trien-Van Leimpt and Van Alphen, 1980; Wang et al., 2019). In our experiment, local larval parasitoids (*Leptopilina*

spp.) may have oviposited inside *G. kimorum*-parasitized hosts, leading to multiparasitism and subsequent interspecific competition for the same host. Laboratory assays have demonstrated that *L. japonica* does not discriminate against hosts previously parasitized by *Ganaspis* spp. and are able to outcompete them through physical combat or physiological suppression (Wang et al., 2019). This competition could explain at least to some extent, the reduction of *G. kimorum* observed in T groups. In solitary parasitoids such as *Leptopilina* and *Ganapsis* spp., only one adult can emerge from a single host (Bakker et al., 1985). A key competitive advantage of *L. japonica* is its faster development. Its first larval instar emerges from the egg case approximately 48 hours after oviposition, whereas *Ganapsis* spp. require up to 120 hours (Wang et al., 2019). Given our experimental design, when blueberries were exposed in the field, *G. kimorum* larvae were still embryos within the egg shells. *Leptopilina* spp. locating the blueberries during the exposure period, could have outcompeted *G. kimorum* due to their more rapid development. This hypothesis is supported by emergence timing. Although *G. kimorum* oviposited in the laboratory up to 48 hours before exposure, the first insects to emerge in the T group were *Leptopilina* spp. (21 days vs 23 days) (Table 3.1).

This study also revealed that already infested blueberries (T) were more attractive to drosophilids (Ds) compared to the negative un-infested control (N). This aligns with the olfactory behaviour and ecological niches of *Drosophila* species. Whereas *D. suzukii* oviposits in ripening fruit and is primarily attracted to CO_2 (Krause Pham and Ray, 2015), other *Drosophila* spp. prefer to oviposit into over-ripened fermenting fruit, are drawn to yeast-derived volatiles and are repelled by CO_2 (Billeter and Wolfner, 2018; Turner and Ray, 2009). The T groups were also significantly more attractive to *Leptopilina* spp., particularly *L. boulardi*, likely due to the higher abundance of other drosophilids, such as *D. melanogaster* and *D. simulans*, which are their primary host species in Europe (Fleury et al., 2004).

Interestingly, plant position did not explain the variance observed in insect group emergence. Within the *Leptopilina* spp., neither *L. boulardi* nor *L. japonica* showed preference for canopy or ground locations. On the contrary, *L. heterotoma* was found exclusively in ground samples. Patch location in *L. heterotoma* is mediated by the presence of yeast and fermentation products in decaying infested fruit, but also by previous oviposition experience (Quicray et al., 2023). The primary hosts of *L. heterotoma* include *D. melanogaster*, *D. hydei* and *D. suboscura*, while only few individuals can successfully develop into *D. suzukii* and *D. immigrants*. These findings suggest that *L. heterotoma* primarily scavenge in decaying fruit on the ground, where their primary hosts reside, and are unlikely to be major competitors to *G. kimorum*.

Overall, our study demonstrated that *G. kimorum* residing in *D. suzukii* infested fruits faces significant competitive pressure. This is most likely driven by secondary infestation of *Drosophila* spp. and by parasitoids of the genus *Leptopilina* that can outcompete *G. kimorum* due to their faster development. While this study specifically focused on competition occurring at the larval stage, future research should also investigate the role of pupal parasitoids of *D. suzukii* in shaping parasitoid-host dynamics.

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CHAPTER 4. OVERWINTERING STRATEGY

Investigating the overwintering strategy of *Ganaspis kimorum*, a biological control agent of *Drosophila suzukii*

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Abstract

The larval parasitoid *Ganaspis kimorum* Buffington (Hymenoptera: Figitidae) is a classical biological control agent of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). This study investigated the overwintering strategy of *G. kimorum* under field conditions in Switzerland and Northern Italy. Field experiments conducted from 2021 to 2024 involved exposing *G. kimorum* to naturally variable temperatures in seven sites with different climates. Results indicate that diapause induction may be mediated by temperature, with exposures after late September successfully triggering diapause. Dissections revealed that diapause occurs in the early larval instar (L1) residing within early pupal stages of *D. suzukii*. In four out of seven sites, *G. kimorum* was able to survive winter conditions and emerged as adult. Survival rates varied significantly across sites (0.4-17.6%). The analysis revealed significant negative associations between insect survival and the count of days with temperatures below 0°C and above 30°C. Analysing the variation of Degree Days needed until adult emergence confirmed that *G. kimorum* arrests its development as a first-instar larva and suggests

a lower thermal threshold of 9°C, with 329±6 post-diapause Degree Days needed to develop to the adult stage. The study provides valuable insights into the biology of the parasitoid that may be helpful for optimizing field releases and enhancing the effectiveness of *G. kimorum* as a biological control agent of *D. suzukii*.

Keywords:

Ganaspis kimorum, Drosophila suzukii, Biological Control, Diapause, Overwintering, Thermal threshold, Degree days

4.1 Introduction

As poikilothermic organisms, the development and fitness of insects are strongly influenced by temperature, which shapes their distribution and abundance (Angilletta, 2009; Nedvěd, 2009; Vieira et al., 2020). For insect predators and parasitoids, understanding the environmental limitations that influence their potential geographic distribution and their overwintering capacity is crucial for their successful application as biological control agents (BCAs) (Hondo et al., 2006; Hughes et al., 2010; Wang et al., 2018b).

Insects often enter dormancy to cope with abiotic stress, such as cold temperatures. Dormancy may involve quiescence, an immediate response to climatic thresholds, or diapause, a hormonally mediated, genetically programmed state characterized by metabolic suppression, developmental arrest, and reproductive inactivity (Denlinger, 2002; Koštál, 2006). Environmental triggers like photoperiod and temperature often influence diapause initiation, but their effects vary among species. For example, diapause in *Anastatus japonicus* Ashmead (Hymenoptera: Eupelmidae) is regulated by photoperiod and temperature (Zhao et al., 2021), whereas photoperiod primarily triggers diapause in *Colpoclypeus florus* (Walker) (Hymenoptera: Eulophidae), and temperature plays a dominant role for *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae) (Li et al., 2008; Milonas and Savopoulou-Soultani, 2000).

This study investigates the overwintering capacity and diapause of the larval parasitoid *Ganaspis kimorum* Buffington (Hymenoptera: Figitidae), a biological control agent targeting the invasive spotted-wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). Native to East Asia, *D. suzukii* has become a global pest since the early 2000s, spreading across the Americas, Europe, and Africa (Andreazza et al., 2017; Asplen et al., 2015). Its serrated ovipositor (Atallah et al., 2014), broad host range (Burrack et al., 2013; Kenis et al., 2016), rapid development cycle (Hamby

et al., 2016) and high dispersal ability (Tait et al., 2020), enabled this pest to quickly exploit new ecological niches, resulting in significant agricultural losses worldwide (De Ros et al., 2021; Farnsworth et al., 2017; Tait et al., 2021).

Monitoring in the native area and specificity tests (Biondi et al., 2021; Daane et al., 2021; Girod, 2017; Seehausen et al., 2020; Wang et al., 2018a, 2020), strongly suggest that the ecological host range of *G. kimorum* is limited to *D. suzukii* residing in ripening fruit (Stahl et al., 2024), and for this reason was selected as BCA in both Europe and the U.S. (Fellin et al., 2023; Gariepy et al., 2024; Lisi et al., 2022). Initial reports suggest that *G. kimorum* can overwinter in introduced regions (Fellin et al., 2023), but exact overwintering strategies and potential diapause remain unknown.

The thermal performance and cold tolerance of *D. suzukii* have been extensively studied. Flies overwinter as adults (Dalton et al., 2011; Enriquez and Colinet, 2017; Shearer et al., 2016; Zerulla et al., 2015), especially when expressing the winter-acclimated phenotype (Colinet and Kustre, 2024; Leach et al., 2019; Sario et al., 2023; Stephens et al., 2015). Adults undergo a reproductive diapause in response to shorter day lengths and cooler temperatures (Grassi et al., 2018). On the other hand, *D. suzukii* larvae are freeze intolerant and die quickly after short-term exposure to relatively mild temperatures (Jakobs et al., 2017; Stockton et al., 2019).

Studies on *D. suzukii* pupal parasitoids such as *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) and *Trichopria drosophilae* (Perkins) (Hymenoptera: Diapriidae) showed their ability to survive up to three months at constant 12°C under laboratory conditions (Wang et al., 2018b). and under extended natural winter conditions as immature stages (Amiresmaeili et al., 2020; Häner et al., 2022). Research on larval parasitoids focused on *Leptopilina* spp. and Ganaspis lupini (previously *Ganaspis* cf. *brasiliensis* G3 according to Sosa-Calvo et al. (2024)) and found population-specific cold tolerance associated with geographic origin (Gibert et al., 2001). Both species

showed a prepupal diapause, with G. lupini having a low thermal threshold (TL) ranging between 5 and 9°C, and *Leptopilina japonica* Novković & Kimura initiating diapause at 15 or 18°C (Hougardy et al., 2019; Murata et al., 2013).

However, most studies on parasitoid diapause and overwintering are conducted under laboratory conditions or rely on temporary field exposures (Amiresmaeili et al., 2020; Häner et al., 2022; Hougardy et al., 2019; Li et al., 2008; Vieira et al., 2020; Wang et al., 2018b) limiting their ecological relevance. Fluctuating temperatures in natural settings can cause physiological and ecological effects that differ from those predicted under constant temperatures (Colinet et al., 2015), and for this reason should be better investigated.

Here, we present findings from two experiments, conducted in winter 2021/2022 and 2023/2024 in Switzerland and Northern Italy, respectively. We exposed *G. kimorum*-parasitized *D. suzukii* larvae to natural conditions at seven sites across different altitudes, monitoring emergence, survival, overwintering stages, and Degree Day (DD) accumulation. DD analysis is a reliable tool for predicting insect development and emergence (Sridhar and Reddy, 2013), and it is particularly valuable in the context of pest control programs (Herms, 2004). By leveraging this approach, our findings provide critical insights into the capacity of *G. kimorum* to establish in release sites (Dietschler et al., 2023; Jenner et al., 2010) and contribute to modelling host-parasitoid interactions.

4.2 Material and methods

4.2.1 Insect colonies

Ganaspis kimorum specimens originated from a population collected in 2016 near Tokyo, Japan (Girod et al., 2018a). Parasitoids were reared at CABI in Delémont, Switzerland for experiment 1 and at Edmund Mach Foundation (FEM) in S. Michele all'Adige, Italy for experiment 2. Rearing followed

the protocol described by Girod et al. (2018b) and Rossi-Stacconi et al. (2022), under controlled conditions (16:8 light:dark (L:D) photoperiod, 22 ± 2°C and 70 ± 10% relative humidity (RH)).

Drosophila suzukii colonies were established from locally collected adults and maintained in climatic chambers with a photoperiod of 16:8 L:D at 23 ± 2°C and 60 ± 10% RH.

4.2.2 Experiment 1

A first experiment was conducted in 2021 in field cages in Switzerland to do preliminary investigations into diapause induction and the overwintering stage of *G. kimorum* under near/natural conditions. This study was part of the large-arena field cage experiment to assess the host-specificity of *G. kimorum* (Seehausen et al., 2022) and was conducted in two locations, Delémont (DEL) and Cadenazzo (CAD) (Table 4.1) (Supplementary Figure S4.1). In Delémont, a small insect rearing cage (47.5 × 47.5 × 47.5 cm, MegaView Science Co., Taiwan) was installed within a larger polyester netting cage (200 × 200 × 160 cm). From August 20 to September 30, 2021, about twenty freshly emerged *G. kimorum* adults (males and females) were released weekly into the smaller cage. Blueberries infested with one-day old *D. suzukii* larvae were exposed to the parasitoids every week for 3 days. Thus, a total of five parasitoid releases and exposures of blueberries were conducted for the experiment. Following exposure, blueberries were transferred to ventilated plastic containers (10 x 5 cm), closed with a foam lids, and kept in the cages. Because of a shortage of parasitoids, in one case, *D. suzukii* larvae were exposed to parasitoids under laboratory conditions (August 27-30) before being placed in the field cages (E1). A second exposure occurred directly in field between September 10 and 13 (E2). Parasitoid emergence was monitored weekly to determine development time and the onset of winter diapause.

On December 13, 2021, the containers with the blueberries and potentially parasitized *D. suzukii* were equally divided, placed in two plastic boxes with a mesh window (to allow exposure to natural temperatures), and moved in the ground at the two locations so that the lid was at ground level. Holes in the bottom of the box prevented rain or melting snow from flooding the overwintering individuals. Temperature inside the overwintering boxes was recorded every hour using data loggers (HOBO® Pendant MX, Onset®, USA).

Between February 25 and March 11, 2022, ten *D. suzukii* puparia were removed from the overwintering container in Delémont and dissected under a microscope to determine the overwintering stage of *G. kimorum* according to morphology and size (Wang et al., 2019). The remaining overwintering individuals were transferred on February 25 and March 11 to a growth chamber and reared at 20°C until parasitoid emergence.

4.2.3 Experiment 2

Following the preliminary findings of Experiment 1, a second experiment was conducted in 2023 to further evaluate the overwintering strategy of *G. kimorum* under natural conditions. This experiment took place in Trentino-Alto Adige in Northern Italy, the first region in Europe where the BCA was released (Fellin et al., 2023). Five sites were selected in forest areas bordering cultivated land at different altitudes: Faedo (FAE), San Genesio (GEN), Laimburg (LAI), Novale di Fié (NOV) and San Michele all' Adige (SMA) (Table 4.1) (Supplementary Figure S4.1). Before taking them into the field, fruit samples were prepared according to the following method: blueberries from the same batch were soaked in a 2% bleach solution for five minutes, rinsed three times, dried, and placed in sterilized 9 cm petri dishes. Blueberries were transferred to *D. suzukii* rearing cages for oviposition (4 hours), followed by exposure to *G. kimorum* parasitization for 48 hours under laboratory conditions as described above for both species. Blueberries were portioned (150 ± 1 g) in plastic containers (11

x 14 cm), covered with a mesh and transferred to overwintering sites. Control containers were maintained under the same laboratory conditions (16:8 L:D photoperiod, 22 ± 2°C and 70 ± 10% RH).

At each site, samples were kept in large metal cages (5 mm mesh) to prevent external interference (e.g., predation) and monitored for weather parameters using climatic loggers (HOBO® Pendant MX, Onset®, USA). Over nine weeks (September 27 to November 22, 2023), one container per site was exposed weekly, resulting in nine replicates, referred to as exposures (E1–E9). Exposed fruit samples were retrieved between May 1 and 14, 2024, and placed in controlled conditions (16:8 L:D photoperiod, 23.5 ± 1.5°C and 70 ± 10% RH) for 30 days to monitor insect emergence. Every container was monitored weekly to check insects' emergence.

At 63 and 112 days after exposure (DAE), field samplings were conducted to assess insect development stages. *Drosophila suzukii* juveniles (larvae or puparia) were collected from the fruit samples and taken to the laboratory. The number of sampled juveniles varied depending on availability, ensuring some remained for natural spring emergence. Half of the samples were dissected under a stereomicroscope (LEICA Microsystems, Wetzlar, Germany) to identify developmental stages and vital status. Insects were considered alive if they presented mobility, or if their body shape was intact and coloration clear. Contrary, dead insects did not show mobility and had sign of postmortem discoloration (browning). The categorization of *G. kimorum* development stages were based on the morphology of closely related species (Melk and Govind, 1999). As only parasitoid larval stages were observed throughout the experiment, they were categorized into: first larval instar (L1), second larval instar (L2) and third larval instar (L3). *Drosohila suzukii* juvenile stages were grouped into larvae, early pupae and late pupae based on Chyb & Gompel (2013). Remaining samples were acclimated (12:12 L:D photoperiod, 15 ± 2°C and 70 ± 10% RH) for a week and transferred to rearing

conditions (16:8 L:D photoperiod, 22 ± 2°C and 70 ± 10% RH) to assess successful emergence and survival rate (emergence tests).

Overwintering *G. kimorum* adults emerging from the second sampling (112 DAE) were used in parasitization and longevity bioassays conducted at rearing conditions (16:8 L:D photoperiod, 22 ± 2°C and 70 ± 10% RH). Both overwintering and control adult wasps were isolated into Dutscher rearing tubes (2.85 x 9.5 cm) containing a cellulose plug hydrated with 2 mL distilled water and honey applied to the inner lid. Mated females that were 6 ± 1 day old were exposed to 10 ± 0.5 g of blueberries infested with *D. suzukii* larvae (6 blueberries with 5-15 eggs per berry) for 72 hours in a cylindrical plastic container (6 x 8 cm). After parasitization, wasps were returned to rearing tubes for assessing their longevity. Mortality was recorded every two days, water and honey were replenished weekly.

4.2.4 Data analysis

The analysis focused on the first five exposures (E1-E5) because no successful development to the adult stage was observed from E6 onward. From each site, daily parameters of maximum temperature (T_{max}), minimum temperature (T_{min}), and average temperature (T_{ave}) were extracted. As the data did not meet the assumption of normality (as assessed by Shapiro-Wilk test), Tave from different sites were statistically compared with a non-parametric Kruskall-Wallis rank sum test followed by a Dunn's post-hoc pairwise test. Tests were performed in R (4.3.0) (R Core Team, 2020) using the "dunn.test" package (Dinno, 2024). Cumulative time below freezing ($Ct_{<0}$) was calculated as the number of consecutive days where daily Tave was ≤ 0°C. Extreme climatic events were calculated as the count of days where $T_{min} \leq 0$°C ($D_{T<0}$) or Tmax exceeded 30°C ($D_{T>30}$).

To evaluate the temperatures conducive to diapause induction, the weekly average temperature for each exposure and site was calculated as the mean of the daily temperatures during the first seven days of exposure. Degree days (DD) were calculated using the single sine method described by Zalom et al. (1983) according to the following formula:

$$DD = \frac{1}{\pi}\left\{\left(\frac{T_{max} + T_{min}}{2} - T_L\right)(\theta_2 - \theta_1) + \alpha[cos(\theta_1) - cos(\theta_2)] + (T_U - T_L)\left(\frac{\pi}{2} - \theta_2\right)\right\}$$

$$\theta_1 = sin^{-1}\left[\left(T_L - \frac{T_{max} + T_{min}}{2}\right) \div \alpha\right]$$

$$\theta_2 = sin^{-1}\left[\left(T_U - \frac{T_{max}+T_{min}}{2}\right) \div \alpha\right],$$

where T_{max} and T_{min} are daily maximum and minimum temperatures, T_U is the upper thermal threshold, and T_L is the lower thermal threshold. A T_U of 29.3°C was selected, based on data for the closely related species *Ganaspis lupini* (Hougardy et al., 2019). The lower thermal threshold (T_L) was estimated by doing a sensitivity analysis of the Coefficient of Variation ($CV = \sigma/\mu$, where σ is the standard deviation and μ the mean (Lovie, 2005)) as follows: First, DDs were calculated at T_L values ranging from 0°C to 15°C for two overwintering scenarios: 1) *Ganaspis kimorum* does not have a true diapause but only a quiescence. Therefore, DD accumulation starts at the time of exposure in autumn and ends at adult emergence in the following spring, assuming that the parasitoid continues accumulating DD throughout the entire exposure period. 2) *Ganaspis kimorum* has a true diapause. Thus, an arrestment of development is induced in autumn and terminates after certain time of exposure to cold temperatures, after which DD begin to accumulate. This analysis was conducted with three starting dates of DD accumulation: December 1, January 1 and February 1.

For each scenario, the T_L with the lowest CV was accepted as the developmental threshold in the above-described calculation of DDs (Chmiel and Wilson, 1979), which was conducted using the "degday" package (Lyons, 2022) in software R (R Core Team, 2020). The DD requirements until adult emergence were statistically compared separately for scenario 1) control vs. overwintering parasitoids at T_L of non-diapausing parasitoids, scenario 2) control vs. overwintering parasitoids at T_L of diapausing parasitoids, and a scenario 3), which is as scenario 2 but with an addition of DDs required for the parasitoid to reach the L1 stage. This third analysis was to confirm the diapausing instar that was observed through the above-described dissections. The number of DDs to reach the L1 stage was estimated based on developmental parameters established for *G. lupini* by Wang et al. (2019). At 22°C, *G. lupini*'s L1 larval stage emerges from the egg case at 122 hours and transitions to the L2 stage at approximately 142 hours (±6 days). Thus, the resulting number of DDs to reach the L1 stage was estimated to 78. Because the residuals of the ANOVAs did not follow the assumptions of normality (as assessed by Shapiro-Wilk test) and homogeneity of variance (as assessed by Levene's test), the statistical analysis was conducted with a non-parametric Mann-Whitney U test in the software R (4.3.0) (R Core Team, 2020).

Parasitoid overwintering survival was calculated for each site and exposure as the total number of adults emerged either after overwintering exposure or from samples collected during winter (emergence tests). The survival rate (SR) was expressed as the absolute emergence corrected by control group, using the formula: $SR\ (\%) = (A_{0w}/A_c) \times 100$, where A_{OW} is the number of overwintering adults and A_C is the number of adults that emerged under control conditions without overwintering exposure (Häner et al., 2022).

A logistic regression was used with the "glm" function of the stats package in R (4.3.0) (R Core Team, 2020) to analyse the influence of $D_{T<0}$ and $D_{T>30}$ as independent variables on the survival

of *G. kimorum* assessed through the dissections conducted during samplings. Survival as the binary dependent variable indicated whether parasitoid larvae examined during dissections (n = 102) were alive or dead.

The longevity of post-diapausing *G. kimorum* adults were statistically compared separately for sexes (control vs. male and control vs. females) with a Mann-Whitney U test on R software (4.3.0) (R Core Team, 2020), as the data did not meet the assumptions of normality and homogeneity of variances.

Parasitization bioassays measured parasitism using the Apparent Parasitism Rate (APR), defined as the proportion of parasitoid offspring among all emerged insects. APR was calculated for each female using the formula: $\mathrm{APR} = n_p/n_t$, where n_p is the number of parasitoid offspring and n_t is the total number of flies and parasitoids emerging (Girod et al., 2018b).

Table 4.1: Site description.

Full name	Site	Coordinates	Altitude (m)
Cadenazzo	CAD	46.1616 N ; 8.9325 E	225
Delémont	DEL	47.3732 N ; 7.3256 E	515
Faedo	FAE	46.1950 N ; 11.1670 E	660
Laimburg	LAI	46.3848 N ; 11.2865 E	294
Novale di Fié	NOV	46.5312 N ; 11.4985 E	655
S. Genesio	GEN	46.5615 N ; 11.3244 E	1233
S. Michele A/A	SMA	46.1894 N ; 11.1365 E	216

4.3 Results

Overall average temperatures varied significantly across sites ($\chi^2 = 61.1$, df = 6, $P < 0.05$) (Supplementary Figure S4.2-S4.3), with highest temperature of 8.5°C recorded in LAI and lowest temperature of 4.9°C in GEN (Table 4.2). The highest T_{max} of 36.6°C occurred in NOV on May 9

2024, while LAI recorded the second highest (34.2°C) on September 30, 2023. The lowest T_{min} values were observed in GEN (-11.9°C on January 20, 2024) and CAD (-8°C on January 12, 2024) (Table 4.2). Analysis of extreme climatic events revealed notable site-specific trends (Supplementary Figure S4). LAI and NOV had the highest number of $D_{T>30}$ where T_{max} exceeded 30°C (21 and 22 days, respectively). Meanwhile, CAD and GEN recorded the highest $D_{T<0}$ values, with 106 and 118 days, respectively (Table 4.2).

Table 4.2: Temperature data recorded for the different sites. T_{ave} refers to the average temperature during the entire winter exposure period. Different letters indicate significance according to the post-hoc pairwise Dunn's test ($P < 0.05$). Cumulative days with average temperatures below 0 ($C_{T<0}$). Extreme climatic events: number of days with T_{min} below 0 ($D_{T<0}$), and number of days with T_{max} above 30°C ($D_{T>30}$).

Site	n	T_{ave} ± SEM (°C)	T_{max} (°C)	T_{max} (date)	T_{min} (°C)	T_{min} (date)	$C_{t<0}$ (days)	$D_{T<0}$ (days)	$D_{T>30}$ (days)
CAD	196	6.6 ± 0.4c	33.8	Sep. 8	-8	Jan. 12	4	106	10
DEL	196	7.1 ± 0.4bc	33.8	Sep. 8	-6.4	Jan. 27	5	85	10
FAE	217	6.7 ± 0.3c	26.1	Apr. 14	-4.7	Jan. 21	5	36	0
GEN	230	4.9 ± 0.3d	28.6	Oct. 9	-11.9	Jan. 20	11	118	0
LAI	230	8.5 ± 0.4a	34.2	Sep. 30	-6.5	Jan. 22	6	56	21
NOV	230	7.3 ± 0.4bc	36.6	May. 9	-8.1	Jan. 22	8	74	22
SMA	217	8.2 ± 0.4ab	30.8	Apr. 7	-4.4	Jan. 14	4	43	1

Weekly average temperatures following the first exposure (E1) ranged from 19.9 ± 0.4°C in LAI to 15.9 ± 0.5°C in DEL. Apart from DEL, where the second exposure (E2) had a higher average temperature of 18.3 ± 0.3°C, other sites exhibited a steady decline across subsequent exposures, reaching a minimum of 8.4 ± 0.5°C in GEN (Table 4.3).

Table 4.3: Weekly average temperature (°C) ± Standard Error Mean (SEM) for all the sites, measured after exposures. Asterisks (*) indicate sites where adults emerged after winter.

Sites	Exposures

	E1	E2	E3	E4	E5
CAD	15.9 ± 0.5	18.3 ± 0.3	-	-	-
DEL	15.9 ± 0.5	18.3 ± 0.3*	-	-	-
FAE	16.0 ± 0.4*	15.6 ± 0.3*	13.2 ± 1.3	11.1 ± 0.7	10.5 ± 0.5
GEN	15.8 ± 0.6	15.3 ± 0.5	11.0 ± 1.5	9.2 ± 0.6	8.4 ± 0.5
LAI	19.9 ± 0.4	19.5 ± 0.4	15.7 ± 1.4	12.8 ± 0.7	12.0 ± 0.5
NOV	18.4 ± 0.7*	17.7 ± 0.3	14.1 ± 1.4	11.5 ± 0.6	10.7 ± 0.6
SMA	17.6 ± 0.4*	17.3 ± 0.4*	15.2 ± 1.2*	12.8 ± 0.7*	12.3 ± 0.5

4.3.1 Experiment 1

From the weekly exposures between August 20 to September 30, 2021, *G. kimorum* emerged before overwintering only from *D. suzukii* that were exposed under laboratory conditions from August 27 to 30. A first male parasitoid was found in the vial on October 25, and two additional males and two females on November 9. In none of the other samples (which were all parasitized in the field cages) did parasitoids emerge before winter. Dissections of *D. suzukii* puparia conducted on February 25 and on March 11, 2022, revealed the presence of second instar larvae (about 1 mm in length and without tail) in the pupal cases. Overwintering survival was confirmed in DEL but not in CAD. Three *G. kimorum* males emerged from samples that were exposed in DEL from September 10-13, 2021. Development from the overwintering stage to the adult parasitoid took in a growth chamber at 20°C an average of 25 days (23-28 days).

4.3.2 Experiment 2

During the 63 DAE sampling, 182 juvenile stages of *D. suzukii* were dissected. The highest number of living *G. kimorum* was observed in FAE (22), followed by SMA (12) and LAI (11) (Supplementary Table S4.1). Among the recorded larval stages of *G. kimorum*, L1 was the most abundant (n = 58), followed by L2 (n = 15) and L3 (n = 3). No later development stages of the

parasitoid, such as pupae or adults were detected. Early pupal stages of *D. suzukii* contained the largest number of *G. kimorum* L1 individuals (n = 32), whereas L2 stages were more frequently observed in later pupal stages (n = 12) (Figure 4.1). The rate of living *G. kimorum* L1 within early pupae of *D. suzukii* was 94%, the highest among the groups analysed (limited to those with > 3 samples) (Figure 4.1).

During the 112 DAE sampling, 64 juvenile stages of *D. suzukii* were dissected. The highest number of living *G. kimorum* was observed in FAE (n = 9), followed by SMA (n = 8) and NOV (n= 3) (Supplementary Table S4.2). Out of the 26 *G. kimorum* that were found, 25 (96%) were L1, and one was L2; no L3 larvae were recorded. The rate of living L1 *G. kimorum* within early pupal stages of *D. suzukii* was 82% (Figure 4.1).

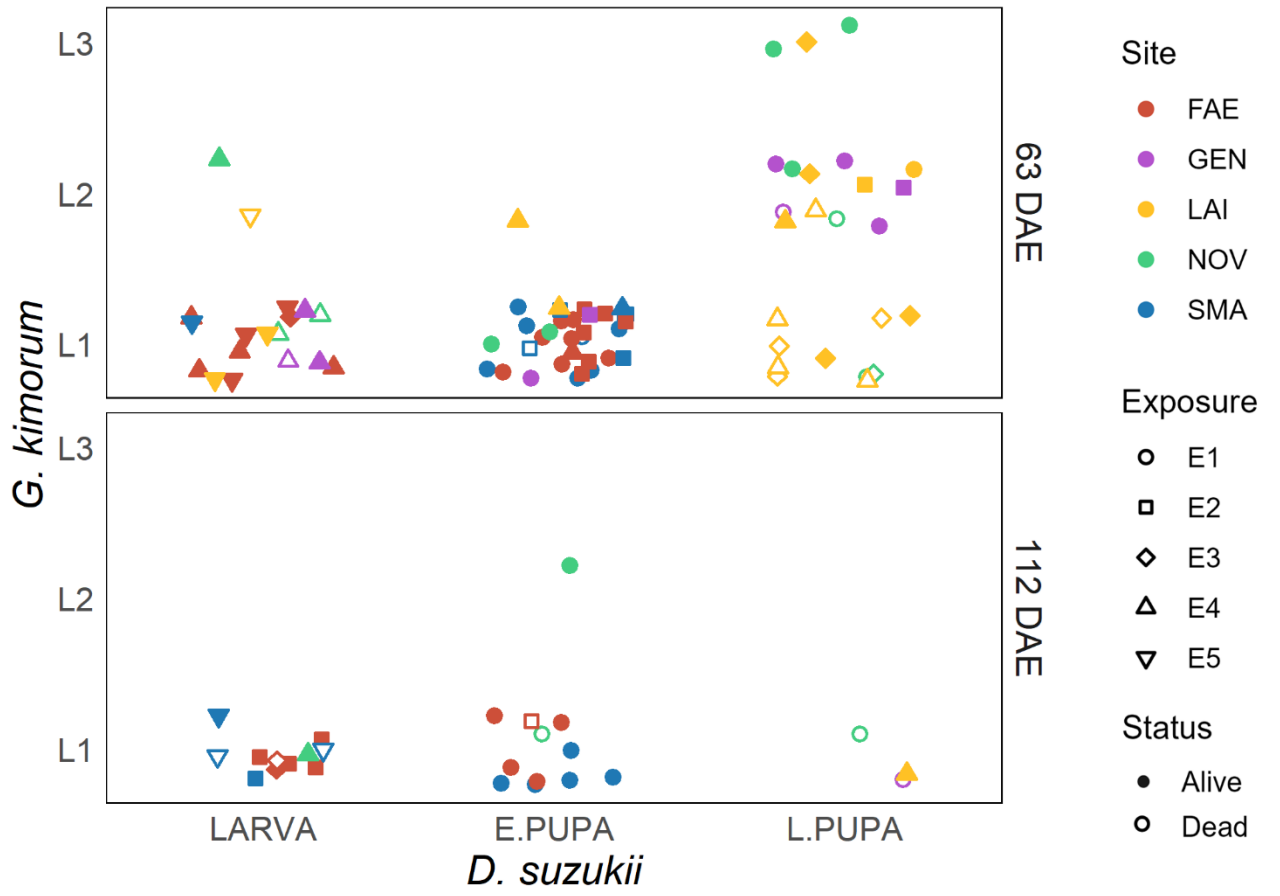

Figure 4.1: Correlation between juvenile stages of *Ganaspis kimorum* (L1, L2, L3 larval stages) and *Drosophila suzukii* (larva, early pupa, late pupa) for samples collected at (upper panel) 63 and (lower panel) 112 days after exposure. Numbers on the side of groups (with samples size larger than three) indicate the percentage of alive parasitoids for that specific development stages.

Emergence data showed a gradual reduction of survival for consecutive exposures for both host and parasitoid (Figure 4.2). Survival rates of *D. suzukii* decreased with advancing exposure dates, with adults emerging only up to 67 days after exposure (Supplementary Figure S4.5) or from puparia collected at 63 DAE during the first sampling. No emergence occurred from samples collected after 112 DAE or in spring. *Ganaspis kimorum* emerged from samples exposed in three sites out of five and exclusively in spring or from samples taken during the winter (Supplementary Table S4.3). Relative to non-overwintering laboratory-reared controls, the highest survival rate was recorded in FAE (E1: 17.6%, E2: 6.6%) and SMA (E1: 6.3%, E2: 5.5%, E3: 3.3%, E4: 1.2%; E5: 0.4%), when compared to the other sites (Figure 4.2). In FAE, *G. kimorum* adults emerged from E2 on April 12,

May 8, 10, and 12. From the same site, adults emerged from E1 on May 8, 10, and 12. In NOV, one adult from E1 emerged on May 29. In SMA, adult parasitoids emerged on April 10 (E3), 13 (E1-E2), and 18 (E4) (Supplementary Figure S4.2). Survival of parasitoids assessed through dissections was significantly decreased by both the number of days below 0°C ($D_{T<0}$) (χ^2= 4.3320; df=1,99; P= 0.03740) and above 30°C ($D_{T>30}$) (χ^2=3.9166; df=1,99; P=0.04781).

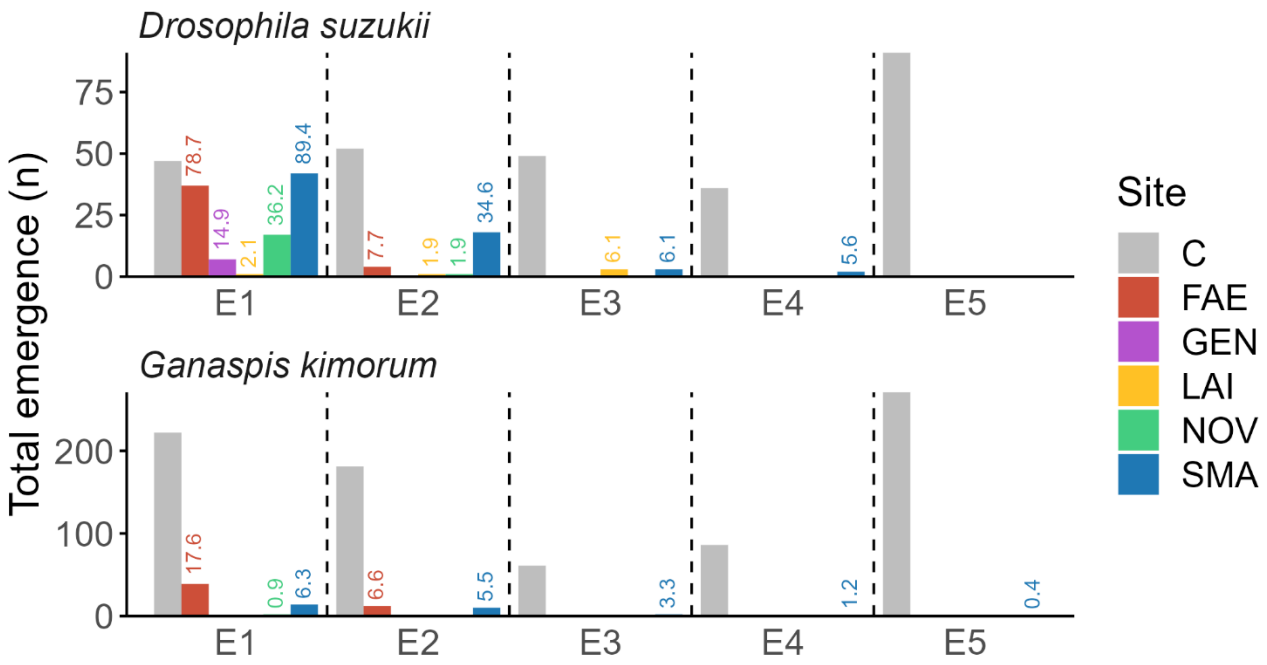

Figure 4.2: Overwintering survival in Experiment 2, expressed as total emergences of (upper panel) *Drosophila suzukii* and (lower panel) *Ganaspis kimorum* for the five exposure dates (E1-E5). Grey bars represent the emergences recorded from the non-overwintering laboratory-reared controls (C), while coloured bars those recorded in the field sites. Numbers on top of the bars indicate percentage of survival relative to the controls.

The sensitivity analysis suggested a lower thermal threshold (T_L) of 7°C for the accumulation of DDs without a true diapause, with the lowest coefficient of variation (CV) being 0.129. For the accumulation of DDs by diapausing parasitoids, the analysis suggested a T_L of 9°C, with the lowest CV being 0.153, regardless of the starting date considered (Figure 4.3). For convenience, January 1 was chosen as the starting date for the subsequent calculations of DDs.

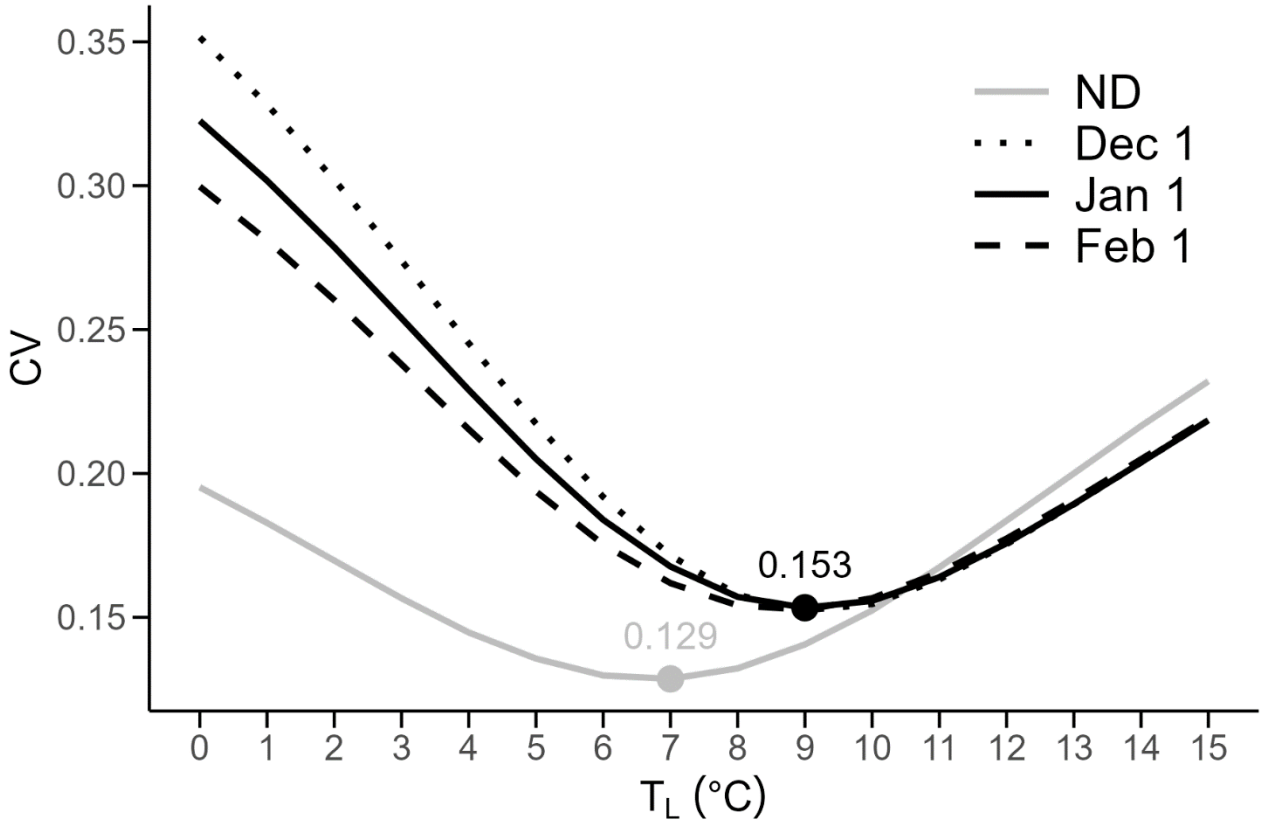

Figure 4.3: Sensitivity analysis to find the lower thermal threshold (T_L) of *Ganaspis kimorum*, by using the Coefficient of Variation (CV) for degree day (DD) requirements of the parasitoid to adult emergence. The grey line indicates the change in CV at different T_L for a scenario of non-diapausing parasitoids. The black lines indicate the change for post-diapausing parasitoids, with DD accumulating at three different starting dates. Numbers above the points indicate the lowest CV values, corresponding to the selected T_L.

The comparison of accumulated DDs between control and non-diapausing overwintering parasitoids at T_L=7°C showed that overwintering parasitoids accumulated significantly more DDs compared to the control, with an increase of 55.2% (W = 948, df = 484, $P < 0.05$; Figure 4.4a). In contrast, the comparison between control and diapausing *G. kimorum* at T_L=9°C showed that the overwintering group accumulated significantly fewer DDs compared to control, with an average reduction of 19.6% (W = 23662, df = 484, $P < 0.05$; Figure 4.4b). The DDs required for the parasitoid to reach the end of the diapausing stage (L1) was calculated as 78 DDs. Adding these DDs to the ones

of diapausing parasitoids resulted in no significant difference between overwintering (409±4 DD) and control adults (407±6 DD) (W = 15183, df = 484, *P* > 0.05; Figure 4.4c).

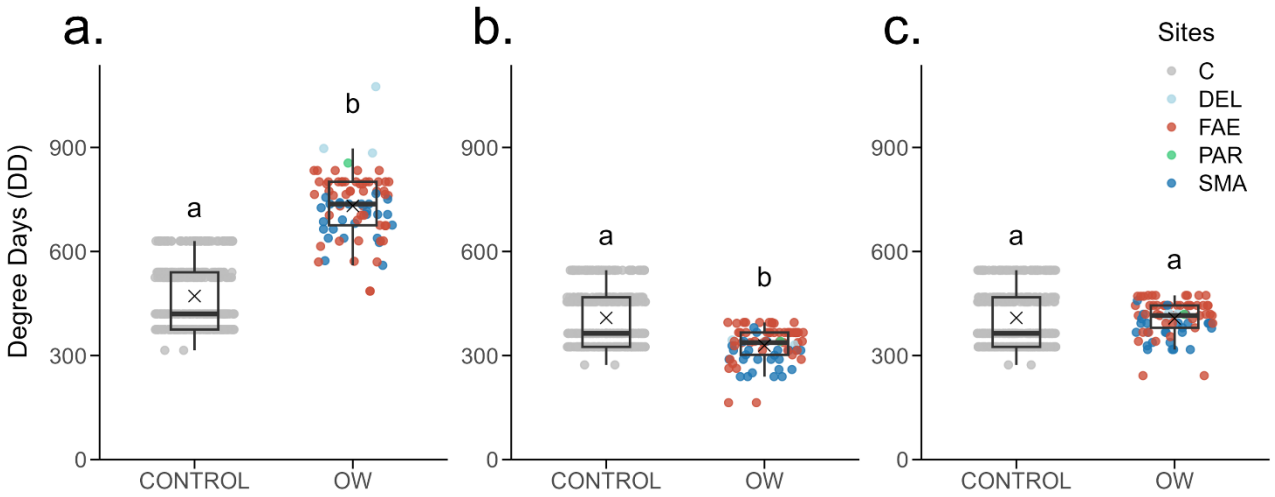

Figure 4.4: Degree days needed for development to the adult stage compared between laboratory reared non-diapausing (CONTROL) and overwintering (OW) *Ganaspis kimorum* considering three scenarios: a non-diapausing parasitoids with **(a)** lower thermal threshold (T_L) of 7°C and DD accumulation starting at the date of field exposure in autumn, **(b)** diapausing parasitoids with a T_L of 9°C and starting DD accumulation on January 1, and **(c)** diapausing parasitoids as in b but with the addition of DDs needed to reach the diapausing stage determined through dissections (first larval stage). Lower case letters above the boxplots indicate significant differences according to the Mann-Whitney *U* test.

The pairwise comparison showed that overwintering males had a 29% shorter life span compared to laboratory-reared males (W = 53, df = 15, P < 0.05; Figure 4.5). The longevity of overwintering females was not statistically different from laboratory-reared ones (W = 11, df = 9, P > 0.05; Figure 4.5). Parasitisation bioassays conducted on overwintering insects showed that two out of five females (40%) were able to successfully parasitize the host, with an apparent parasitism level of 46% and 6%. Only one out of five (20%) of the control females were able to parasitize the host, with an apparent parasitism level of 38%.

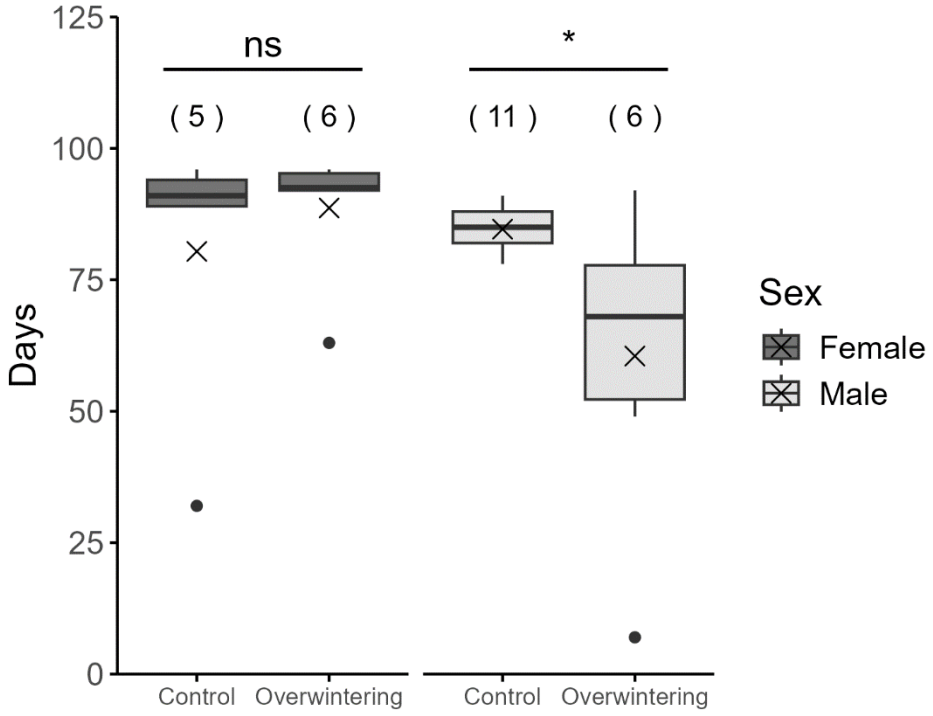

Figure 4.5: Longevity of laboratory-reared control and overwintering *Ganaspis kimorum* adults. Boxplots represent the distribution of lifespan for both sexes showing median (line), interquartile range (box) and mean (x). Whiskers extend to the smallest and largest values within 1.5 × IQR. Outliers are indicated as individual points. Number in parenthesis are adults tested, asterisk indicate significant difference at $P < 0.05$ and ns that there is no significant difference according to Mann-Whitney *U* test.

4.4 Discussion

Our study confirms that *G. kimorum* is able to overwinter under field conditions in Northern Italy and possibly also in Switzerland. However, the timing of exposures and environmental conditions in the different field sites were critical for diapause induction and survival. Early exposures of parasitized *D. suzukii* conducted in late August in Switzerland led to emergence of adults in late October and early November, indicating that these parasitoids did not encounter the environmental cues required for diapause induction. In contrast, exposures from late September to mid-October resulted in successful overwintering, with emergence of adult parasitoids occurring only after the

winter period. This suggests that diapause induction is strongly influenced by the timing of the exposition and associated environmental conditions.

In the second experiment, only the first five of the nine exposures (E1-E5), occurring between late September and late October, yielded insects. This period appears crucial for diapause induction and marks the last activity phase of both host and parasitoid. In Northern Italy, *D. suzukii* females with mature eggs are present until late October (Grassi et al., 2018), and Winkler et al. (2021) estimated that oviposition ceases at a minimum temperature of 13.2°C, matching conditions in our study area during the same period. According to our 2023 monitoring data (M.V.R.S., unpublished), *G. kimorum* was detected in fresh fruit samples collected in the first week of October, indicating activity at least until this date (no further collections were made). Thus, the first five exposures (late September–late October) represent a realistic scenario in which both host and parasitoid coexist and remain ovipositing.

Dissections revealed that the overwintering stage of *G. kimorum* was predominantly the early larval instar (L1), residing inside early host pupae. Our results indicate that *G. kimorum* employs eudiapause, a facultative developmental arrest, likely triggered by environmental factors such as temperature. This dormancy within the endoparasitic phase appears to exploit the lipid-rich resources of the host's early pupal body (Kühnlein, 2012; Liu and Huang, 2013), providing insulation during winter and a nutritional reserve for spring emergence (Sinclair and Marshall, 2018).

Interestingly, differences between our findings and those of Hougardy et al. (2019) might reflect divergence in biology between *G. kimorum* and its closely related species *G. lupini*, which appears to enter diapause at the prepupal stage. This divergence would further support the recent redescription of *G. kimorum* as a distinct species (Sosa-Calvo et al., 2024), or could be related to the experimental protocol adopted. In our study, later larval instars (L2 and L3) were observed to be present in host

larvae and puparia 63 days after exposure to autumn and winter temperatures in the field, particularly in warmer sites and early exposures; however, these stages failed to survive prolonged exposure. After 112 days of exposure, first-instar larvae dominated across all sites, further supporting the hypothesis that diapause occurs in the L1 stage within early stages of host puparia. Also, the presence of L2 stages during samplings conducted in Switzerland in late February and March support our findings, as can be explained by the parasitoids having resumed their development at this time of the year. Since *G. kimorum* diapauses as early larval instar, it needs to arrest host development. This process may be regulated by injected venom proteins, a common mechanism among *Drosophila* parasiotids to suppress the host immune response and manipulate its development (Poirié et al., 2014). This has been observed in both pupal and larval parasitoids. For instance, *T. drosophilae* uses venom to arrest pupal development for nutrient utilization (Pang et al., 2024), while *Leptopilina* spp. use venom to suppress host immune response (Huang et al., 2021). Among the various components of parasitoid venoms, those produced by *Ganaspis* spp. contain metalloprotease enzymes, which are believed to play a role in host development manipulation (Poirié et al., 2014). We hypothesize that *G. kimorum* also manipulates its host through venom, extending the pupal stage to align with its own developmental needs. Supporting this hypothesis, our dissections predominantly revealed *D. suzukii* in the early pupal stage when parasitized by living *G. kimorum*. This suggests that *G. kimorum* may suppress further host development, maintaining it in this phase, its preferred stage for diapause.

Diapause induction in insects is regulated by token stimuli such as photoperiod and temperature (Gill et al., 2017; Koštál, 2006). Other drosophilid parasitoids like *G. lupini*, *L. japonica*, and *Asobara tabida* (Nees) (Hymenoptera: Braconidae) exhibit temperature-mediated diapause regardless of photoperiod (Hougardy et al., 2019; Kraaijeveld and van Alphen, 1995; Murata et al., 2013). In our field study, photoperiods longer than 14:10 (L:D) in late August failed to induce diapause, while

exposures from September onward (13:11 to 11:13 L:D) did not result in premature emergence. This result does not exclude a possible role of photoperiod in diapause induction, but should be further investigated at constant temperatures to uncouple these two factors.

The temperatures triggering diapause appear to range between 13°C and 18°C (see Table 3). In LAI, where average temperatures during the first two exposures exceeded 19°C, diapause was not induced, and dissections revealed the presence of later larval stages (L2 and L3). These findings align with earlier studies, indicating that Japanese strains of *L. japonica* cease development between 15°C and 18°C (Murata et al., 2013). Similarly, *G. lupini* and *L. japonica* have been reported to enter diapause at 11.8°C and 17.2°C, but not at temperatures above 19°C (Hougardy et al., 2019).

Survival of *G. kimorum* was influenced by extreme climatic events, with both low and high-temperature negatively impacting insect's survival. This association was also observed across different sites. Sites with milder winter temperatures, such as SMA, NOV, and FAE, exhibited higher survival rates, whereas cold temperatures in CAD and GEN resulted in no successful overwintering. For example, in GEN, even though insects were exposed at permissive temperatures (15.8°C and 15.3°C for E1 and E2 respectively) the cumulative days of average temperatures below 0°C (11 days) and the days where T_{min} was below 0°C (118 days) likely exceeded the cold tolerance of *G. kimorum*. By comparison, sites with shorter periods of sub-zero temperatures (e.g., SMA and FAE) supported higher survival rates. These findings align with studies conducted on larval parasitoids, such as *Leptopilina spp.*, which can only withstand a limited period at 0°C (Murata et al., 2013), compared to more cold-tolerant pupal parasitoids (Häner et al., 2022). Also, high temperatures and reduced humidity could have compromised overwintering survival (Colinet and Boivin, 2011; Danks, 1987; Gill et al., 2017; Koštál, 2006). In fact, *D. suzukii* immatures cannot survive constant temperatures of 29.3°C, but can withstand short exposures at temperature above 30.6°C (Hougardy et al., 2019). The

hot days recorded in LAI, where maximum temperatures reached 34.2°C could have posed a threat for both hosts and parasitoids residing in the fruit samples and could explain the recorded low survival rates. In LAI and CAD, dry conditions likely contributed to the reduced survival of both hosts and parasitoids. Low humidity has been shown to decrease survival rates in other parasitoids (Murata et al., 2013), and desiccation during overwintering may have further exacerbated mortality in these sites.

The survival of *G. kimorum* ranged from 0.4 to 17.6%, with decreasing probabilities recorded with later exposure dates. The SMA site offers some evidence for this trend, showing survival of 6.3%, 5.5%, 3.3%, 1.2% and 0.4% for E1, E2, E3, E4 and E5 respectively. This probability of survival is in accordance with the 4% recorded for *L. japonica* strain collected in Sapporo (Northern Japan) and exposed to 0°C for 90 days (Murata et al., 2013). Similarly, larval stages of the pupal parasitoid *T. drosophilae* had a survival of 2% when exposed to semi-field conditions for 101 days (Amiresmaeili et al., 2020). While the survival calculated in the present study is in accordance with the literature and the recorded climatic data, it may be an overestimation, because it was calculated including the emergence of adults from the samples taken 63 and 112 days after exposure (emergence tests). On the other hand, including solely the emergence occurring in spring would underestimate survival, as a significant number of puparia were removed from the fruit samples to conduct both emergence tests and dissections. Juveniles removed for dissections had the potential to emerge as adults, and their removal possibly compensated the overestimation resulting from the addition of adults from emergence tests. For additional information we suggest to refer to the supplementary material provided (Table S4.1, S4.2 and S4.3).

The sensitivity analysis to find the lower thermal threshold (T_L) using the Coefficient of Variation and the analysis of the DD accumulation provided insights into the overwintering strategy of *G. kimorum*. Under the non-diapausing scenario (T_L = 7°C), the overwintering group exhibited an

unrealistically excessive accumulation of DDs. In contrast, the diapausing scenario (T_L = 9°C) presented a realistic outcome, with post-overwintering development accounting for 80.5% of the DDs required for the laboratory-reared control. Incorporating the DDs needed to reach the diapausing stage, the overwintering group achieved 99.6% of the DDs observed in the laboratory-reared control group, confirming the lower thermal threshold found through the sensitivity analysis, and that *G. kimorum* arrests its development at the late L1 larval stage and therefore undergoes a winter diapause.

Overall, the variance observed in development time (and resulting DD) in the overwintering group was comparable to that of the control group and can be attributed to the plasticity in temperature-dependent development that occurs among individuals of the same species (Colinet and Boivin, 2011). The greater variability noted in FAE, where the first natural emergence and subsequent emergence under laboratory conditions occurred 26 days apart, may be attributed to the effect of fluctuating temperatures within a permissive range. Such fluctuations are known to enhance performance compared to constant temperatures (Colinet et al., 2015) and could have accelerated the timing of the initial emergence.

Cold exposure often imposes significant fitness costs due to chilling effects, starvation, or their combination (Colinet and Boivin, 2011; Ellers and Van Alphen, 2002; Hance et al., 2007). The most notable impacts are typically observed on survival and reproduction. Parasitoids capable of overwintering often experience a reduced lifespan, as fat reserves become a limiting factor. For example, in several *Aphidius* spp., adult survival declines linearly with duration of cold exposure (Colinet et al., 2006; Ismail et al., 2010). In our study, overwintering males of *G. kimorum* exhibited a reduced lifespan, similarly to what was observed in *G. kimorum* larvae in cold storage (Lisi et al., 2024). On the other hand, females were unaffected, successfully parasitizing hosts and producing viable offspring. This sexual difference might be related to sexual allocation. Species like *G. kimorum*,

which use haplodiploidy for sex determination (Boulton et al., 2015; Small et al., 2012) can manipulate sex ratio based on host size, favouring females (King, 1993; Seidl and King, 1993). According to this theory, females likely have had access to more nutritional resources hence a longer lifespan compared to males. Another plausible explanation is that females are generally larger in size (Hurlbutt, 1987), possess greater fat reserves, and have the capacity to resorb eggs for additional energy when required (Papaj, 2000; Rosenheim et al., 2000). Consequently, following resource-intensive periods such as winter, females may exhibit a physiological advantage over males. However, this bioassay was conducted on a limited number of parasitoids, as survival rate after overwintering exposures was relatively low. Further studies should be conducted to confirm this theory and support our preliminary results.

To the authors' knowledge, this is the first study to examine the overwintering behaviour of *G. kimorum* under natural conditions, providing important insights that support classical biological control programmes. Specifically, the study establishes a lower thermal threshold and identifies climatic conditions that can guide the selection of optimal release sites to ensure successful establishment. Additionally, it provides a starting point to make predictions of parasitoid emergence in spring under field conditions and to model host-parasitoid population dynamics. In Northern Italy, *D. suzukii* terminates its reproductive diapause in March and shows high egg maturation by mid-April (Grassi et al., 2018). Our findings indicate that *G. kimorum* emergence coincides with this period, highlighting the tight coevolution that has shaped the host-parasitoid interactions.

In conclusion, our findings provide important insights into the overwintering biology of *G. kimorum*, confirming its ability to successfully overwinter in introduced area as early larval instars within the early pupal stages of *D. suzukii***.** The study underscores the pivotal role of environmental conditions, particularly temperature, in diapause induction and survival. By establishing a lower

thermal threshold for development and revealing the parasitoid's diapause strategies, this research lays a strong foundation for the effective use of *G. kimorum* in classical biological control programmes.

4.5 Supplementary information

4.5.1 Supplementary tables

Table S4.1: *Ganaspis kimorum* collected during the sampling 63 days after exposure at the five sites (FAE, GEN, LAI, NOV, SMA) and for the five exposures (E1-E5). Semicolon (;) separates the insects identified as alive (left) and dead (right). Number in parenthesis is the total number of *Drosophila suzukii* juveniles collected.

	E1	E2	E3	E4	E5	∑
FAE	7;0 (8)	6;0 (9)	1;0 (6)	5;0 (6)	3;0 (5)	22;0 (34)
GEN	4;1 (10)	2;0 (10)	0;0 (4)	2;1 (9)	-	8;2 (32)
LAI	1;0 (4)	1;0 (5)	4;3 (8)	3;4 (10)	2;1 (10)	11;8 (37)
NOV	5;2 (10)	0;0 (10)	0;1 (3)	1;2 (10)	0;0 (10)	6;5 (43)
SMA	6;1 (9)	4;1 (7)	0;0 (3)	1;0 (6)	1;0 (11)	12;2 (36)

Table S4.2: *Ganaspis kimorum* collected during the sampling 112 days after exposure at the five sites (FAE, GEN, LAI, NOV, SMA) and for the five exposures (E1-E5). Semicolon (;) separates the insects identified as alive (left) and dead (right). Number in parenthesis is the total number of *Drosophila suzukii* juveniles collected.

	E1	E2	E3	E4	E5	∑
FAE	4;0 (4)	4;1 (6)	1;1 (5)	0;0 (6)	-	9;2 (21)
GEN	0;1 (5)	-	0;0 (4)		-	0;1 (10)
LAI	-	-	-	1;0 (5)	0;0 (2)	1;0 (6)
NOV	2;1 (5)	-	-	1;0 (4)	0;0 (1)	3;1 (10)
SMA	5;0 (5)	1;0 (2)	0;0 (3)	0;0 (3)	2;1 (4)	8;1 (17)

Table S4.3: Overwintering survival of *Drosophila suzukii* (Ds) and *Ganaspis kimorum* (Gk) for the five overwintering sites (FAE, GEN, LAI, NOV, SMA, and control, CON) at different exposures (E1-E5). #Daylight ratio (L:D) at exposition time. Numbers are the total adult's emergence after exposition. Numbers followed by asterisk (*) are adults emerging from samples collected during the emersion tests. The percentage in parentheses is the total survival compared to survival in the laboratory-reared control.

	Exposures

Sites	Species	E1 Sep. 27 11.9/12.1[#]	E2 Oct. 4 11.5/12.5[#]	E3 Oct. 11 11.1/12.9[#]	E4 Oct. 18 10.8/13.2[#]	E5 Oct. 25 10.4/13.6[#]
CON	*Ds*	47	52	49	36	91
	Gk	222	181	61	86	271
FAE	*Ds*	37 (78.7%)	1+3* (7.7%)	-	-	-
	Gk	27+12* (17.6%)	6+6* (6.6%)	-	-	-
GEN	*Ds*	7 (14.9%)	-	-	-	-
	Gk	-	-	-	-	-
LAI	*Ds*	1 (2.1%)	1 (1.9%)	3 (6.1%)	-	-
	Gk	-	-	-	-	-
NOV	*Ds*	17 (36.2%)	1 (1.9%)	-	-	-
	Gk	1+1* (0.9%)	-	-	-	-
SMA	*Ds*	42 (89.4%)	18 (34.6%)	0+3* (6.1%)	0+2* (5.6%)	-
	Gk	1+13* (6.3%)	5+5* (5.5%)	1+1* (3.3%)	1 (1.2%)	0+1* (0.4%)

4.5.2 Supplementary figures

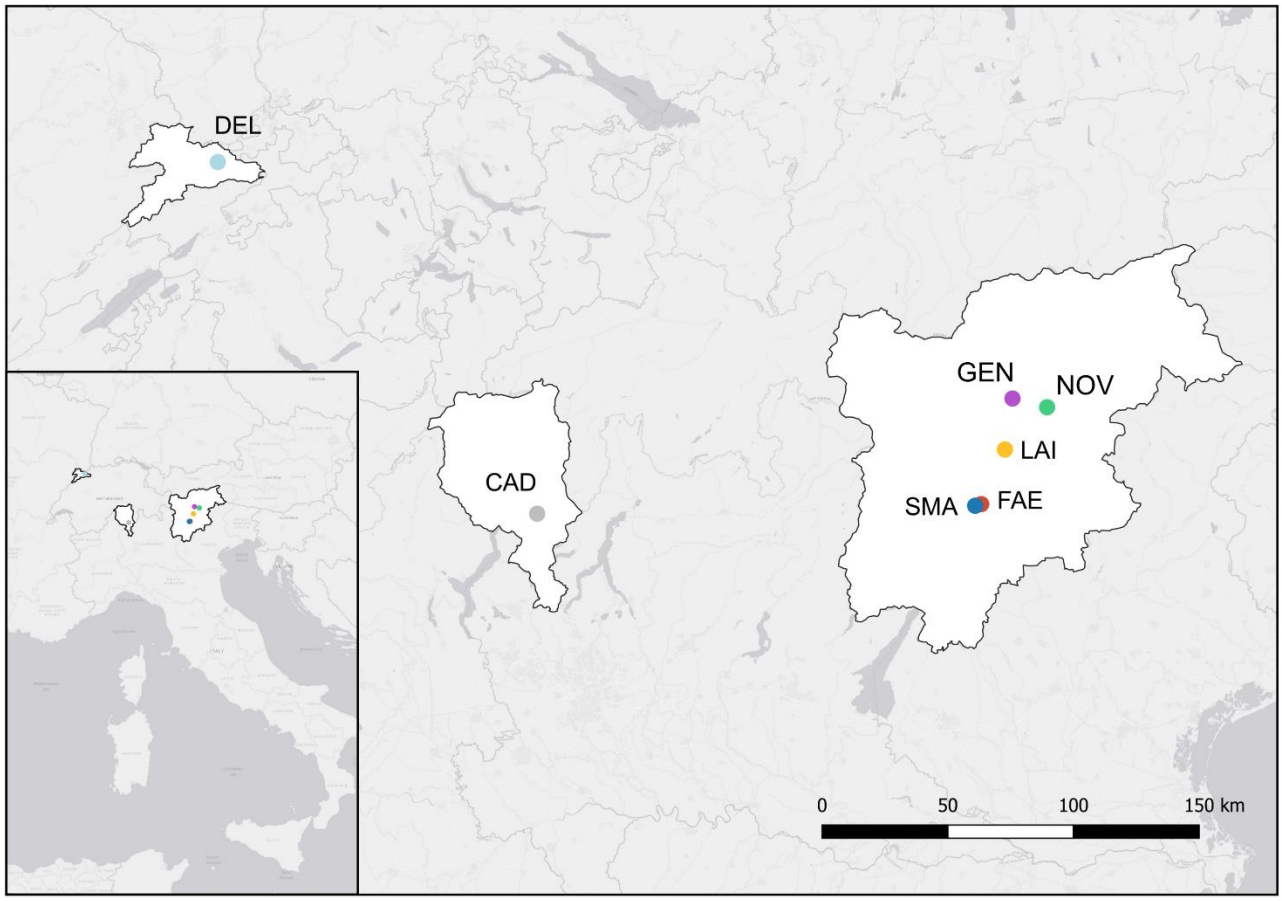

Figure S4.1: Locations of the field sites in the Swiss cantons Jura (DEL) and Ticino (CAD), and the Italian region Trentino Alto Adige (GEN, NOV, LAI, SMA, and FAE). *Map lines delineate study areas and do not necessarily depict accepted national boundaries.*

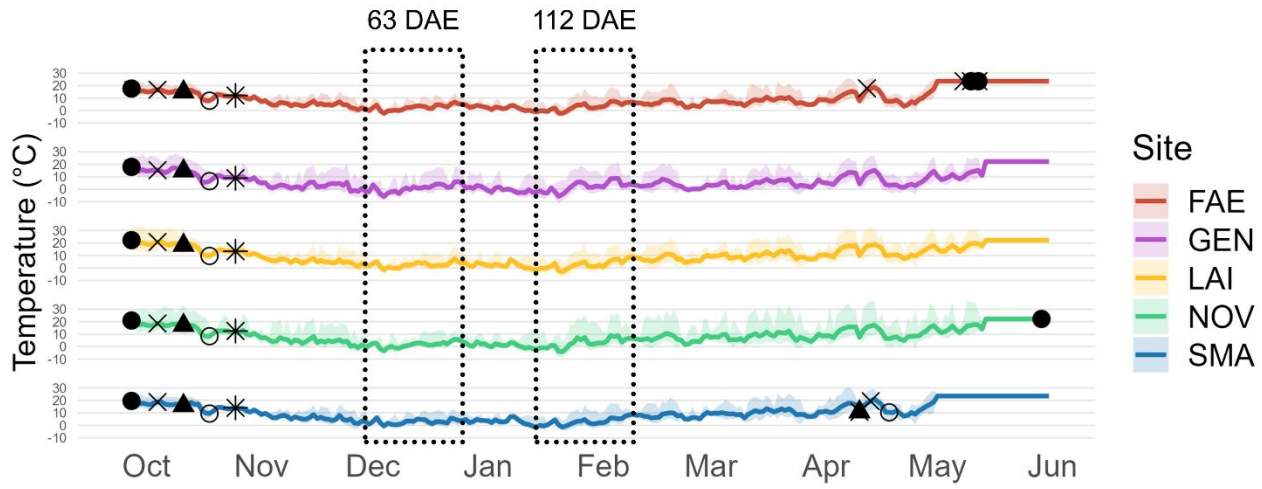

Figure S4.2: Overwintering periods and temperature ranges for all the sites of Experiment 2. Lines represent daily average temperatures, and shades the range between maximum and minimum temperatures. Symbols indicate different exposure dates. Repeating symbols on the same line, indicate successful emergence of *Ganaspis kimorum*. Dotted rectangles indicate the periods of first (63 DAE) and second (112 DAE) samplings.

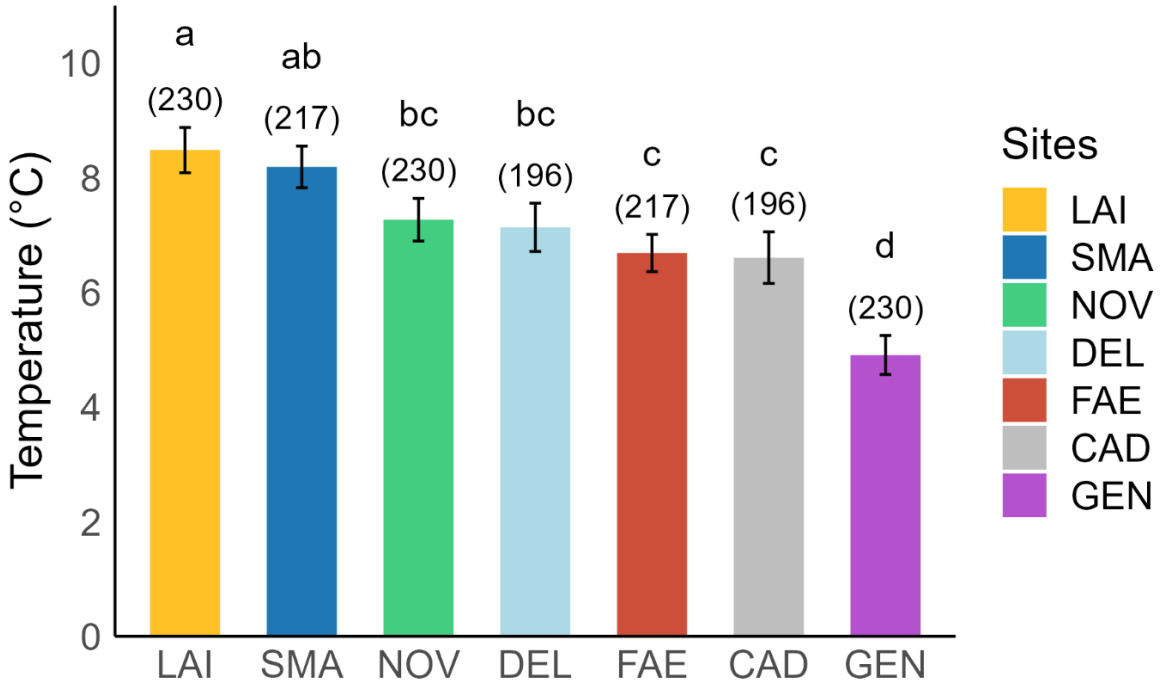

Figure S4.3: Average temperatures recorded throughout the entire field exposure. Numbers in parenthesis are the exposure period expressed in days. Lowercase letters above the bars indicate significant differences between means according to Dunn's post-hoc test at $P < 0.05$.

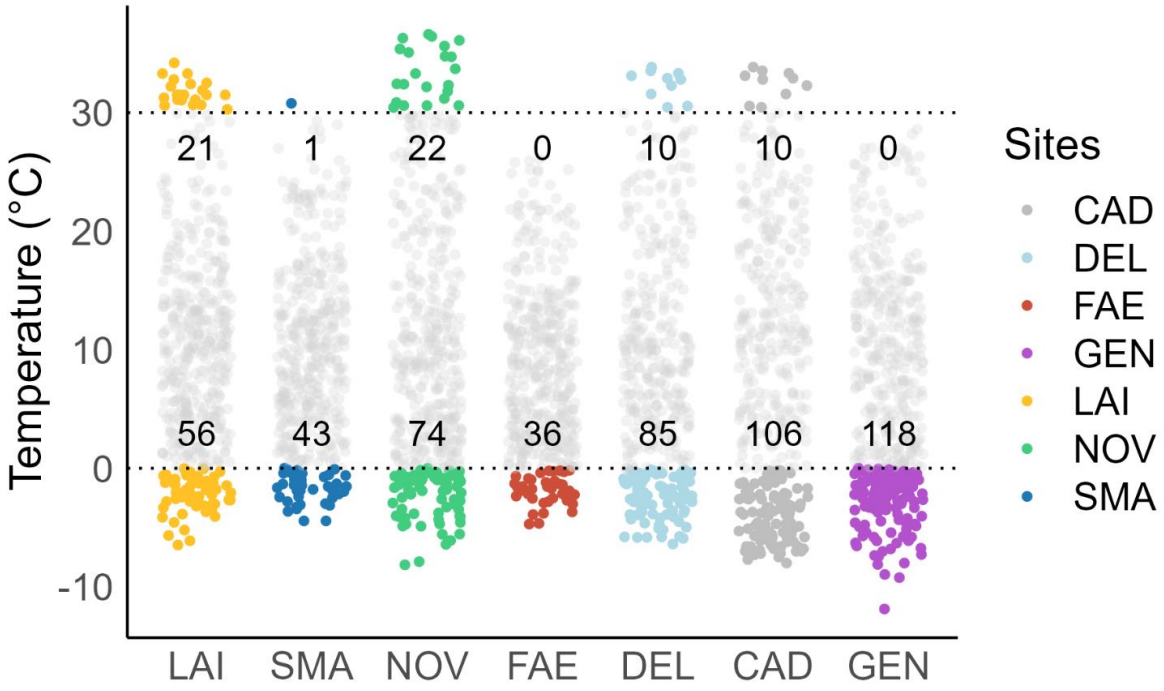

Figure S4.4: Extreme climatic events in the field sites. Dotted lines indicate the thermal thresholds set at (below) 0°C and (top) 30°C. Coloured dots represent days where maximum temperatures were above 30°C or minimum temperatures were below 0°C. Correspondent numbers indicate the count of related events.

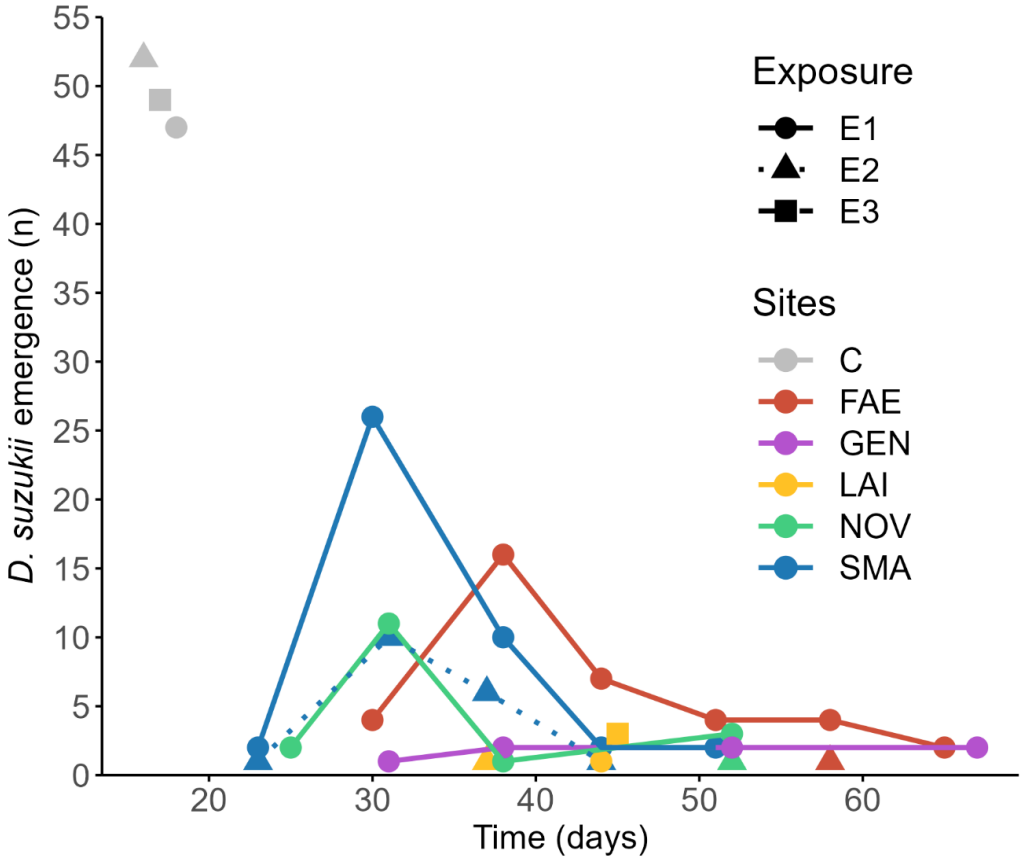

Figure S4.5: Development time of exposed *Drosophila suzukii* (coloured) and laboratory-reared control (grey). Shapes and trendlines identify different exposures. Different colours identify sites.

4.6 References

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CHAPTER 5. HOST SEARCHING: CHEMICAL ECOLOGY

Foraging behavior of *Ganaspis brasiliensis* in response to temporal dynamics of volatile release by the fruit - *Drosophila suzukii* complex

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Abstract

The lineage G1 of *Ganaspis brasiliensis* is a larval parasitoid of the worldwide pest *Drosophila suzukii* and one of its most effective natural enemies in the native area. Because of its high degree of host specificity, *G. brasiliensis* G1 is considered a suitable species for introduction in areas invaded by *D. suzukii* following a classical biological control approach. Indeed, the release of the parasitoid is currently implemented in the USA and Italy. G1 females attack only host larvae developing in ripening fresh fruits on the plant and not larvae that develop in decaying fruits. To date, virtually no information is available on the cues regulating the foraging behavior of G1. In this study, we therefore aimed to find out whether chemical cues are exploited by G1 females to: (i) locate host fruits; (ii) locate suitable host larvae within infested fruit; (iii) discriminate between infested fresh fruits and infested rotting ones. We used a model system composed of blueberries and *D. suzukii* tested in two-choice olfactometer bioassays (with *D. suzukii*-infested fruits, healthy fruits, and pure air as odor targets),

followed by the collection and the characterization of volatile organic compounds (VOCs) released by the tested targets. The results showed a clear time-dependent choice made by G1 females of infested versus healthy fruits related to the concomitant development of *D. suzukii* larvae and fruit degradation. Attraction to infested fruits was recorded during the early stages of infestation, followed by a repellent phase coinciding with fruits largely degraded by larval feeding. We found that the attractiveness of *G. brasiliensis* G1 towards fruits infested by young larvae was associated with the detection of VOCs released by the infested blueberries, and host's cuticular hydrocarbons. Conversely, the repellence of older and deteriorated fruits hosting developed *D. suzukii* larvae was associated with the detection of a fermentation compound produced by microorganisms likely carried inside the fruit by the flies. The response of G1 females to the temporal dynamics of chemical cues emitted by the fruit–host larvae complex was consistent with the high degree of specificity of the parasitoid towards the ripening host fruits and towards *D. suzukii*.

Keywords:

Classical biological control, Blueberry, GC–MS, Olfactometer, Larval parasitoids, VOCs

5.1 Introduction

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae), commonly known as the spotted-wing drosophila (SWD), is native to East Asia and starting from 2008 has become a serious economic threat to the production of berries and stone fruits worldwide (Cini et al., 2012, Asplen et al., 2015, Andreazza et al., 2017, Boughdad et al., 2021). The control of *D. suzukii* populations relies primarily on chemical insecticides, sometimes complemented by other local-area management tactics (Tait et al., 2021). These approaches often fail to prevent crop infestation and yield losses because the wild flora surrounding the cultivated fields offers plenty of fruits where *D. suzukii* can reproduce and overwinter, avoiding the impact of insecticide sprayings (Klick et al., 2016, Tonina et al., 2018; Buck et al., 2023). This situation is exacerbated by the lack of effective indigenous natural enemies in all invaded areas worldwide (Lee et al., 2019, Wang et al., 2020). Hence, the development of effective and sustainable control strategies able to provide an area-wide management of *D. suzukii* has become a critical issue (Haye et al., 2016, Wang et al., 2016). From this perspective, classical biological control, *i.e.*, the introduction of natural enemies from the native area of the pest into an area of new colonization, has been considered a valuable strategy to reduce the density of *D. suzukii* populations in non-crop habitats (Daane et al., 2016). This approach aims to increase the effectiveness of management strategies in crops, reducing the negative side effects of the chemical control. Explorations for natural enemies in the native area of *D. suzukii*, including China, South Korea, and Japan, resulted in the collection of three potential biocontrol agents, namely the larval parasitoids *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae), *Leptopilina japonica* Novkovic and Kimura and *Ganaspis brasiliensis* (Ihering) (Hymenoptera: Figitidae) (Daane et al., 2016, Girod et al., 2018a, Giorgini et al., 2019). While *A. japonica* and *L. japonica* are generalist parasitoids of drosophilid species, *G. brasiliensis* showed a narrow host range. Hence, it was chosen as the best

candidate for introduction into Europe and North America hypothesizing limited or null non-target impact (Wang et al., 2020 and references therein).

The thorough characterization of *G. brasiliensis* required for its introduction revealed a high genetic variability of the species, differentiated into five main lineages, each characterized by specific bio-ecological features and host ranges. Therefore, the existence of a complex of cryptic species was hypothesized (Nomano et al., 2017, Giorgini et al., 2019, Seehausen et al., 2020). Among the *G. brasiliensis* lineages, the G1 was found to have the highest degree of host specificity. Ecological observations in the native area suggested that G1 females can attack only *Drosophila* larvae developing in ripening fresh fruits on the plant, thus limiting its parasitic activity towards true carpophagous species belonging to the *D. suzukii* species-group (Nomano et al., 2017, Matsuura et al., 2018, Giorgini et al., 2019). Besides, *G. brasiliensis* G1 was never collected in fruit-baited traps containing decaying fruits hosting drosophilid larvae (Giorgini et al., 2019). Laboratory observations confirmed all these features rendering *G. brasiliensis* G1 a suitable species for introduction within the framework of classical biological control programs. In fact, it was shown that G1 females rarely parasitize *D. suzukii* or other drosophilid larvae within artificial diets or rotting fruits (Seehausen et al., 2020). Furthermore, although the parasitoid could develop in other *Drosophila* species closely related to *D. suzukii* (*i.e.*, *D. melanogaster* Meigen and *D. simulans* Sturtevant) when forced to attack them in fresh fruits, it always preferred *D. suzukii* when given the choice (Seehausen et al., 2020). This result was even more evident in choice tests under semi-field conditions where G1 females were found to parasitize *D. suzukii* larvae in fresh fruits and almost no *D. melanogaster* larvae in rotting fruits (Seehausen et al., 2022). Taken together, all these results suggest that the specific ecological niche occupied by the *G. brasiliesnsis* G1 in the field, *i.e.*, the larva in fresh ripe fruits on plants, is even narrower than the physiological host range. Consequently, the release of *G. brasiliensis* G1 for

classical biological control of *D. suzukii* was approved in Italy (Lisi et al., 2022) and the USA (Beers et al., 2022). A Japanese strain of *G. brasiliensis* G1 (Girod et al., 2018a), provided by the quarantine laboratory at CABI, Delémont, Switzerland, was introduced in both countries since the 2021 (Fellin et al., 2023).

To date, virtually no information is available on the cues regulating the foraging behavior of *G. brasiliensis* G1. While it shows specificity to fresh fruits infested by *D. suzukii* larvae, the parasitoid appears to be quite a generalist regarding fruit species (Girod et al., 2018a, Giorgini et al., 2019). In this study, we consequently aimed to answer the following questions: (i) Is there any chemical cue exploited by G1 females to locate host fruits? (ii) Is there any chemical cue from the *D. suzukii*-infested fruit complex exploited by G1 females to locate suitable host larvae inside the fruit? (iii) Is there any chemical cue involved in parasitoid preference for infested fresh fruits over infested rotting ones? To answer these questions, we used a model system composed of blueberries and *D. suzukii* tested in two-choice olfactometer bioassays against G1 females (with *D. suzukii*-infested fruits, healthy fruits, and pure air as odor targets), followed by the collection and the characterization of volatile organic compounds (VOCs) released by the tested targets.

5.2 Material and methods

5.2.1 Insects

The experiments were conducted in the laboratories of the Fondazione Edmund Mach, San Michele all'Adige, Italy (FEM), the National Research Council, Institute for Sustainable Plant Protection, Portici, Italy (IPSP) and the Department of Agricultural, Forest and Food Science, University of Torino, Italy (DISAFA). The colony of *D. suzukii* was initiated from infested fruits collected in Trento province, Italy, during 2021. Fly larvae were reared on a standard cornmeal-based

artificial diet according to Rossi-Stacconi et al. (2022). A permanent rearing of the G1 lineage of the larval endoparasitoid *G. brasiliensis* was established at the laboratories of FEM, IPSP and DISAFA within the framework of the Italian national classical biological control program against *D. suzukii* (Lisi et al., 2022; Fellin et al., 2023). Parasitoids were reared on *D. suzukii*-infested blueberries (*Vaccinium corymbosum* L.) (Ericaceae) according to the protocol described by Rossi Stacconi et al. (2022). The starting colony of G1 derived from wild individuals collected in Naganuma Park (Hachioji, Tokyo, Japan) from 2015 to 2017 (Girod et al., 2018a), and was provided by CABI's Swiss centre (Delémont, Switzerland) to FEM in 2021. Both *D. suzukii* and *G. brasiliensis* G1 cultures were maintained at the following conditions: 24 ± 1 °C temperature, 70 ± 5 % RH and 16L:8D photoperiod.

5.2.2 Olfactometer bioassays

The foraging behavior of G1 females was investigated in a dynamic Y-tube olfactometer consisting of 1-cm-diameter Y-shaped glass tube with a 9-cm-long base and two 8-cm-long arms as described in detail by Cascone et al. (2019).

Healthy fruits of blueberry variety Brigitta were picked in an organic farm only a few hours before running the olfactometer bioassay. In the field, *D. suzukii* infestation of tested fruits was prevented by covering them in insect-proof net before veraison to avoid any oviposition. Infested fruits were prepared in the laboratory by allowing a hundred mated *D. suzukii* females to oviposit in a fruit sample of 50 g (corresponding to an average of 31 blueberries) for 4 h inside an aerated plastic box (35 × 25 cm and 30 cm high). An infestation of 8.59 ± 4.16 (mean ± s.d.) *D. suzukii* eggs/fruit resulted, leading to an average of 3.68 ± 1.27 (mean ± s.d.) puparia/fruit (some eggs did not hatch and/or some larvae did not complete development).

Parasitoid females were tested against three different targets, healthy (uninfested) blueberry fruits (H-BB), *D. suzukii*-infested fruits (SWD-BB), and pure air (Air) presented in the following combinations: (i) SWD-BB vs H-BB; (ii) SWD-BB vs Air; (iii) H-BB vs Air. The fruit sample tested in the olfactometer consisted of 50 g of infested blueberries (SWD-BB) or heathy blueberries (H-BB). To assess the effect of the temporal combination of host development and fruit degradation level on the behavioral response of G1 females, the same bunch of SWD-BB or H-BB fruits was tested at seven time points: immediately after *D. suzukii* oviposition (T0) and thereafter, every 24 h for six consecutive days (T1-T6). Under the experimental conditions, blueberries were infested with *D. suzukii* eggs at T0, the first larval instars would have hatched at T1, and only *D. suzukii* larvae would have present in the fruits from T2 onward.

Mated G1 females having no prior oviposition experience were used in olfactometer bioassays. Parasitoid adults were collected as soon as they emerged from parasitized puparia and transferred in aerated plastic boxes (13 cm height and 11 cm diameter) in absence of fruits and host flies and provided with honey and water. Each box contained 30 adults (sex ratio 1:1). Males and females of *G. brasiliensis* mate successfully as soon as they emerged (<12 h old) (Girod et al., 2018b, Girod et al., 2018c), and since the egg load reaches the peak in 6–8 days (Wang et al., 2018), we tested 7-day-old females. These conditions also guaranteed the highest chance of testing mated females. Indeed, female wasps were all fertilized (always produced female progeny) in our experimental setting.

Target fruit samples were placed individually inside a 20-L glass jar, which was subsequently closed tightly for 15–20 min prior to the beginning of a choice test to allow a consistent diffusion of the odor plumes and to allow the fruits to acclimatise within the olfactometer system. Each of the two glass jars was independently connected to an arm of the Y-tube by a Teflon tube. A stream of purified (at 99,99 %) air was split into two equal streams, each set at 100 mL min−1 by a flow meter and

directed to a glass jar. Choice tests were conducted between 12:00 and 16:00 in a laboratory evenly illuminated by fluorescent lights (PPFD of 700 μmol m2s−1) and at 24 ± 1 °C. The position of the jars with fruit samples was swapped every five parasitoids tested to avoid any position bias.

Each olfactometer bioassay included the three two-choice tests (SWD-BB vs H-BB, SWD-BB vs Air and H-BB vs Air) performed using the same fruit samples. Each olfactometer bioassay was replicated 10 times, using a different sample of fruits (SWD-BB and H-BB) in each replicate. Replicates of the olfactometer bioassay were performed one at a time and started on ten different days. Ten or 20 G1 females were tested individually in each replicate of the choice tests. Each female was used only once. Overall, 440 females were tested in each of the three choice tests, divided as follows: 80 (T0), 80 (T1), 80 (T2), 60 (T3), 50 (T4), 40 (T5), 50 (T6). The choice of each parasitoid female was recorded within 5 min from the release in the common arm of the olfactometer. A choice was considered made when the parasitoid arrived inside one of the two trapping bulbs positioned at the end of the Y-tube arms. Wasps not making any choice within 5 min were scored as non-responsive and excluded from the analysis. Overall, only 91 out of 1320 individuals (6.9 %) were non-responsive.

5.2.3 VOC collection and identification

Fruit samples for gas chromatography-mass spectrometry (GC–MS) analyses were obtained from a single fruit batch of ripe blueberries (cv Brigitta), picked from the field (where fruits were covered by insect-proof net) and cold-stored at 1 °C for one week. Prior to VOC collection, the blueberry batch was divided into three subsamples, corresponding to different treatments: *D. suzukii*-infested blueberries (SWD-BB), artificially pierced blueberries (AP-BB) and healthy blueberries (H-BB). SWD-BB fruits containing 8 to 12 eggs of *D. suzukii* were chosen for this experiment. Artificially pierced blueberries were punctured 10 times in random positions with a fine needle (entomological pin size 000, diameter 0.25 mm) (Austerlitz Insect Pins®, Austerlitz, Czech Republic). Each blueberry

sample (50 g) was placed in a polyethylene terephthalate oven bag (25 × 38 cm) (Anfora et al., 2009), and VOCs were collected using the closed-loop-stripping-analysis (CLSA) method (Boland et al., 1984). A 12 V vacuum pump (DC12/16FK type, Fürgut, Tannheim, Germany) circulated air within the bag to an adsorbent trap loaded with 1.5 mg charcoal (CLSA-Filter, Brechbühler AG, Switzerland). The pump was powered by a 6 V rechargeable battery (RS Components Srl, Italy) and the air flow was set at 0.2 L min^{-1}. Volatile collections from the three fruit treatments were performed in parallel for 3 h at 0, 24, 48, 72 and 144 h after *D. suzukii* oviposition or the artificial piercing (time points T0-T3 and T6 of olfactometer bioassays) and replicated 4 times, totaling 60 VOC collections (*i.e.*, 3 treatments × 4 replicates × 5 collection times). Each blueberry sample was subjected to VOCs collection sequentially at the different time points tested and stored in controlled condition (21 °C and 70 % RH) during consecutive collections.

Once collected, each volatile sample was eluted from the charcoal filter into 1 mL-glass vials using 500 µL of dichloromethane (>99.9 % purity; Merck/VWR, Darmstadt, Germany). After each elution, the CLSA filters were cleaned using a set of solvents at different polarity (dichloromethane, methanol, and acetone) to remove any volatile trace and to avoid contamination of subsequent samples. The eluted volatile samples were reduced to 100 µL using a slow stream of nitrogen and stored at − 20 °C until GC–MS analysis. All GC–MS analyses were carried out using a TSQ Quantum XLS (Thermo Scientific, Austin, TX, USA) equipped with a CTC-PAL3 autosampler. The separation was performed using a 30 m × 0.25 mm ID × 0.25 µm Restek Rx Sil MS w/Integra-Guard column (Restek corporation, Bellofonte, PA, USA). A split − splitless injector was set at 270 °C and 1 µL of solution was injected. The GC separation started at 35 °C, was held for 4 min, then was increased with the following intervals: 35 − 80 °C at 20 °C/min, 4 min at 80 °C, 80 − 100 °C at 2 °C/min, 5 min at 100 °C, 100 − 170 °C at 2.5 °C/min, and finally 170 − 270 at 20 °C/min with a 1 min final isotherm at 270 °C. A 1.2

mL/min helium was the carrier gas of choice. The MS signal was obtained by electron ionization at 70 eV, with the transfer line and the ion source both set at 250 °C using a full scan acquisition mode. All the analytical data were processed using XCalibur 4.5 software (Thermo Scientific, Austin, TX, USA). The putative identities of the compounds were characterized by a comparison with synthetic standards considering their GC retention indices and with mass spectra compared via the Wiley mass spectra database.

5.2.4 Statistical analysis

Statistical analyses of data originated from Y-tube olfactometer bioassays were run in R (R Core Team, 2023). Generalized linear models were built to assess the effect of VOC blends produced by SWD-BB or H-BB samples on attractiveness towards *G. brasiliensis* G1 females at the time points T0-T6. Each choice test (SWD-BB vs H-BB, SWD-BB vs Air, and H-BB vs Air) was analysed individually. Ten replicates for each choice test were included in the analysis. The number of parasitoids that responded to one or the other odor source was included in the model as a dependent variable while the time point (T0-T6, the sequential temporal combination of host development and fruit degradation) was included as an explanatory variable. The data distributions were fitted to a binomial model using the logit link function. Overdispersion was checked by the dispersion test function of the DHARMa package (Hartig, 2022) using non-parametric tests to compare simulated data dispersion with observed data dispersion. Wald Chi-Square statistics were used to assess the overall significance of time on the parasitoid attraction to the volatile blend produced by blueberry samples. Post-hoc tests for multiple comparisons were run using the Šidák method at the confidence level of 95 % to determine statistical differences between the time points T0-T6. Within each two-choice treatment, the two-tailed binomial test was used to assess whether the attraction of G1 females to any odor source in the Y-tube olfactometer differed from a random choice (50:50).

5.3 Results

5.3.1 Olfactometer bioassays

The impact of *D. suzukii* infestation of blueberry samples on the attraction of *G. brasiliensis* G1 females over 6 days is reported in Figure 5.1. In both choice situations where SWD-BB fruits were offered to G1 females, time had a significant effect on attractiveness to the parasitoid (SWD-BB vs Air, Wald-$\chi 2 = 46.60$, df = 6, $p < 0.001$; SWD-BB vs H-BB, Wald-$\chi 2 = 32.38$, df = 6, $p < 0.001$). Parasitoid females were attracted to SWD-BB fruits during the first three days after fly oviposition, with significant differences at T1 and T2 compared to the air treatment (binomial test, $p = 0.037$ and $p = 0.022$, for T1 and T2, respectively), and at T3 compared to the H-BB treatment (binomial test, $p = 0.005$). Conversely, no preference for SWD-BB fruits was observed at T4 to T6. In fact, G1 females showed a significant repellence to SWD-BB fruits tested against the air at time T4, T5 and T6 (binomial test, $p = 0.008$, $p < 0.001$ and $p = 0.008$ for T4, T5, and T6, respectively) and compared to healthy fruits at time T5 and T6 (binomial test, $p = 0.002$ for T5 and $p = 0.019$ for T6). When testing H-BB vs air, G1 females did not show any preference and no effect of the age of the fruits was recorded (Wald-$\chi 2 = 6.66$, df = 6, $p = 0.353$).

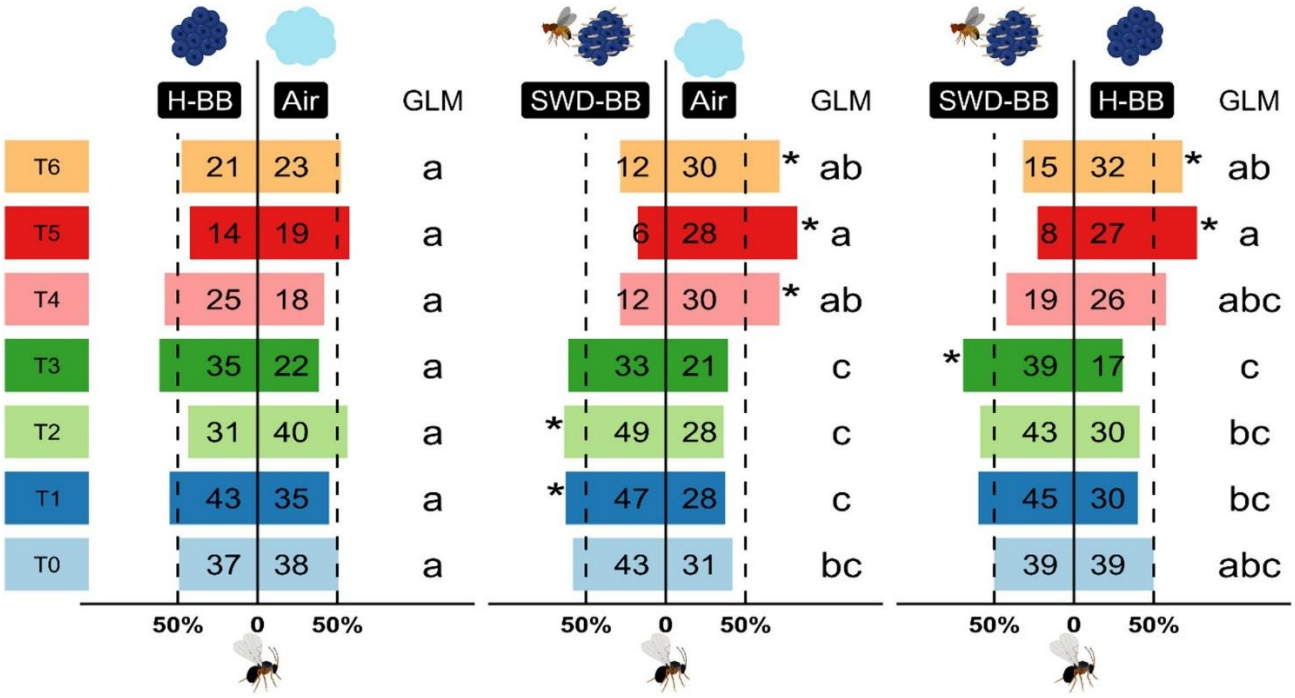

Figure 5.1: *Ganaspis brasiliensis* G1 behavioral response in a two-choice olfactometer bioassay. Percentage of parasitoid females attracted to the two odor sources are reported on the x-axis. SWD-BB: blueberries infested by *D. suzukii*; H-BB: uninfested blueberries. Each two-choice bioassay was carried out over seven consecutive times starting from 0 h (T0) to one-six days (T1-T6) after *D. suzukii* oviposition. Numbers in the bars indicate parasitoids choosing. Asterisks indicate a significant response of *G. brasiliensis* to one of the two odor sources (two-sided binomial test, $p < 0.05$). Significant differences ($p < 0.05$) between times (T0-T6) are indicated with different letters (Šidák post hoc test at the 95 % confidence level after GLM analysis with data distribution fitted to a binomial model using the logit link function).

5.3.2 VOC collection and identification

Analysis of the CLSA extracts revealed 19 VOCs (Table 5.1). Four compounds, tetradecanal, hexadecanal, heneicosane and (Z)-9-tricosene were detected in consistent quantities in the infested blueberries (SWD-BB) at all time points tested (T0-T3, and T6) while no detection or trace amounts (tetradecanal) were recorded from both AP-BB and H-BB samples (Table 5.1, Supplementary figure S5.1-S5.4). Phenylethyl alcohol was not detected or detected in traces in all samples from T0 to T3 (Table 4.1, Supplementary Figure S5.4). At 144 h after exposure to *D. suzukii* (T6), phenylethyl alcohol was detected in much higher proportion in the SWD-BB samples compared to the H-BB and

AP-BB samples (Table 5.1, Supplementary Figure S5.5). The remaining 15 compounds were detected, at different rates, in all treatments and at all times (Table 5.1).

Table 5.1: Volatile organic compounds (VOCs) identified from healthy (H-BB), artificially pierced (AP-BB) or *Drosophila suzukii*-infested (SWD-BB) blueberries. VOCs were collected at 0 h (T0), one to three days (T1-T3), and six days (T6) after *D. suzukii* oviposition. VOCs specifically produced by SWD-BB blueberries are reported in bold.

#	Name	Formula	RT	RI	H-BB					AP-BB					SWD-BB				
T					0	1	2	3	6	0	1	2	3	6	0	1	2	3	6
	Esters:																		
1	Ethyl isovalerate	C7H14O2	4.15	729	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Aldehydes:																		
2	(E)-2-Pentenal	C5H8O	4.15	729	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
3	Hexanal	C6H12O	4.41	773	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
4	Nonanal	C9H18O	10.62	1101	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
5	(E)-2-Nonenal	C9H16O	12.17	1148	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
6	Decanal	C10H20O	13.93	1202	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
7	**Tetradecanal**	C14H28O	26.59	1607											**x**	**x**	**x**	**x**	**x**
8	**Hexadecanal**	C16H32O	32.14	1811											**x**	**x**	**x**	**x**	**x**
	Alkanes:																		
9	Dodecane	C12H26	13.77	1197	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
10	Tridecane	C13H28	17.05	1296	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
11	Eicosane < n->	C20H42	29.11	1697	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
12	**Heneicosane**	C21H44	38.94	2096											**x**	**x**	**x**	**x**	**x**
	Alkenes:																		
13	1-Octadecene	C18H36	26.11	1590	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

14	**(Z)-9-Tricosene**	C23H46	41.59	2277													**x**	**x**	**x**	**x**	**x**
	Monoterpenes:																				
15	1,8-Cineole	C10H18O	4.83	849	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
16	(E)-β-Ocimene	C10H16	5.82	929	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
17	Linalool	C10H18O	10.48	1096	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Alcohols:																				
18	**Phenylethyl alcohol**	C8H10O	10.84	1107																	**x**
	Aliphatic acids:																				
19	Hexadecanoic acid	C16H32O2	35.77	1955	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

5.4 Discussion

The success of oviposition in or on its host by a parasitoid female is a complex phenomenon largely relying on chemical communication becoming increasingly reliable as the foraging parasitoid gets closer to the host. Parasitoids of generalist herbivorous insects can locate their hosts on many plant species and in different environments. Chemical cues from uninfested plants are usually unreliable signals for host location, while herbivore-induced plant volatiles (HIPVs), produced after the insect host starts feeding or laying eggs, represent abundant and reliable cues exploited by parasitoids over long distances (Dicke and Baldwin, 2010, Turlings and Erb, 2018). Additionally, some parasitoids of generalist hosts can also exploit long-range volatiles produced by uninfested plants through associative learning (*i.e.*, after experiencing a cue from the plant and associating it to the host presence) (Geervliet et al., 1998, Segura et al., 2016). At short distances, host-derived chemical cues, including volatile compounds and non-volatile contact compounds, are responsible for orienting parasitoids toward their host. Some highly volatile host-derived compounds (*e.g.*, some sex

pheromones) are also exploited to locate hosts over long distances (Vet and Dicke, 1992, Steidle and Van Loon, 2003).

In this paper, we studied the long-range behavioral response of a specialist parasitoid, the lineage G1 of *G. brasiliensis*, towards the chemical cues produced by its generalist host, *D. suzukii*, developing in blueberries. The results we obtained in olfactometer bioassay confirmed the general theory, showing how host-induced VOCs elicited a foraging response by parasitoid females, while volatiles released by uninfested fruits did not. Additionally, we showed that not only chemical cues are involved in host location but also that the time gap from *D. suzukii* oviposition (and thus the development stage of the host larva) is a key factor for success of host location by G1 females. Taken together, the choice tests with infested fruits (SWD-BB vs H-BB, and SWD-BB vs Air) showed that G1 females were attracted to infested blueberries from the first to the third day after *D. suzukii* oviposition, whilst they were repelled by infested fruits after 4–6 days from host oviposition. Conversely, when G1 females were offered a choice between healthy blueberries and pure air, they did not show any preference, regardless of the age of the fruits tested. The timely response of G1 females to infested fruits reflected the parasitoid requirements for successful oviposition. *Ganaspis brasiliensis* females preferentially parasitize very young host larvae (1–2 days old) (Wang et al., 2018) developing in fresh ripe but not rotting fruits (Seehausen et al., 2020, Seehausen et al., 2022). In our experimental setting, after 4 to 6 days from *D. suzukii* oviposition, infested fruits hosted late instar larvae of *D. suzukii* and were deteriorated by the feeding activity of the fly larvae and their associated microorganisms. Hence, they were recognized as unsuitable by the parasitoid.

In this study, the behavioral response to infested fruits by the specialist *G. brasiliensis* G1 was similar to that previously observed for some generalist parasitoids of drosophilids attacking *D. suzukii* (Biondi et al., 2021, de la Vega et al., 2021). These parasitoid species showed a preference for infested

fruits in respect to healthy fruits or pure air. Conversely, significant behavioral differences emerged between *G. brasiliensis* G1 and the generalist parasitoids towards decaying fruits. In our study, G1 females were repelled by decaying fruits infested by *D. suzukii* (*i.e.*, blueberries after 4–6 days from *D. suzukii* oviposition) when offered as an alternative to pure air or healthy fruits. In the study by Biondi et al. (2021), the generalist parasitoids *L. japonica* and *A. japonica* preferred decaying fruits to pure air and did not show any preference when the choice was between decaying fruits and infested fruits. In fact, these authors also showed that the olfactometer response by *G. brasiliensis* lineage G3 was similar to that of the generalist parasitoids *L. japonica* and *A. japonica*. The different behavior of *G. brasiliensis* G1, here examined, further confirm the very narrow host range of this lineage with respect to the lineage (sister species) G3 (Seehausen et al., 2022).

The results of olfactometer bioassays with G1 females found substantial support in the characterization of VOCs released by the blueberries tested. *Drosophila suzukii*-infested blueberries differed from healthy ones and from the artificially pierced ones by the release of five specific VOCs of which four (tetradecanal, hexadecanal, heneicosane and (Z)-9-tricosene) were detected at all tested times (T0-T6). The fifth compound, phenylethyl alcohol, was detected in consistently high proportion only six days after oviposition (T6). Fourteen VOCs detected in healthy blueberries were also detected in similar proportions in infested and artificially pierced blueberries; they are known to be components of blueberry aroma and flavor (Gilbert et al., 2015, Farneti et al., 2017, Sater et al., 2020) and blueberry cuticular wax (Trivedi et al., 2019, Klavins and Klavins, 2020). The results of the chemical analysis of VOCs clearly showed that infested blueberries release compounds associated with the activity of *D. suzukii* and are not derived from mechanical damage to the fruits. Tetradecanal, hexadecanal, heneicosane and (Z)-9-tricosene might be some of the chemical cues that G1 females exploit to locate fruits infested by a suitable host stage. Tetradecanal and hexadecanal are components of the

pheromone blend that mediate long-range communication of *D. melanogaster* (Lebreton et al., 2017), but they have not been reported for *D. suzukii* (Dekker et al., 2015, Snellings et al., 2018, Lima et al., 2023). Heneicosane and (Z)-9-tricosene are compounds found in the cuticular hydrocarbon profile of some drosophilids including *D. suzukii*; in particular, (Z)-9-tricosene is released by adults of both sexes and has been suggested to play a role in intraspecific communication by acting as a sexual or aggregation pheromone (Dekker et al., 2015, Snellings et al., 2018, Lima et al., 2023).

The compound 1,8-cineole, which was detected in healthy, infested and artificially pierced blueberries, was exclusively found in infested blueberries in a previous study (de la Vega et al., 2021). Also, these authors found a VOC profile of *D. suzukii*-infested (26 volatile compounds) and healthy blueberries (13 volatile compounds) very different from our results with only three compounds in common (ethyl isovalerate, 1,8-cineole and, exclusively in infested fruits, phenylethyl alcohol). These differences may be partially explained by the different blueberry variety used in the two studies highlighting an important issue about the successful behavior of *G. brasiliensis* G1 when foraging on different blueberry varieties attacked by *D. suzukii*.

In addition to qualitative differences, some noteworthy quantitative distinctions deserve attention. For instance, in infested fruits tetradecanal was detected in substantial quantities throughout the entire tested period (from 0 to 6 days after oviposition, therefore in the absence of *D. suzukii* adults). Conversely, both healthy and artificially pierced fruits released no amounts or traces of tetradecanal. The significant increase in tetradecanal production by infested blueberries appears to be a direct consequence of their infestation by *D. suzukii*. Therefore, tetradecanal might be considered a candidate HIPV, potentially exploited by G1 females to locate *D. suzukii*-infested blueberries. However, we cannot exclude that tetradecanal and other chemical cues exploited by the parasitoid may originate from its larval host. The cuticular hydrocarbons of *D. suzukii* detected from infested

fruits are known to be a product of adult flies. Since *D. suzukii* females were allowed to oviposit for only 4 h and the VOCs were collected from infested fruits always in the absence of adult flies, it is likely that heneicosane and (Z)-9-tricosene are deposited on the fruits during oviposition, as occurs for the aggregation pheromones of several *Drosophila* species (Bartelt et al., 1985, Wertheim et al., 2006). Alternatively, they might be produced by the larvae. In fact, the infested blueberries were not attractive towards G1 females at the end of the oviposition time (T0), hence the cuticular hydrocarbons left by the adult flies on the fruit surface would appear to play a minor role in the foraging behavior of the parasitoid. In the generalist parasitoid of drosophilids *Leptopilina heterotoma* (Thomson), the aggregation pheromone of the host is a highly reliable kairomonal cue exploited for substrate selection over a long distance, while other host cues (*e.g.*, cuticular hydrocarbons, feces) are exploited for host location at a closer spatial scale (Wertheim et al., 2003, Quicray et al., 2023). Considering all the features of the volatile blend released by infested fruits, we might hypothesize that highly volatile compounds (*e.g.*, tetradecanal) represent the primary cues exploited for host location over a long distance, while fly cuticular hydrocarbons (*i.e.*, heneicosane and (Z)-9-tricosene), characterized by a lower volatility (Dekker et al., 2015, Snellings et al., 2018), come into play at a short distance. To confirm this hypothesis, it is pivotal to test the behavioral response of G1 females towards the purified compounds identified in this paper and to trace for each one a specific dose–response curve.

The VOC profile detected from blueberries deteriorated by larval feeding 6 days after *D. suzukii* oviposition supports the repellence of infested fruits towards G1 females 4–6 days from *D. suzukii* oviposition. In detail, at time T6, the peak proportion of phenylethyl alcohol dramatically increased, resulting in much higher levels in infested blueberries compared to healthy and mechanically damaged fruits. Similarly, de la Vega et al. (2021) collected phenylethyl alcohol from *D. suzukii*-infested blueberries and not from healthy fruits and artificially pierced fruits. Phenylethyl alcohol is a volatile

that mediates the attraction of *D. suzukii* and other drosophilids to fermented sweet materials and is a fermentation product of yeast and lactic acid bacteria (Cha et al., 2012, Đurovic et al., 2021). Although phenylethyl alcohol can be a small component of the volatile profile of healthy blueberries (Farneti et al., 2017), our results suggest that the high specific production of phenylethyl alcohol from deteriorated infested fruits may be associated with fermentation microorganisms carried by *D. suzukii*. *Ganaspis brasiliensis* G1 might exploit the huge emission of phenylethyl alcohol (and other fermentation volatiles) to discriminate those fruits harbouring late larval instars of *D. suzukii* that are not suitable for parasitization or that harbour larvae of non-host drosophilid species.

The results of this research pave the way to set up tools to be exploited for enhancing the foraging success of *G. brasiliensis* G1 and in turn the efficacy of the biological control of *D. suzukii*. Specific compounds could be profitably used to attract the parasitoid where its activity is most needed, or to bait traps to check its distribution as, for example, after its release in biological control programs. Nonetheless, it now appears fundamental to assess whether the same attractive compounds are released by different host fruits attacked by *D. suzukii* or if specific VOC blends associate with different infested fruits. If this is the case, it then will be interesting to assess the ability of G1 females to locate its host in different host-fruit complexes.

5.5 Supplementary information

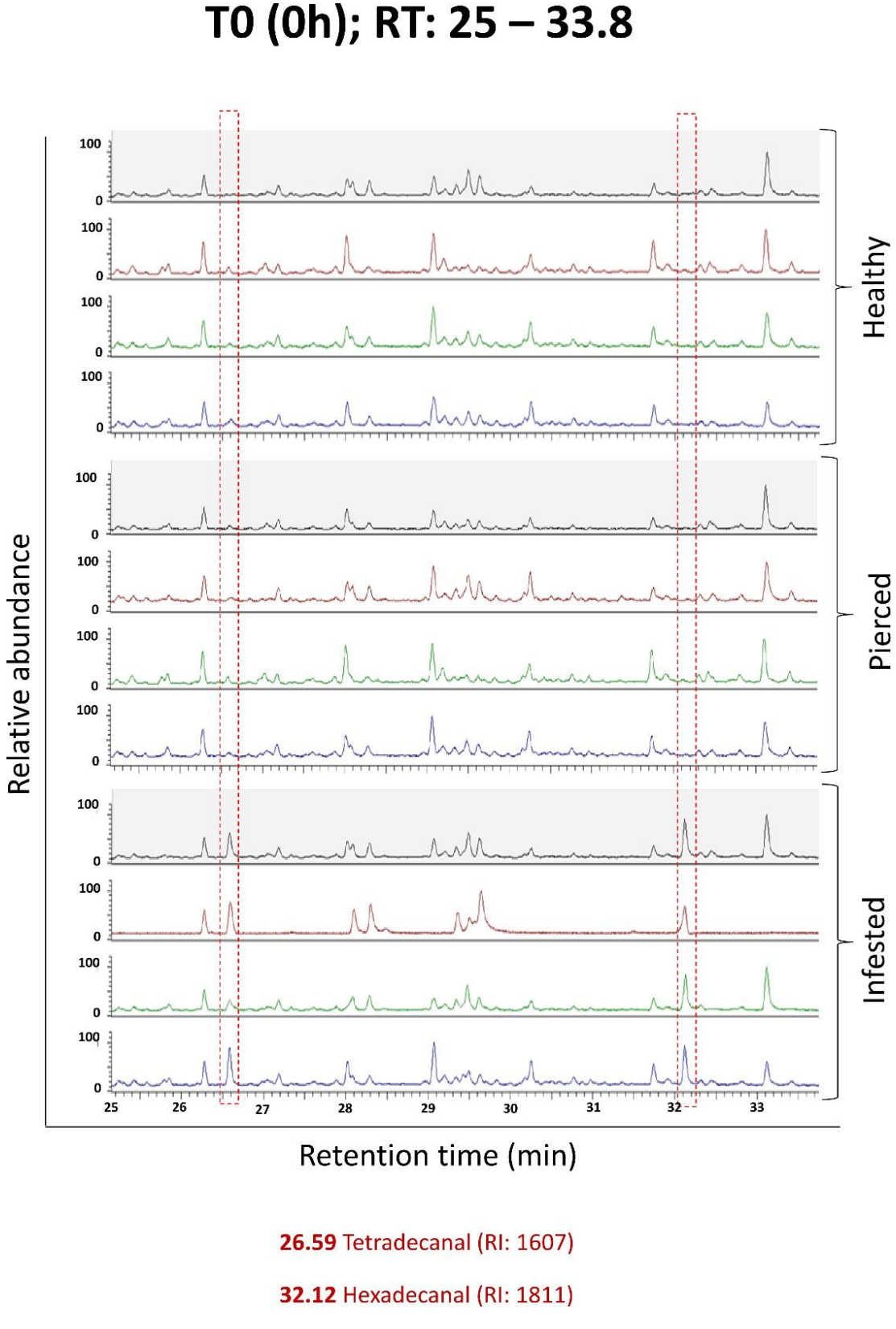

Figure S5.1: Comparison of the peaks localized in the RT 25-33.8 interval for infested, pierced, and healthy blueberries at time T0 (0h). All four replicates are displayed for each treatment. Peaks for tetradecanal and hexadecanal are present only in the infested blueberries.

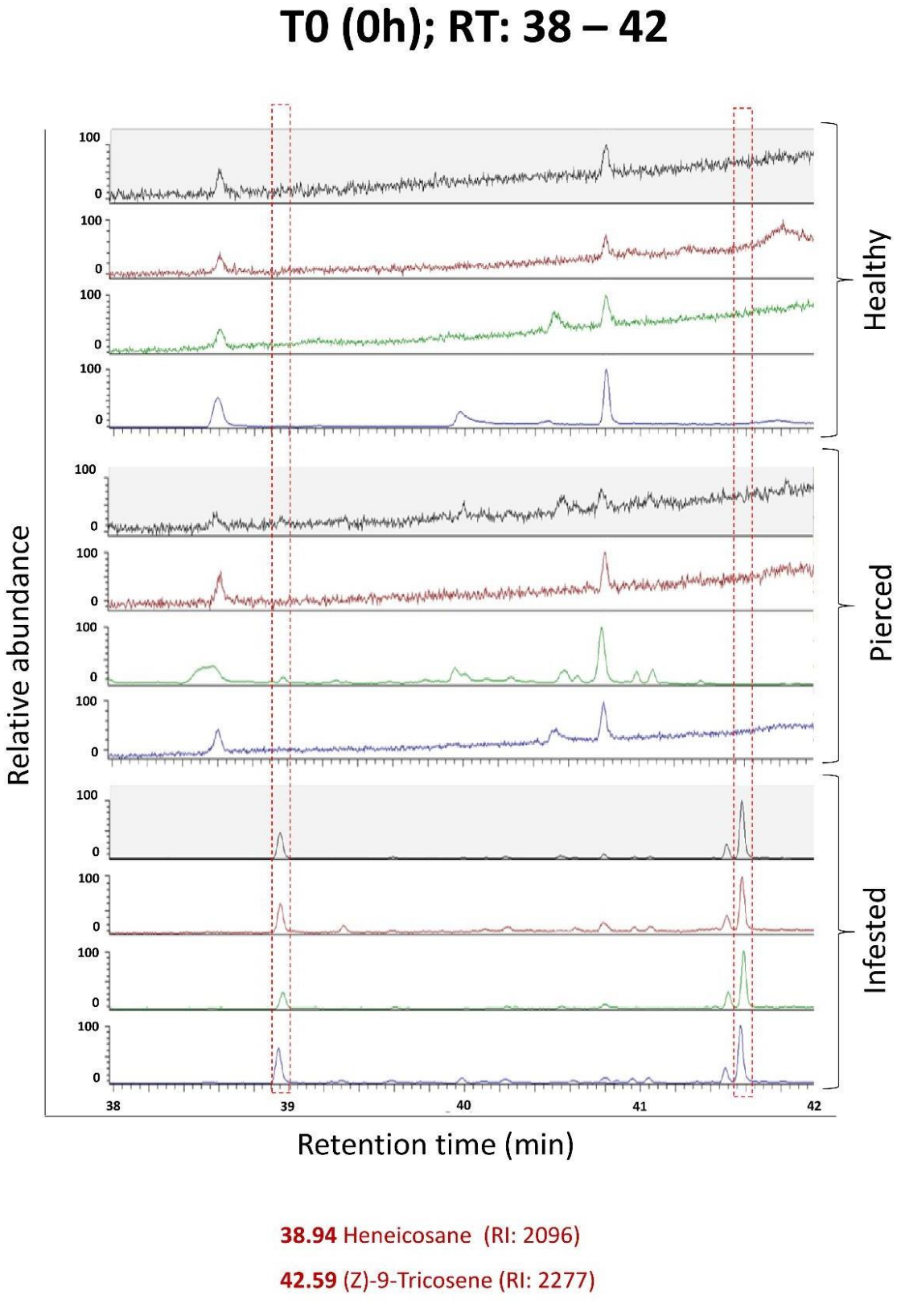

Figure S5.2: Comparison of the peaks localized in the RT 38-42 interval for infested, pierced, and healthy blueberries at time T0 (0h). All four replicates are displayed for each treatment. Peaks for heneicosane and (Z)-9-tricosene are present only in the infested blueberries.

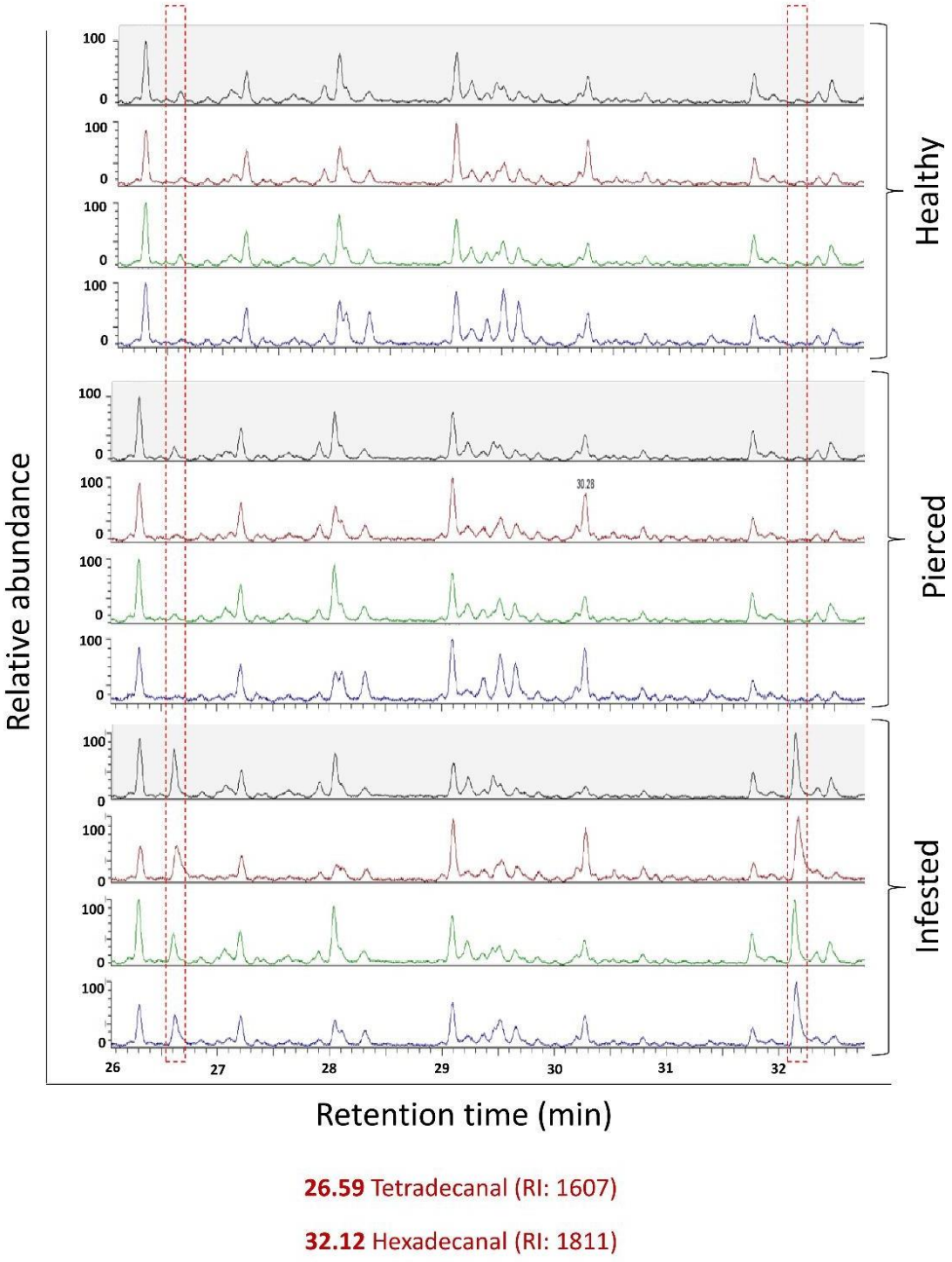

Figure S5.3: Comparison of the peaks localized in the RT 26-32.8 interval for infested, pierced, and healthy blueberries at time T3 (72h). All four replicates are displayed for each treatment. Peaks for tetradecanal and hexadecanal are present only in the infested blueberries.

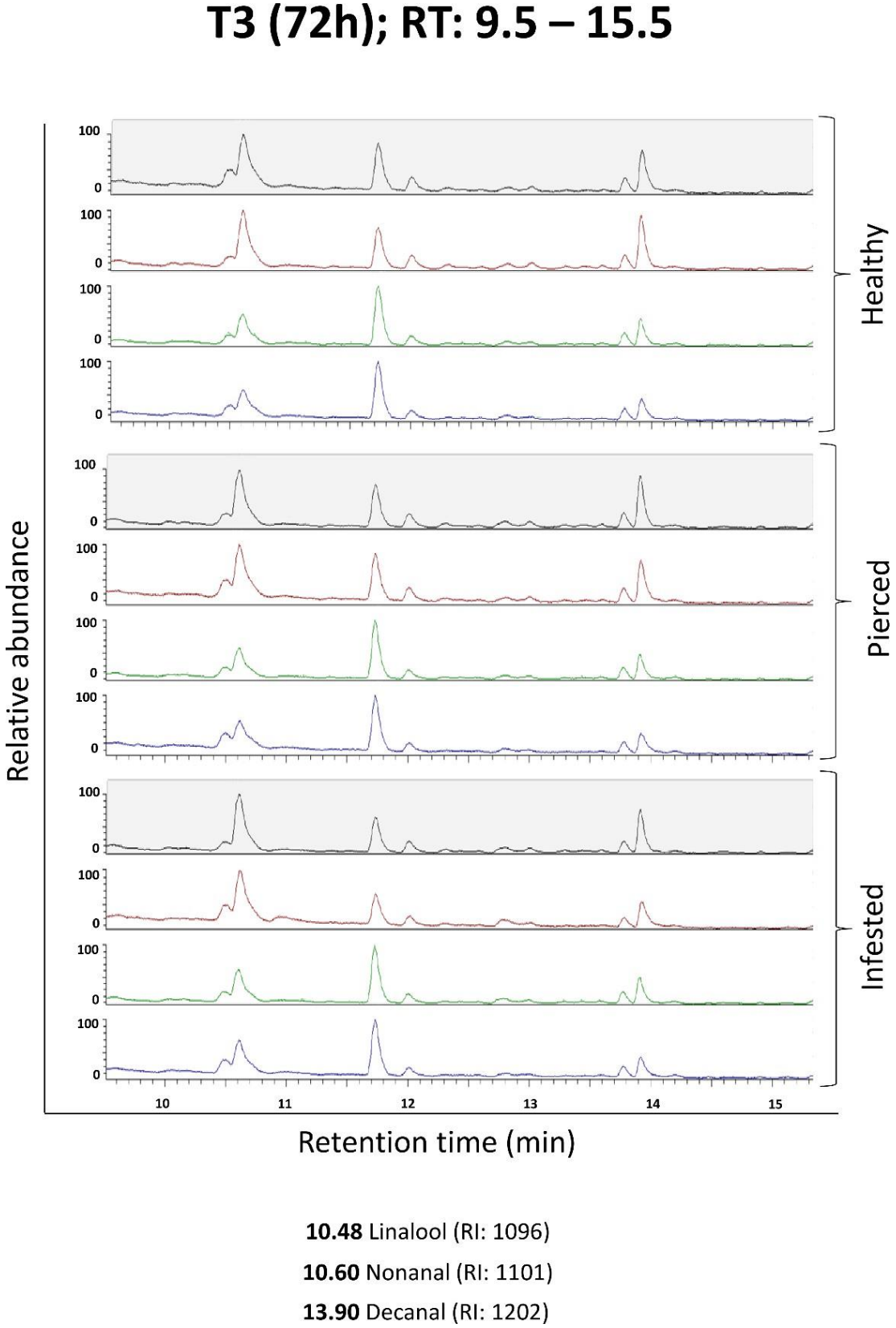

Figure S5.4: Comparison of the peaks localized in the RT 9.5-15.5 interval for infested, pierced, and healthy blueberries at time T3 (72h). All four replicates are displayed for each treatment. No differences in peaks are detected.

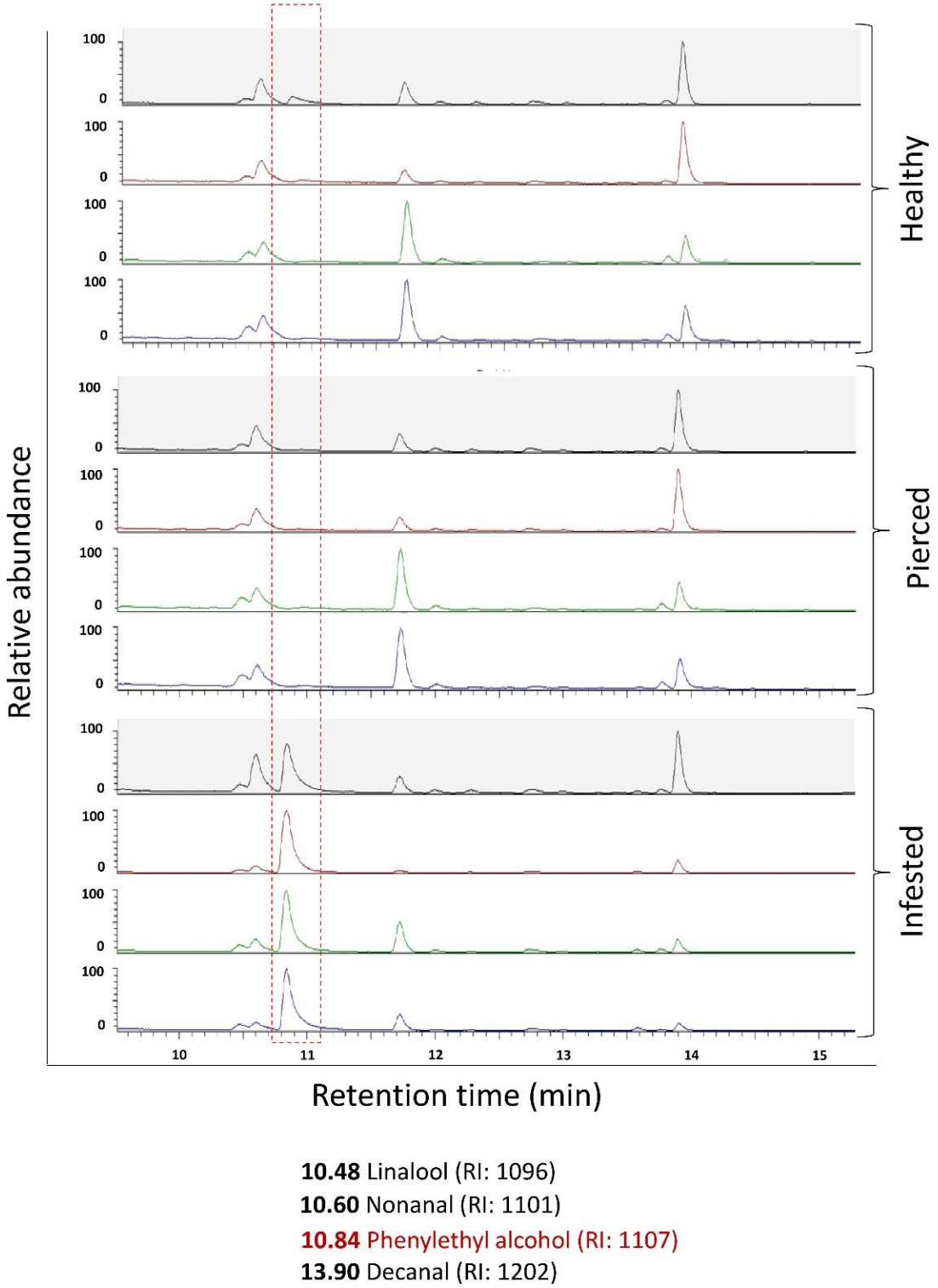

Figure S5.5: Comparison of the peaks localized in the RT 9.5-15.5 interval for infested, pierced, and healthy blueberries at time T6 (144h). All four replicates are displayed for each treatment. Peaks for phenylethyl alcohol are present only in the infested blueberries.

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CHAPTER 6. HOST SEARCHING: BIOTREMOLOGY

Detection and characterization of incidental vibrations from *Drosophila suzukii* in infested fruits

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Abstract

The spotted-wing drosophila (SWD), *Drosophila suzukii* (Matsumura), is an invasive species native to East Asia and now widespread worldwide. Major economic damage is caused by the larvae developing within ripening soft-skin fruit. Currently, larval detection in fruit is limited to destructive methods and post-harvest control strategies heavily rely on the use of chemicals or cold to inhibit egg eclosion and larval growth. Feeding larvae are likely to induce substrate-borne vibrations in the berry that could be exploited as cues by predators or to develop a non-invasive pest detection method, an approach previously applied on leaves and wooden structures, but never on fresh fruit. We used a laser vibrometer to detect and characterize the incidental vibrations produced by *D. suzukii* larvae within fresh blueberries at five different pest age (48, 96, 168, 216 and 264 h). An innovative statistical analysis was performed to assess if infestation level (number of pupae) and pest age (hours after exposure) affect the spectrum and the amplitude of vibrations. The recordings of infested berries were characterized by the presence of a series of broad-band pulses (frequency range 0.1–2 kHz) without a

regular temporal pattern, in an amplitude range between 12.1 and 946 µm/s. Furthermore, the analysis revealed the possibility to distinguish between different pest ages and infestation levels. By a spectral analysis of the recordings, the pest ages can be distinguished among each other, but for the age groups at 168 and 216 h after infestation. The vibration amplitude trend gradually increased up to 168–216 h after infestation, and then decreased until fly emergence. Low-infested blueberries showed a faster *D. suzukii* development time compared to high-infested blueberries. This was reflected into vibrational recordings, as low-infested blueberries exhibited peak amplitude at earlier stage compared to high-infested ones. Results suggest that *D. suzukii* larvae induce detectable vibrations by feeding within berries that are dependent on infestation level and pest age. We discuss the possible ecological role of such vibrations as cues for unintended receivers, such as predators and parasitoids, and their potential for innovative infestation detection methods.

Keywords:

Spotted-wing drosophila, Non-destructive detection, Laser vibrometer, Biotremology

6.1 Introduction

Apart from being important economical commodities and relevant component of human and animal's diet, fruits provide microhabitats to a vast group of insects (Sallabanks and Courtney 1992). This interaction can be mutualistic and have neutral or even beneficial effects for some host plants (Wilson 2008), but from an agriculture perspective, infested fruits are a major economical concern. Among frugivore insects residing in fruit pulp there is an invasive pest causing extensive agricultural damage worldwide, the spotted-wing drosophila (SWD), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). Native to Southeast Asia, since 2008 *D. suzukii* has invaded North America (Hauser 2011), Europe (Cini et al. 2012), South America (Andreazza et al. 2017) and Northern Africa (Ouantar et al. 2020) thanks its high fecundity (Emiljanowicz et al. 2014; Hamby et al. 2016), wide host range (Lee et al. 2015; Kenis et al. 2016) and thermal adaptability (Ryan et al. 2016). *Drosophila suzukii* is among the very few *Drosophila* species able to lay eggs in ripening fruit (Atallah et al. 2014). Development parameters are temperature and humidity-dependent (Tochen et al. 2014, 2016). Under optimal laboratory condition at 22 °C, eggs take 12 to 14 days to reach adulthood, passing through three larval instar stages (5.8 days) and a pupal stage (6 days) (Emiljanowicz et al. 2014; Tochen et al. 2014). While larvae reside most of their life in fruit pulp, pupation can also occur on fruit surface, or detached outside (Kanzawa 1939; Hamby et al. 2016; Woltz and Lee 2017; Bezerra Da Silva et al. 2019). Crop damage is caused by larvae feeding in ripening fruit and serious economic impacts have been reported to raspberry (Farnsworth et al. 2017), blueberry (Yeh et al. 2020), strawberry and blackberry (De Ros et al. 2015), Swiss cherry, plum and grape (Knapp et al. 2021) cultivation.

Pest detection methods rely on monitoring adults utilizing traps and different lures (Cloonan et al. 2018). A less common monitoring method is larval count. that has the advantage of providing immediate information to growers to adjust the most appropriate control strategies depending on the

real-time infestation status (Tait et al. 2021). However, at the moment this technique is time-consuming as it requires several steps such as fruit crushing, salt or vacuum extraction and filter collection (Van Timmeren et al. 2017; Babu et al. 2023). A successful non-contact detection method of *D. suzukii* not only could favor early pest detection, but also post harvesting sector that now heavily relies on chemical application and cold storage to suppress the pest (Walse et al. 2012; Kraft et al. 2020; Mostafa et al. 2021). Insects are known to produce substrate-born vibrations while residing in or on their substrate (Hill et al. 2019; Virant-Doberlet et al. 2023). These vibrations can either be produced by specialized organs, such in the case of mating signals of Hemiptera (Laumann et al. 2011; Virant-Doberlet et al. 2011), or they can be incidental, such in the case of vibrations produced by movement (Devetak 2014; Oberst et al. 2017) or feeding (Kollasch et al. 2020; Turchen et al. 2022). These vibrations have been exploited to detect invertebrates 'presence within different substrates (Mankin et al. 2011), initially with accelerometers, piezoelectric sensors and microphones (Mankin et al. 1997, 2004; Castellanos and Barbosa 2006; Pearson et al. 2007; Devetak 2014), and more recently utilizing more accurate laser Doppler vibrometers (Zorović and Čokl 2015; Liu et al. 2017; De Luca and Vallejo-Marin 2022). This methodology has been already applied to assess incidental vibrations produced by insects within wood (Zorović and Čokl 2015), and extensively on plant's leaves (Kollasch et al. 2020; Turchen et al. 2022), but to the authors' knowledge, never on fruits. Even if most of juvenile insect stages, such as larvae, nymphs and pupae, are substrate-bound and likely depend on perception of vibratory signals, their vibroscape, described as their natural vibrational environment (Šturm et al. 2021), has been rarely studied and it remains poorly understood (Yack and Yadav 2022). While *Drosophila* adults are known to utilize substrate-born vibrations during courtship, where male tremulations result into female immobilization (Fabre et al. 2012; Mazzoni et al. 2013; McKelvey et al. 2021), little is known about their larval stage. Moreover, the identification and characterization of these incidental vibrational signals are necessary steps to unravel their ecological

role. In particular to assess if they function as cues in host-parasitoid interactions, as previously assumed (Vet and Bakker 1985; Girod et al. 2018a), but never assessed thoroughly. Eavesdropping is a common scenario in the animal realm, where numerous and diverse signals (chemical, visual, acoustic, electrical, tactile) are exploited by unintended receivers (Peake 2005; Hughes et al. 2012). This technique is adopted by predators and parasitoids to locate preys and hosts (Broad and Quicke 2000; Takanashi et al. 2016; Yack and Yadav 2022). As biological control (BC) is becoming a relevant practice adopted to suppress this pest population (Lee et al. 2019; Tait et al. 2021), it is fundamental to deepen our knowledge on BC agents host searching, which might not rely solely on chemical, but also on physical cues such as substrate-born vibrations (Higham and Hebets 2013; Nieri et al. 2022).

In this study we used a laser vibrometer to detect and characterize incidental vibrations produced by larvae of *D. suzukii* feeding within fresh fruit pulp. We hypothesized that infested blueberries are characterized by the presence of larval feeding vibrations. We first evaluated the presence of vibrations by comparing the recordings of fruit infested with living larvae and un-infested fruit. We, then, characterized the vibrations and applied innovative statistical methods to test the influence that infestation level and pest age had on the amplitude and spectral parameters of recorded vibrations.

6.2 Material and methods

6.2.1 Insect rearing and infestation

Drosophila suzukii individuals used for fruit infestation were mass reared according to the protocol specified in Rossi Stacconi et al. (2022) and derived from multiple field collections of live adults at different locations in the Trento Province (Italy), during 2020 and 2021 seasons. Considered that traits of recorded substrate play an important role into defining spectra we ensured that every blueberry had similar weight and shape. Fresh blueberries from the same batch were selected and

weighted to ensure a standardized weight of 1.5 ± 0.1 g using a precision scale (D-72336, Kern and Sohn, Balingen, Germany). Rotten, soft, deformed or damaged fruits were discarded. Un-infested blueberries were used as control, while the others were placed in *D. suzukii* mass rearing cages (30 × 30 × 70 cm, Bugdorm BD4F3074, MegaView Science Co., Ltd, Taichung, Taiwan) to allow oviposition. Every 15 min, infested blueberries were removed from the cages and checked under stereoscope (M80, Leica Microsystem, Germany) to count the number of laid eggs and ensure the absence of other living organisms such as mites. Blueberries with 5 to 30 fly eggs were selected for recordings and maintained individually in a plastic cylinder container (diameter 6 cm, height 8 cm) with absorbent cotton at the bottom and a lateral hole (diameter 2 cm) covered with fine mesh for aeration. A small fragment of reflective tape (2 × 2 mm2) was attached to the surface of each blueberry in the same position, near the calyx, to minimize differences in vibrational recordings. For the duration of the experiment, distilled water (3 ml) was poured weekly on the cotton in each container to avoid sample desiccation. Containers were maintained in controlled conditions at 21 ± 2 °C, 70% RH, and 16:8 L:D.

6.2.2 Drosophila suzukii development parameters

The containers holding the infested blueberries were monitored for adults' eclosion every 48 h. Emerging *D. suzukii* adults were counted and stored in 70% ethanol solution. One week after last adult emergence, the samples were hydrated and pupae were isolated and counted. The total number of pupae was used as a proxy to estimate blueberries' infestation level. Blueberries were grouped into two categories: low infestation (L) containing 1 to 6 pupae (n = 14), and high infestation (H) containing 7 to 13 pupae (n = 16). To evaluate *D. suzukii* development the following parameters were used: the adult survival rate, expressed as the percentage of eggs laid that reached adulthood, the

average emersion time (AEM), the first emersion time (FET) and the last emersion time (LET), as the number of days from the infestation date to adult emergence.

6.2.3 Vibrational recording and analysis

Vibrational recordings were taken in a soundproof room between February and April 2022. The blueberries were placed in calyx-up position and individually tested on an anti-vibration table (Astel s.a.s, Ivrea, Italy). Vibrations were recorded using a laser Doppler vibrometer (PDV 100, Polytec, Germany) focused on the reflective tape previously positioned on the blueberry. Recordings were digitized using the software BK Connect (Brüel and Kjær Sound and Vibration A/S, Nærum, Denmark) at 6.4 kHz sample rate and 24-bit depth resolution through a data acquisition device (LAN XI type 3050-B-040, Brüel and Kjær Sound and Vibration A/S, Nærum, Denmark), and stored directly onto a computer hard drive. For each blueberry, vibrations were recorded for 3 min at five different time intervals: at 48, 96, 168, 216 and 264 h after *D. suzukii* infestation. Control un-infested blueberries were recorded at the same time. Recordings were analyzed using the software BK Connect (Brüel and Kjær Sound and Vibration A/S, Nærum, Denmark) with a Fast Fourier Transformation (FFT), Hanning type, window size of 400 Hz and 66.7% overlap for the frequency range 100–2000 Hz. The amplitude of vibrations was measured as velocity of substrate displacement. To characterize the single pulses induced by the feeding larvae, the peak frequency and the corresponding amplitude were measured for three pulses per recording at three infestation time (28 recordings at 96 h after infestation, 30 at 168 h, and 29 recordings at 216 h). A pulse was defined as a physically unitary or homogeneous sound, composed of a brief succession of sine waves (Alexander 1967). To evaluate the influence of infestation, its level, and pest age, on the entire length of the recording, the spectra averaged for the three-minutes recordings were compared.

6.2.4 Statistical analysis

All statistical analyzes have been carried out in R (4.2) (R Core Team 2020) with the following packages: fda.usc (Febrero-Bande and Oviedo De La Fuente 2012), fdANOVA (Gorecki and Smaga 2018), permuco (Frossard and Renaud 2021), together with custom code developed by the authors and available upon request. To compare *D. suzukii* development parameters a Shapiro–Wilk normality test followed by non-parametric Mann–Whitney was performed. To compare vibrational recording, following a functional data analysis (Ramsay and Silverman 2005) approach, we analyzed the average spectra for each three minute recording and considered, for each blueberry and time interval, a functional observation of the amplitude of vibrations as a function of the frequency. These curves have then been compared by means of point-wise and global non-parametric two-way functional ANOVA (Zhang and Liang 2014), testing, in particular, the effect of infestation, pest age and infestation level. For the point-wise analysis, we compared the between- and within-groups variances at each frequency, producing a sequence of statistical tests and corresponding *p* values. The globalizing test statistic (GPF) (Zhang and Liang 2014), was then obtained integrating the point-wise statistic. The corresponding *p* value was computed through a permutation test (Good 2000). All the statistical analysis described henceforth have been performed for the single frequencies (point-wise tests) and integrating over the frequencies (global tests). To avoid inconsistencies between the results of the two families of tests, *p* values were always computed through permutation tests, with 100,000 permutations. To test the effect of pest age on recording intensity in infested blueberries, we performed a post-hoc analysis by means of Quade's test (Conover 1999). Quade's test is a non-parametric test based on ranks for multiple treatments (represented here by pest age) in randomized complete block designs. To test the effect of the level of infestation on recorded vibrations, always controlling for the effect of pest age, we added different covariates, numeric variables, to the categorical factors in our functional ANOVA model. This resulted in an Analysis of Covariance (ANCOVA) with the following covariates: number of eggs at the infestation time, number of developed pupae, and pupae/eggs rate

as a measure of the survival rate of the pest's larvae. We also examined the interaction between the amounts of eggs and pupae, both by allowing for the interaction in the model and by adding explicitly to the model the ratio among the two variables. For more details about tests and models, see the Supplementary file.

6.3 Results

6.3.1 Drosophila suzukii development rate

On average, high infested blueberries had more than twice the number of pupae compared to low infested blueberries (Table 6.1). Adult survival rate and FET did not differ significantly between the two groups (Table 6.1). On the contrary, the LET was significantly delayed in high infested samples (3.8 days on average, Table 6.1), indicating a lower development rate of the larvae in the high infested berries.

Table 6.1: *Drosophila suzukii* mean development parameters for control and the two treatments: Low (L) and High Infestation level (H). Egg, pupae and emerging adults are expressed as mean number (n ± SEM), First Emersion Time (FET), Last Emersion Time (LET) and Average Emersion Time (AET) are expressed in days after infestation (days ± SEM). Means within a column followed by different lowercase letters are significantly different according to the performed Mann-Whitney test for pairwise comparison. *U* represent the Mann-Whitney test results. *p*-values are corrected by Monte-Carlo permutation.

	Egg	Pupae	Adults	Survival	FET	LET	AET
Low (*n = 14*)	11.7 ± 1.5a	3.6 ± 0.4a	3.5 ± 0.5a	37 ± 6%a	15 ± 0.3a	17 ± 0.5a	16.3 ± 0.3a
High (*n = 16*)	21.3 ± 1.4b	8.3 ± 0.4b	8.1 ± 0.3b	40 ± 3%a	15.9 ± 0.3a	20.8 ± 0.4b	18.1 ± 0.3b
U:	27.5	1	1	93.5	69.5	22	23
p-value:	0.0005	< 0.0001	< 0.0001	0.4540	0.1023	0.0002	0.0002

6.3.2 Vibrations induced by feeding larvae

Infested samples' recordings were characterized by the presence of a series of different sized broad-band pulses that displayed no regular temporal patterns (Figure 6.1). The amplitude of single

pulses ranged from 33.2 to 312.6 µm/s at 96 h ($n = 28$), from 23.7 to 672.6 µm/s at 168 h ($n = 30$), and from 12.1 to 946 µm/s at 216 h after infestation ($n = 29$). The dominant frequency of pulses was on average 1096, 662 and 651 Hz at 96, 168 and 216 h, respectively. No pulses were ever detected in uninfested blueberries. Moreover, the three minutes average spectra had significant higher intensities for all the frequencies (100–2000 Hz) in infested blueberries compared to control ones (analysis of variance, $\alpha = 0.05$) (Figures 6.2, 6.3).

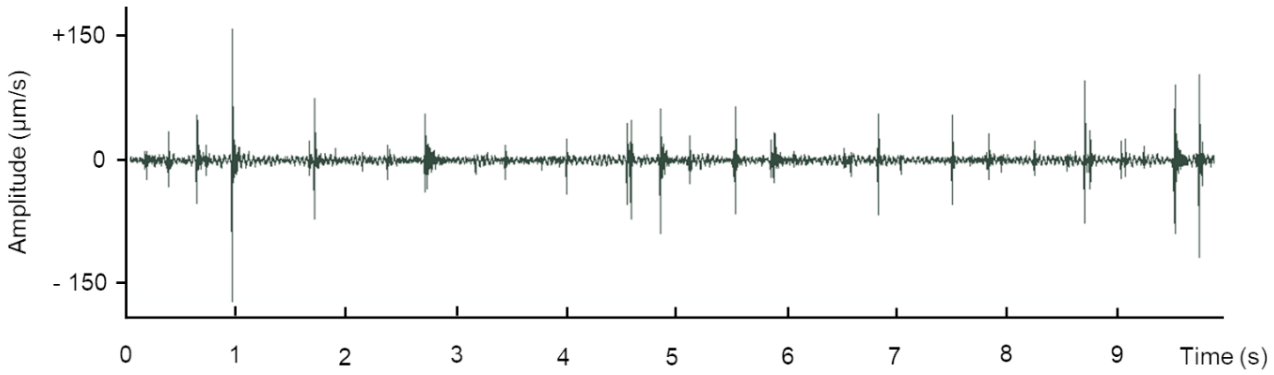

Figure 6.1: Waveform of feeding vibrations of *Drosophila suzukii* larvae developing within fresh blueberry pulp 168 h after infestation. The 10 s lapse shows a series of pulses.

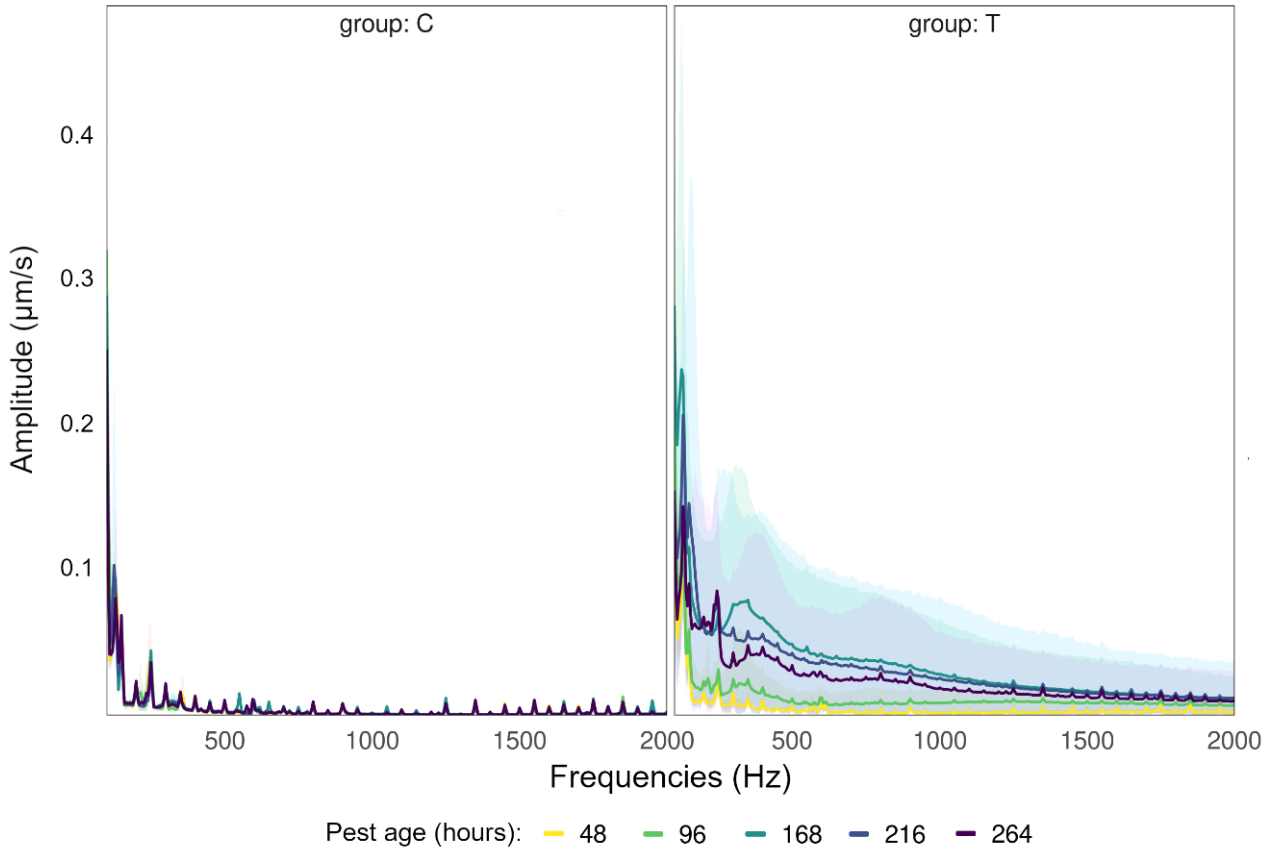

Figure 6.2: Visual representation of the three-minutes spectra for control uninfested blueberries (left) and infested blueberries (right). The infested samples displayed higher recorded intensities. Bold lines represent the average of the samples (n = 30) recorded at different pest age time. Shades represent Standard Deviation values.

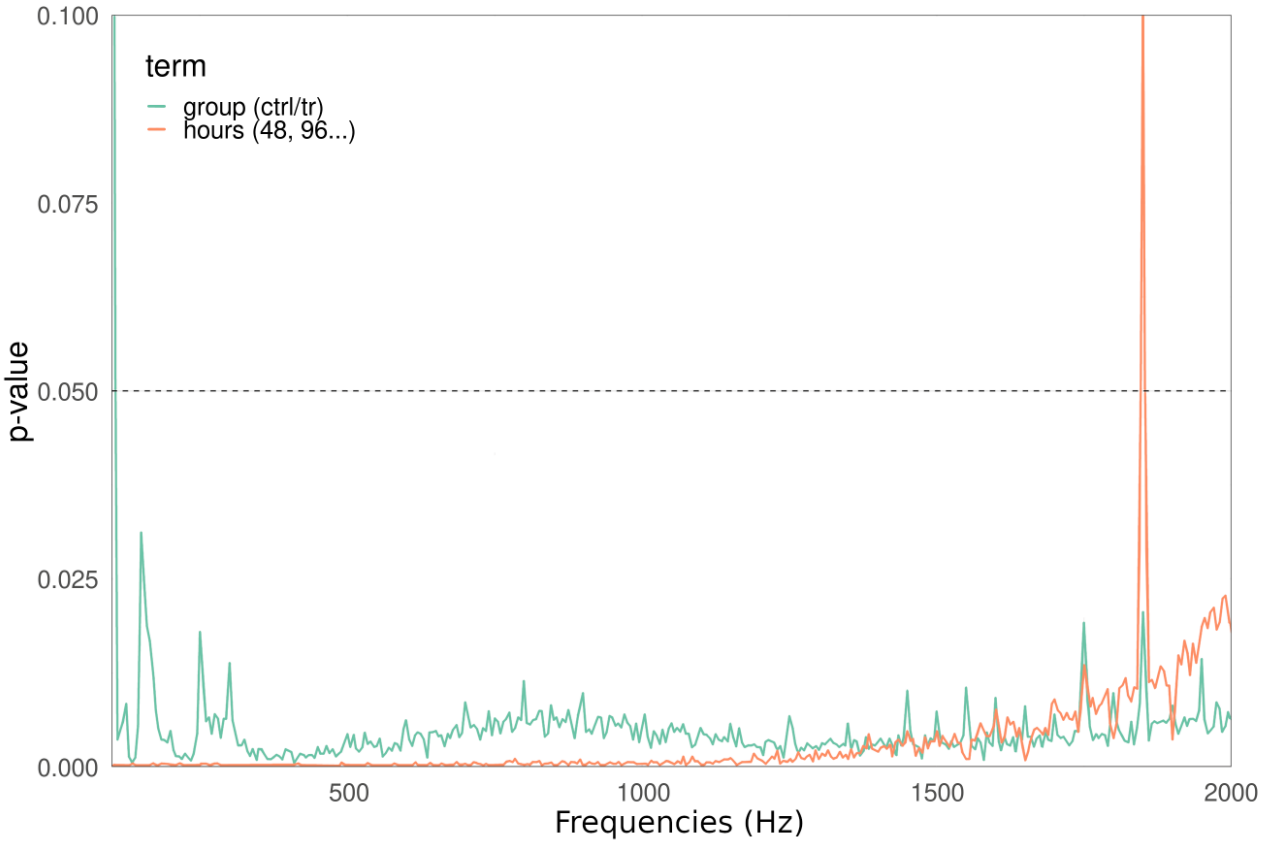

Figure 6.3: *p* values along the frequencies resulting from ANOVA obtained by a permutation method. At level of significance of 95% ($p = 0.05$) both factors under analysis (control and treatment, and the five pest ages) show a significant effect on amplitude for almost all frequencies. Y axis has been cut at 0.1.

6.3.3 Influence of pest age on vibration amplitude

Among infested blueberries, the pest age had a significant effect on the vibration amplitude for almost all frequencies ($\alpha = 0.05$). Only at around 1800 Hz the effect was not significant ($p > 0.05$) (Figure 6.3). The Globalising Pointwise Quade test revealed that over all frequencies, all pest ages could be discriminated among each other ($p < 0.001$), except for two groups, 168 and 216 h (*T-statistic*: 0.84, *p* value = 0.12) (Table 6.2). The pairwise pointwise Quade test (Figure 5.4) revealed that the amplitude of vibrations recorded at 48 h was significantly different from the one recorded at other pest ages ($\alpha = 0.05$) for almost all frequencies, except at very low frequencies (100–125 Hz),

and few more frequencies (upper limit 600 Hz) in comparison with the recordings performed at 96 h. The vibration amplitude at 96 h was significantly different on average from the one recorded at 168 and 216 h for frequencies under 1200 Hz ($\alpha = 0.05$). The pairwise comparison of the vibration amplitude between the 96 h and 264 h groups was not significantly different for most of the frequencies ($p > 0.05$) (Figure 6.4).

Table 6.2: Globalising pointwise quade test results for all time (pest age) comparisons.

Comparison	Statistic (*T)*	*p*-value
48 vs 96	3.69	0.00
48 vs 168	7.14	0.00
48 vs 216	6.34	0.00
48 vs 264	3.94	0.00
96 vs 168	3.45	0.00
96 vs 216	2.66	0.00
96 vs 264	1.00	0.00
168 vs 216	0.84	0.12
168 vs 264	3.20	0.00
216 vs 264	2.40	0.00

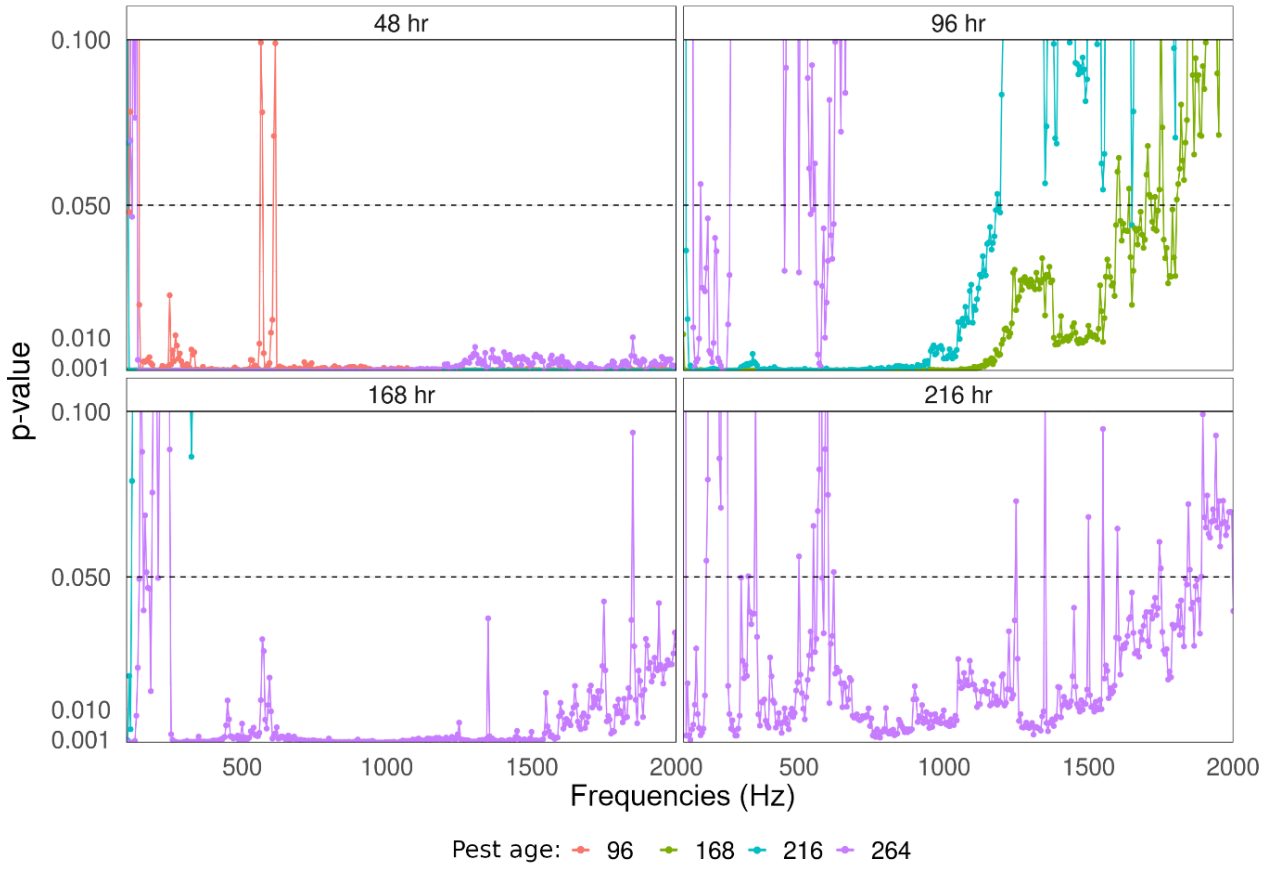

Figure 6.4: *p* value for multiple pairwise Quade test obtained by a permutation method. The tests have been performed for each frequency and for multiple comparisons between different pest ages. Y axis has been cut at 0.1.

6.3.4 Influence of infestation level on vibration amplitude

The analysis of variance confirmed that different infestation levels (Control, Low and High) had a significant effect on the vibration amplitude for all the frequency range ($\alpha = 0.05$) (Figure 6.5). Overall, we observed an increase of the vibration amplitude at increasing infestation levels: the higher the infestation level the higher the vibration amplitude (Figure 6.6). The vibration amplitude trend over time was characterized by a gradual increase (up to 168–216 h after the infestation) followed by a gradual decrease until flies' emergence. The average maximum amplitude was recorded at 0.104 µm/s after 168 h for samples with low infestation, whereas samples with high infestation recorded the average maximum amplitude at 0.189 µm/s after 216 h. The functional analysis of covariance

(ANCOVA) performed on infested berries confirmed that, among infestation level factors considered into the analysis, the number of pupae better explained the observed vibration amplitude recorded for all frequencies ($\alpha = 0.05$) (Figure 6.7). The interaction between egg and pupae resulting from the model was unreliable at low frequency range (100–400 Hz) and above 1500 Hz ($\alpha = 0.05$). Both the number of laid eggs and the ratio between pupae and eggs, did not explain the vibration amplitude for any frequency ($p > 0.05$) (Figure 6.7).

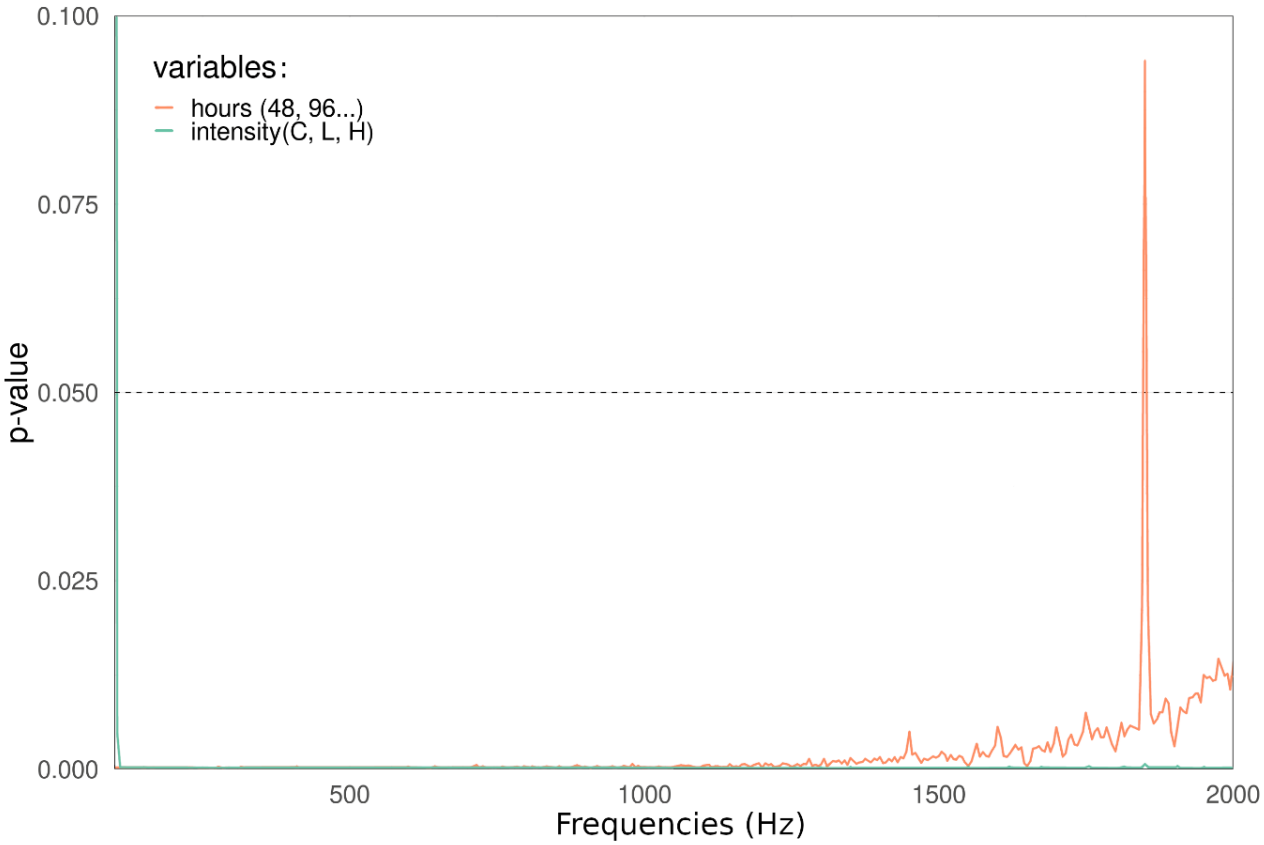

Figure 6.5: *p* values along the frequencies resulting from ANOVA and permutation test. At level of significance of 95% ($\alpha = 0.05$ the infestation levels (C, L and H) show a significant effect on amplitude for all frequencies but those lower than 168 Hz.

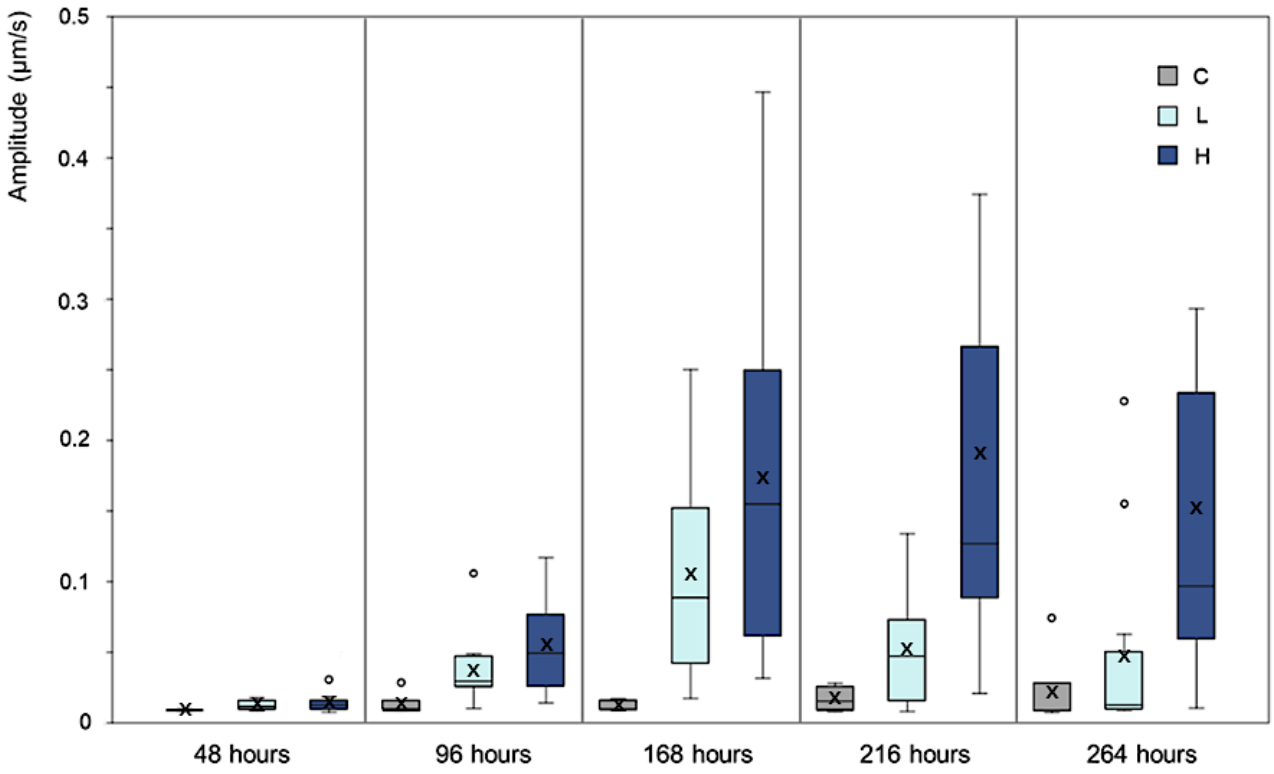

Figure 6.6: Visual representation of vibrations amplitude recorded at different pest age and infestation level (C Control; L Low infestation; H High infestation) previously tested with ANOVA (Figure 5.5). For each box, the central line represents the median, the limits of the box are the 25th and 75th percentiles. The cross symbol (x) represents the mean. Whiskers extending outside the box illustrate data range, while dots are considered outliers.

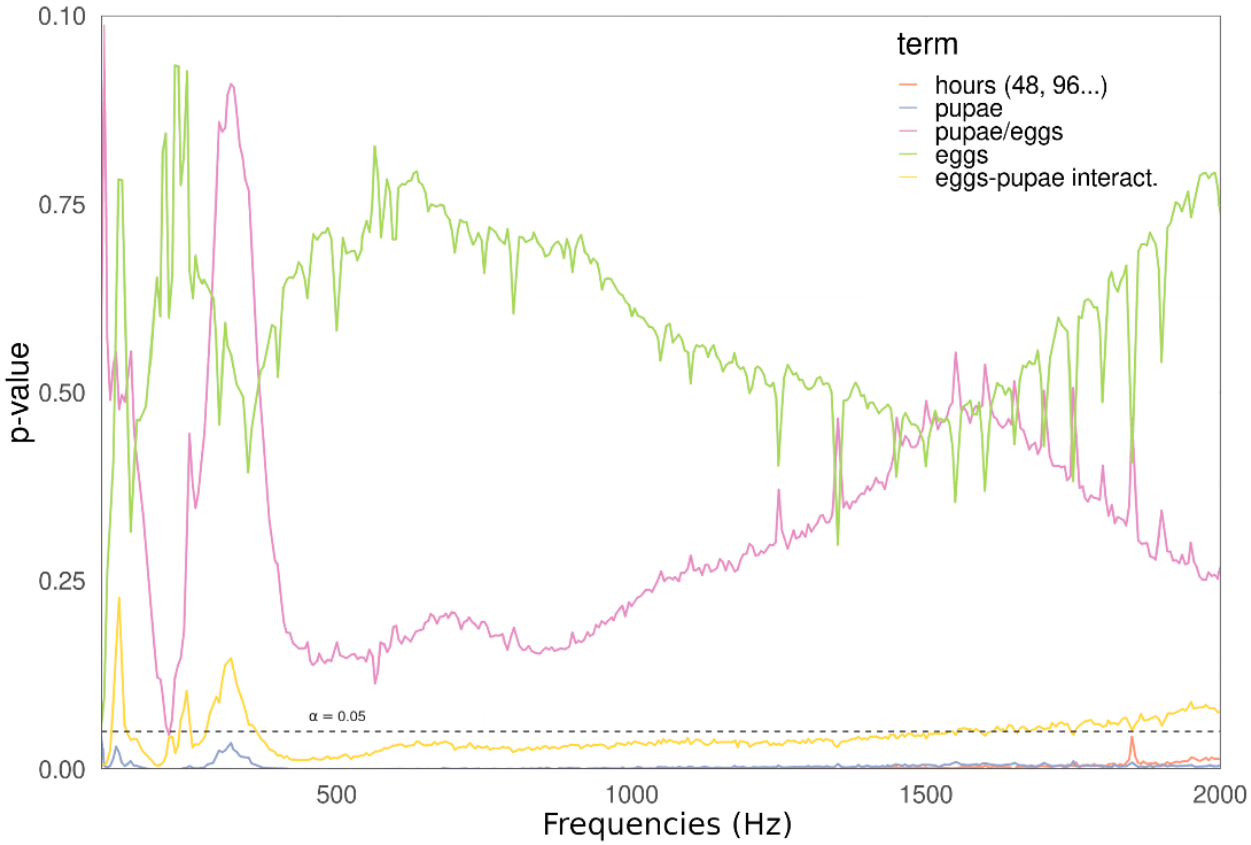

Figure 6.7: *p* value resulting from the functional Analysis of covariance (ANCOVA) obtained by a permutation method. Pupae represent the number of pupae that developed into the sample, eggs represent the number of counted eggs at infestation time, pupae/eggs represent the ratio of the variables, and eggs-pupae interaction represent their interaction resulting from the model.

6.4 Discussion

Infested samples were characterized by the presence of broad-band pulses that are within the sensitivity range of the species (Mamiya et al. 2018). The spectral analysis revealed the possibility to distinguish infested from un-infested samples. Additionally, in our standardized context we were able to distinguish different pest ages and infestation levels. Vibrations produced by hymenopteran and lepidopteran larvae feeding on leaves exhibit similar wave forms to those we described (Kollasch et al. 2020), supporting the hypothesis that the recorded pulses are *D. suzukii* larvae's mandibles chewing the fruit pulp. Kollasch et al. (2020) described peak amplitude of pulses ranging between 1 and 3

mm/s, whereas our recorded amplitudes ranged from 12.1 to 946 µm/s. Such lower values could be explained by several factors, as the difference in size of the emitters used in the two studies, the location of the emitters with respect to recording point on the substrate and the shape and type of the substrate (Oberst et al. 2019) In fact, the insect size is directly related to the amplitude of produced vibrations (Vick et al. 1988; Mankin et al. 2010); and while *D. suzukii* larval length ranges from 1 mm (1st instar) to 4 mm (3rd instar) (Van Timmeren et al. 2017), the lepidopteran and hymenopteran larvae tested by Kollasch et al. (2020) were all late instars (3rd to 5th) ranging from 10 to 30 mm in length. Furthermore, amplitude of vibrations is inversely related to the distance of the emitters with respect to the recording point. In our study the larvae were embedded in a spherical substrate (blueberries), whereas recordings by Kollasch et al. (2020) were performed on larvae feeding on the surface of a flat substrate (leaves); thus, in our case it was impossible to know the exact distance between the emitting larva and the recording point on the blueberry surface. Probably this is the reason behind the variability of amplitude of the recorded pulses. Although *D. suzukii* infestation was carried out at the same time to reduce biases, different larvae could have displayed different development time (therefore size) and were simultaneously active within the substrate, at different distance from the recording point. Interestingly, the amplitude range we observed is very similar to that of the vibrations produced by the larvae of the Asian longhorned beetle (Anoplophora glabripennis Motschulsky) feeding within wood logs (10–960 µm/s) (Zorović and Čokl 2015), despite such larvae can measure up to 50 mm in length (CABI 2020). This may suggest a major influence of the location of the emitter with respect to the substrate (on or within) compared to the effect of the insect. In modern berries processing, quality check traditionally performed by human operators is leaving place to automatized imaging systems that allow to effectively sort and grade fruit based on its appearance (Wang et al. 2021). These detection technologies mainly rely on physical aspects of the fruit surface (Patel et al. 2012; Blasco et al. 2017), and cannot detect the presence of pests located within fruit pulp.

Our results demonstrate that vibrational detection is possible and its integration into fruit processing systems can potentially give a more comprehensive picture of fruit status. One constrains of vibrational detection is the need to isolate the recorded sample to reduce the interference of background noises. This issue could be tackled coupling the recording with other detection systems that are generally placed in controlled area, such as computer vision systems and odor detection (E-Nose) (Wang et al. 2021), or filtering the noise digitally (Bentler and Chiou 2006). Considering that currently post-harvest suppression of *D. suzukii* requires costly long exposure to CO2, not always effective to completely kill the immature stages (Mostafa et al. 2021), a preliminary pest detection could limit its application, reducing both environmental and economical associated costs.

Besides detection for monitoring or control purposes, vibrations recorded in this study are potentially relevant in the *Drosophila* ecology. In fact, feeding vibrations could have both an intraspecific and interspecific function. Intraspecific communication in *Drosophila* relies also on vibrational stimuli (Fabre et al. 2012; Mazzoni et al. 2013; McKelvey et al. 2021). In particular, *Drosophila* femoral chordotonal organs neurons (FeCO) are sensitive to low amplitude vibrations in the 200–2000 Hz range. In particular, it has been shown that frequency that corresponds to the highest sensitivity is 400 Hz at 0.9 µm/s, and 800 Hz at the smaller amplitude of 0.054 µm/s (Mamiya et al. 2018). The pulses we recorded are above this threshold and the peak frequency is within the sensitive range, suggesting that *D. suzukii* could exploit the vibrations induced by feeding larvae as a cue to identify infested berries. Host searching behavior and oviposition site selection in *D. suzukii* is regulated by visual, mechanical and chemical cues (Cloonan et al. 2018; Kidera and Takahashi 2020) but vibrations could represent an additional modality to acquire insights on the suitability of host patches. Specific odors emitted by plants or microorganisms and the color of ripening berries are important for long-range location of suitable hosts, indeed these cues are used for traps' design

(Cloonan et al. 2018; Alawamleh et al. 2021). At short-range, surface secretions produced after oviposition have an aggregative function and stimulate conspecific females to lay eggs shortly after infestation (Tait et al. 2020). However, this effect rapidly decreases (a few hours) as the chemical cues left by the ovipositing female start degrading (Tait et al. 2020). We hypothesize that vibrational cues could be involved in oviposition site selection at advanced stages (> 12 h after infestation), when eggs hatch and larvae start producing incidental vibrations. At this stage, the chemical cues deriving from previous oviposition events could be outcompeted by the vibrational cues induced by larvae developing within the fruit. This would be consistent with the fact that multiple concurrent ovipositions on a single fruit increase offspring survival through different cooperation mechanisms (Tait et al. 2020), whereas ovipositing on fruit where older larvae are already present could be disadvantageous due to intraspecific competition (i.e., cannibalism of eggs and younger larvae) (Bezerra Da Silva et al. 2019). Moreover, if flies perceive the differences in the spectra that we identified in our analysis, vibrations induced by larvae could provide adult flies with information about both the infestation level and the larval stage. Future studies on *D. suzukii* perception of mechanical stimuli and the behavioral response to vibrational playbacks are needed to test this hypothesis. Incidental vibrations produced by developing *D. suzukii* larvae could also serve as a cue for parasitoids searching for their host (Broad and Quicke 2000). Several parasitoid wasps have documented to attack *D. suzukii* both in its native and invaded area (Girod et al. 2018a, b; Lee et al. 2019; Daane et al. 2021), some of which are currently being reared and released as biological control agents (Rossi Stacconi et al. 2022; Fellin et al. 2023). Larval parasitoids in particular could benefit from vibrational detection, for these species both the host location within the substrate and the development stage are crucial for successful parasitization (Wang et al. 2018). For instance, *G. brasiliensis* have a preference toward young larvae of 1–2 days old (Wang et al. 2018) Larval parasitoids' exploitation of vibrotaxis for host detection has been previously assumed (Vet and Bakker

1985; Girod et al. 2018a), but never assessed thoroughly. In several hymenopteran groups (bees, wasps and ants), evaluation on responses of chordotonal organs to vibrational stimuli revealed that the sensitivities range between 1.8 and 80 µm/s (Strauß et al. 2021), suggesting that the signals we recorded in infested samples could be detected by parasitoid wasps, such as larval parasitoids of *D. suzukii*.

Our analysis revealed the possibility to discriminate larvae age based on recordings amplitude. As spectral parameters are influenced by the recorded substrate (Oberst et al. 2019), this was possible because in our experiment blueberries had similar weight and shape. Different fruit traits should be evaluated in future studies to assess how they influence the spectra. These data could lead to the creation of a model that accounts for both pest age and fruit characteristics. Our recordings suggests that vibrations not only carry binary information regarding host presence or absence, but potentially also relevant information regarding their developmental stage. If proven, the eavesdropping performed by parasitoids could contribute to explain their selectivity toward the different host stages of *D. suzukii* (Rossi Stacconi et al. 2015; Wang et al. 2018). The trend of the amplitude of vibrations over time after infestation can be explained by *D. suzukii* life cycle, and in particular by pupation. Once pupal stage is reached, *D. suzukii*, as all insects, does not feed anymore and it is almost immobile, thus the gradual decrease of vibration amplitude observed in our experiment can be explained by the reduction in individual actively feeding. In fact, the highest amplitude values have been observed at 168 h after infestation, which is when larvae probably were close to pupation according to the observed emersion rates, which are in agreement with those of similar experiments conducted on blueberries in comparable climatic conditions (Tochen et al. 2014, 2016; Winkler et al. 2020). In previous studies, the development time from egg to adult took on average 14 ± 0.1 days and larvae pupated approximately eight days after infestation (192 h) (Tochen et al. 2014, 2016), a time at which

we observed signal amplitude plateau and its gradual decrease. The increase in amplitude from 24 to 168 h can be explained by the increase in size of the larvae, for the previously mentioned effect on size over vibrations amplitude. Among the two infestation level groups, L blueberries better represented a natural scenario, in which low numbers of larvae develop within a single berry (Burrack et al. 2013). The later occurrence of the average maximum amplitude observed in H blueberries and its slower decrease compared with L blueberries reflect an overall lower development rate observed in blueberries where larval density was higher. Also emersion times, AET and LET, in highly infested samples was significantly delayed, most likely due to the occurrence of higher intraspecific competition pressure and relative limited availability of food source, both factors that have been proven to extend development time (Hardin et al. 2015; Bezerra Da Silva et al. 2019). The ANCOVA revealed also an important aspect about the development rate and success of *D. suzukii* infestations. Contrary to the number of pupae, the eggs number was not a good indicator to explain changes in the recorded spectra. This evidence supports the fact that egg count is an unreliable proxy to preliminary quantify infestation level. Egg count can be easily performed, even automatized (Waithe et al. 2015) and still a valid method to assess insect fecundity and oviposition preference (Tochen et al. 2014; Ng'oma et al. 2018; Kidera and Takahashi 2020; Fowler et al. 2022), but it poorly represents the number of larvae developing in the infested fruit. Many factors affect *D. suzukii* development from egg to adulthood, such as temperature and humidity (Winkler et al. 2021), interspecific competition (Bezerra Da Silva et al. 2019) and host plant (Olazcuaga et al. 2019). This makes it difficult to estimate accurately infestation level from egg count, especially in samples taken from field and uncontrolled environment. Vibrational recording on the other hand could be a more practical alternative that does not rely on sample history.

Laser vibrometer recordings have been proven a successful non-destructive methodology for early detection of incidental vibrations induced by *D. suzukii* larvae feeding in fresh fruit pulp. Furthermore, the applied analysis has been proven useful to compare vibrational spectral parameters and discriminate both pest ages and infestation level at standardized berry weight. This analysis could be useful in further studies aiming to investigate the effect of broad-band vibrations on other organisms. Moreover, this study paves the way for development of automated monitoring systems that could use a similar comparison analysis to not only detect the occurrence of vibrations but also quantify or identify the infestation level. Additionally, we provided new insights on the ecology of *Drosophila* species, showing that not only adults, but also larvae are emitters of vibrational cues and signals. Which could play a role in both intra and interspecific communication.

6.5 References

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CHAPTER 7. HOST SEARCHING: BIOTREMOLOGY

Influence of vibrational cues on *Ganaspis kimorum* host searching behaviour

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Abstract

The larval parasitoid *Ganaspis kimorum* Buffington (Hymenoptera: Figitidae) has been selected as the best candidate to release for classical biocontrol programs of spotted-wing drosophila (SWD). The parasitoid shows high specificity and prefers to attack 1st and 2nd instar host larvae within freshly infested fruits. While long- and medium-range host location heavily rely on chemical stimuli, the detection of the host in its microhabitat is likely mediated by the vibrations produced by the larvae inside the fruit pulp. We performed bioassays to investigate whether *G. kimorum* uses mechanical vibrational cues to discriminate between suitable and unsuitable host patches. The results suggests that when vibrational cues are present, *G. kimorum* adult females spend significant less time grooming and significantly more time ovipositing. In fact, in the absence of vibration, duration of ovipositor extrusion was reduced by 54%, while frequency and duration of oviposition insertion were reduced by 35% and 78% respectively. We discuss our findings in relation to the parasitoid's behavioural ecology.

7.1 Introduction

In the context of animal communication, eavesdropping is defined as the use of information from signals by individuals other than the primary target (Peake, 2005). These signals (chemical, visual, acoustic, electrical, tactile) are exploited by unintended receivers such as predators and parasitoids to locate preys and hosts (Broad and Quicke, 2000; Takanashi et al., 2016; Yack and Yadav, 2022).

As discussed in Chapter 6, *Ganaspis kimorum* Buffington (Hymenoptera: Figitidae) uses chemical cues to locate its host at long-range distances, with females attracted to *D. suzukii*-infested fruit in the first few days after infestation (Giorgini et al., 2024). However, it remains unclear which cues the parasitoids exploit at short range to locate the host inside the fruit. Based on observations of *Drosophila* parasitoids behaviour, Vet and Bakker (1985) hypothesized that *Ganaspis* spp. rely on vibrational cues (vibrotaxis) to locate host larvae within the media, but their assessment was limited to only five individuals, and it is unclear which *Ganaspis* species was texted (due to its origin and host specificity, it was most likely a G5 strain, now classified as *Ganaspis brasiliensis*).

After confirming that *D. suzukii* larvae produce detectable incidental feeding vibrations (Fellin et al., 2024), we conducted an additional experiment to determine whether the host searching behaviour of *G. kimorum* is influenced by these vibrations. We conducted observations and quantified parasitoids host searching behaviours when in contact with both infested and un-infested blueberries.

7.2 Material and methods

7.2.1 Insects colonies

Ganaspis kimorum specimens originated from a population collected in 2016 near Tokyo, Japan (Girod et al., 2018) and were reared at the Edmund Mach Foundation (FEM) in Italy, according to the protocol described by Rossi-Stacconi et al. (2022). The colony was mantained under controlled conditions: a 16:8 light:dark (L:D) photoperiod, 22 ± 2°C and 70 ± 10% relative humidity (RH).

Drosophila suzukii colonies were established from locally collected adults and maintained in climatic chambers with a photoperiod of 16:8 L:D at 22 ± 2°C and 70 ± 10% RH.

7.2.2 Treatments

Blueberries weighting 2 ± 0.5g were selected and placed into *D. suzukii* rearing cages for approximately one hour. Infested blueberries with 9 ± 1 *D. suzukii* eggs (BL-I) were selected under stereomicroscope (M80, Leica Microsystem, Germany) and used for the assays. Un-infested blueberries from the same batch were used as control (BL-C). 48 hours after infestation, a portion of the BL-C and BL-I were placed in a refrigerator at -80°C for 30 minutes and then maintained at 22°C for one hour. This process was used to rapidly kill the larvae present in the infested fruits, creating a sample without the feeding vibrations produced by larval feeding (FBL-I). Control blueberries were also frozen (FBL-C), to assess whether the freezing process affected their attractiveness. Before conducting behavioural observations, all blueberries were tested with a laser Doppler vibrometer (PDV 100, Polytec, Germany) following the method described by Fellin et al. (2024) to confirm that vibrations were detectable in the BL-I samples and absent in BL-C, FBL-C and FBL-I samples.

7.2.3 Behavioural observations

Experiments were conducted in a soundproof room on an anti-vibration table (Astel s.a.s, Ivrea, Italy) between June and July 2022. Behavioural observations were conducted in a cylindrical plastic arena (2 cm x 5 cm ø) beneath which was mounted a rotary plate that allowed the surface to rotate

around its stationary centre. A single blueberry was placed in the centre of the arena and the rotary mechanism allowed constant recording of the parasitoid's behaviour using a HD camera (HTC-TM700, Panasonic, Japan) mounted on a tripod. The arena featured a hole at the top for the insertion of parasitoids. For each trial, ten 8±1 days-old *G. kimorum* adult females were placed inside the arena. Recording began when the first parasitoid made contact with the blueberry surface. All the other parasitoids were removed from the arena after the first contact.

The first parameter evaluated was *patch residence time* (PRT), which was defined as the total time the parasitoid spent between its first contact with the host patch (blueberry) and its departure. Parasitoids were given 15 minutes (900 seconds) to reach the blueberries. If no parasitoids reached the blueberry within that time, the patch residence time was considered to be 0. From the time of contact, behaviour was recorded for an additional 15 minutes (900 seconds). After this time, the parasitoid was removed from the arena, and the observation was considered finished.

Once the observation ended, the video files were transferred to a PC and edited to increase zooming (dynamic cropping), enhance contrast (+20%) and apply a negative filter (+100% colour inversion) to facilitate observation (Figure 7.1). Video files were analysed using BORIS, an event-logging software (Friard and Gamba, 2016). Behaviours were evaluated in terms of frequency (number of events) and duration (seconds), and categorized as follows:

- Movement: The insect moves forward without antenning.
- Antenning: The insect uses its antennae to tap or swing on the surface of the blueberry.
- Grooming: The insect uses its appendages to clean its head, antennae, wing or abdomen.
- Ovipositor extrusion: The insect extrudes the ovipositor and probes the fruit surface.
- Ovipositor insertion: The insect inserts the ovipositor tip into blueberry flesh.

- Pumping: The insect contracts the valvifers and valvulae to push the egg (oviposition).

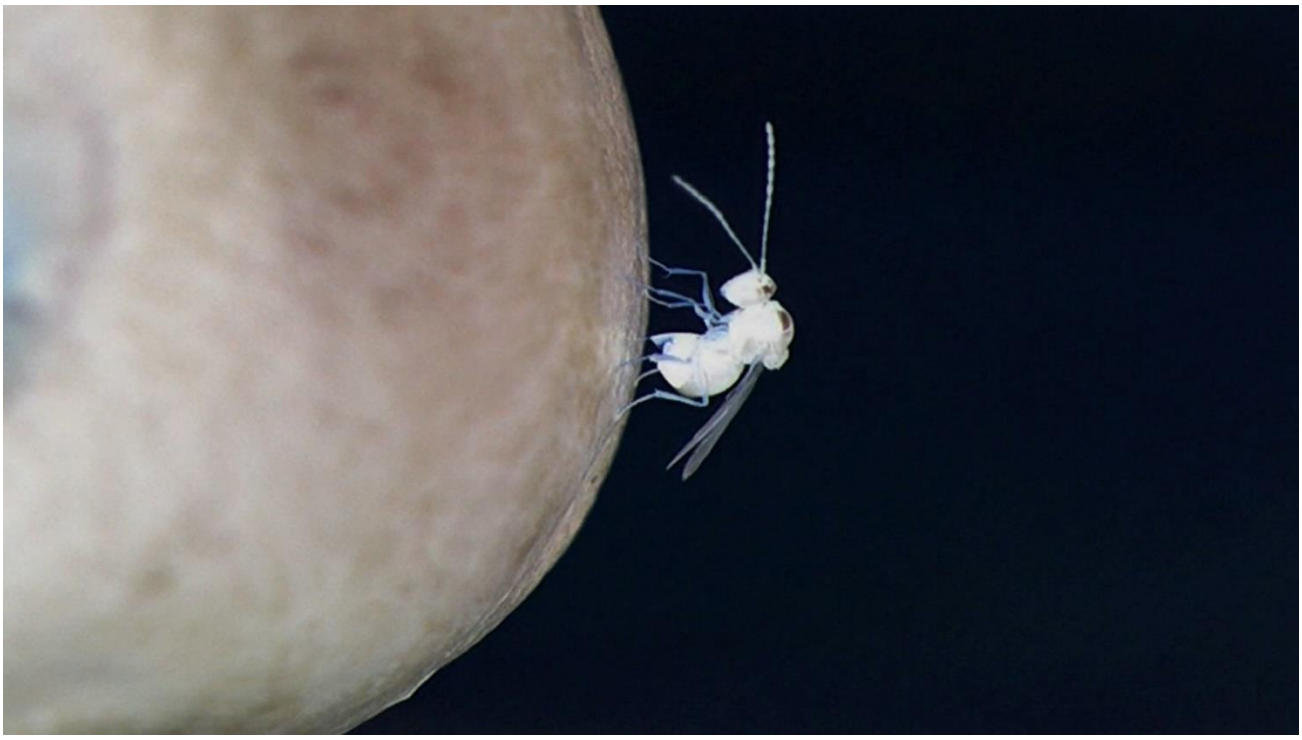

Figure 7.1: Frame extracted from an edited video recording *Ganaspis kimorum* behavior. The editing consisted on a dynamic cropping (zooming to the insect), a complete color inversion (negative filter) and contrast increase. In the figure is possible to see *G. kimorum* inserting the ovipositor tip through the blueberry skin.

7.2.4 Statistical analysis

Since the data did not meet the assumption of normality (as assessed by Shapiro-Wilk test), PRT from different treatments (BL-C, BL-I, FBL-C and FBL-I) were statistically compared with a non-parametric Kruskal-Wallis rank sum test, followed by a Mann-Whitney U's post-hoc pairwise test with Bonferroni correction. As parasitoids exhibited active host searching behavior only towards infested samples (BL-I and FBL-I), the analysis focused on these two groups. Frequency and duration of behaviors for the BL-I (n=31) and FBL-I (n=26) treatments were compared using Mann-Whitney U test with Monte Carlo permutation. All statistical analysis and plotting were conducted using R statistical software (4.3.0) (R Core Team, 2020).

7.3 Results

7.3.1 Patch residence time

The analysis revealed significant differences in residence times among treatments ($\chi^2 = 42.4$, df = 3, $P < 0.05$). Infested blueberries (BL-I and FBL-I) had significantly higher PRT compared to control blueberries (BL-C and FBL-C) (Table 7.1). Based on these findings, subsequent behavioral observations focused exclusively on the infested treatments.

Table 7.1: Patch residence time (PRT) expressed as seconds±SE (Standard Error) spent by *Ganaspis kimorum* on different blueberry treatments: BL-C: Control un-infested blueberries; FBL-C: Frozen control un-infested blueberries; BL-I: Infested blueberries; FBL-I: Frozen infested blueberries. Different letters beside PRT values indicate significance according to the Mann-Whitney pairwise test with Bonferroni correction (P<0.05).

	BL-C	FBL-C	BL-I	FBL-I
Replicates (n)	15	15	15	15
PRT (sec)	24.1±9.3a	45.1±20.9a	886.8±13.2b	770.2±73.7b

7.3.2 Quantitative behavioural analysis

Parasitoids exposed to BL-I and FBL-I showed no differences in movement behavior, both in terms of frequency and duration ($P > 0.05$) (Figure 7.2). Antenning frequency was also comparable between the two treatments ($P > 0.05$) (Figure 7.2a). However, antenning duration was significantly higher in the FBL-I group, with parasitoids spending an average of 164 seconds antenning, compared to 109 seconds in the BL-I group (+51%) (U: 271.5, $P < 0.05$, Figure 7.2b).

Parasitoids exposed to FBL-I spent significantly more time grooming, both in frequency and duration, compared to those exposed to BL-I. On average, they groomed 12.2 times compared to 6.2

times (+95%) (*U*: 247.5, *P* > 0.05), with an average event duration of 202 seconds compared to 53.5 seconds (+277%) (*U*: 239, *P* < 0.05, Figure 7.2).

Oviposition behavior, which includes ovipositor extrusion, insertion and pumping, was notably reduced in *G. kimorum* exposed to FBL-I. In terms of frequency (Figure 7.2a), parasitoids exposed to FBL-I performed significantly fewer insertions than those exposed to BL-I, with an average reduction of 35% (*U*: 258, *P* < 0.05). Pumping events were also significantly lower, with only two events recorded in FBL-I compared to 125 in BL-I (*U*: 116, *P* < 0.05, Figure 7.2a). In terms of duration, ovipositor extrusion and insertion times were significantly shorter in FBL-I, with an average reduction of 54% and 78%, respectively (*U*: 155.5, *U*: 99, *P* < 0.001, Figure 7.2b).

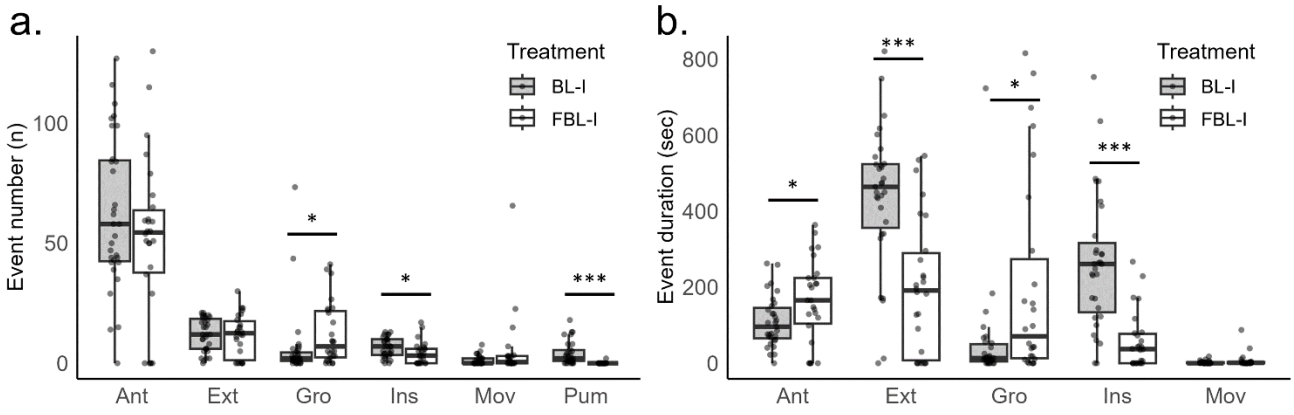

Figure 7.2: Pairwise comparison of *Ganaspis kimorum* behaviors recorded when exposed to infested blueberries expressing larval feeding vibrations (BL-I) or frozen infested blueberries not expressing vibrations (FBL-I). The plot on the left (**a**) shows behavior frequency (numbers of events), while the plot on the right (**b**) shows behavior duration (seconds). The observed behaviors include: Antenning (Ant), Ovipositor Extrusion (Ext), Grooming (Gro), Ovipositor Insertion (Ins), Movement (Mov) and Pumping (Pum). In each boxplot, the central line represents the median, the box limits correspond to the 25th and 75th percentiles, and whiskers indicate data range, Dots represent outliers. Asterisks indicate levels of statistical significance according to the Mann-Whitney U test with Monte Carlo permutation: *: *P* < 0.05 (*), *P* < 0.01 (**), *P* < 0.001 (***).

7.4 Discussion

The results obtained from the patch residence time assays, underline that only infested blueberries are attractive, despite the presence or absence of vibrations. This emphasize the important role of

chemical cues, that are fundamental and necessary to initiate host searching. In fact, behavioral observations were taken at 48 hours after infestation, when olfactometry bioassays confirmed the attractiveness of *G. kimorum* towards infested samples (Giorgini et al., 2024).

Observations clearly showed that host searching behaviour is altered when larvae were dead in the blueberry and did not produce any vibrations. In particular, *G. kimorum* females exposed to FBL-I spent more time grooming and less time using the ovipositor (extrusion and insertion).

Grooming behaviour in insects serves multiple functions, including the maintenance of sensory organ functionality (Zhukovskaya et al., 2013). For instance, a study on American cockroaches demonstrated that antennal grooming removes environmental pollutants and self-produced chemicals, thereby maintaining the sensitivity of olfactory receptors essential for environmental perception (Böröczky et al., 2013). The prolonged grooming observed on FBL-I samples suggests that *G. kimorum* females, in the absence of all necessary cues for successful host detection, prioritized cleaning their sensory organs essential for host searching. The presence of chemical cues but the absence of vibrational ones may have triggered the need to sharpen their sensory organs, possibly due to difficulty in perceiving host movement inside the fruit.

The observed reduction in ovipositor extrusion and insertion by *Ganaspis kimorum* in the FBL-I treatment suggests that vibrational cues play a role in host detection and assessment. Since both BL-I and FBL-I blueberries were assumed to be chemically identical, the absence of larval feeding vibrations in FBL-I likely contributed to the lower probing activity. Vibrational signals may provide essential information about the presence, location, and condition of the host, guiding the parasitoid's decision to extrude and insert the ovipositor. Without these cues, parasitoids exposed to FBL-I may have experienced greater uncertainty regarding host availability, leading to reduced probing and, consequently, fewer oviposition attempts. This highlights the potential importance of multimodal

sensory integration in *G. kimorum* host-searching behavior, where chemical and vibrational cues together facilitate efficient host localization and acceptance.

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CHAPTER 8. INTEGRATION TO AGROECOSYSTEM

Assessment of non-target toxicity of insecticides on *Ganaspis brasiliensis* (Ihering) in laboratory and field conditions

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Abstract

BACKGROUND. G1 strain *Ganaspis brasiliensis* (Ihering) has been recently released in both Europe and America as biological control agent of the spotted wing *Drosophila*, *Drosophila suzukii* (Matsumura). In initial phases of classical biological control programs, it becomes imperative to evaluate the susceptibility of parasitoids to insecticides, to identify the best alternatives to adopt in an integrated pest management and organic perspective. In this study, we evaluated lethal and sublethal effects of topical application of five different insecticides classes: neonicotinoids, diamides, pyrethroids, organophosphates and spinosyns. Additionally, we tested residual toxicity in field trials in vineyards and sweet cherry orchards.

RESULTS. Adult wasps' susceptibility to different insecticides' classes was consistent between laboratory and field. Spinosad exhibited the highest toxicity, with a median lethal concentration (LC50) of 0.00372 of the maximum field dose, and the highest knock-down effect in field trials, causing 92.5 ± 5% of mortality at T0. λ-cyhalothrin showed sublethal effects on both male and female insects' longevity when applied at LC30. In field trial, deltamethrin showed the highest persistence, causing significant parasitoid mortality up to 14 days after treatment. Conversely, cyantraniliprole was the least toxic active ingredient according to both topical and residual bioassays, even though its residues caused mortality up to 7 days after the treatment in the field.

CONCLUSION. Our results indicate that spinosad and λ-cyhalothrin are highly toxic to *G. brasiliensis*, making them incompatible with classical biological control programmes. Cyantraniliprole exhibited lower toxicity, and may be considered a selective pesticide for the integrated management of *D. suzukii*.

Keywords:

Biological control, sublethal effects, IPM, Spinosad, Cyantraniliprole, *Drosophila suzukii*

8.1 Introduction

The spotted-wing *Drosophila*, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), currently stands as the most destructive pest of small fruits and cherries, leading to significant economic losses globally (De Ros et al., 2021). Farmer revenue losses are tied to the unsuitability of berries for the market due to the fly's preference for laying eggs on healthy and ripe fruits, where larvae develop causing direct and indirect damages on fruit (Cini et al., 2012).

In response to *D. suzukii* infestation, growers often resort to the application of conventional and broad-spectrum insecticides sprayed according to calendar schedules (Shawer, 2021; Van Timmeren and Isaacs, 2013). While a long-term management solution is still elusive, integrated pest management (IPM) strategies strive to minimize chemical reliance, thereby enhancing the sustainability and cost-effectiveness of controlling *D. suzukii* (Tait et al., 2021). Currently, the most promising approach involves importing larval parasitoids from the native regions of the pest, Southeastern and Eastern Asia, given the lack of effective indigenous natural enemies in the invaded areas (Lee et al., 2019).

Foreign explorations for co-evolved parasitoids in Asia have demonstrated that the parasitism of *D. suzukii* is primarily supported by three larval parasitoid species: *Asobara japonica* Belokobylskij (Hymenoptera, Braconidae), *Ganaspis brasiliensis* (Ihering), and *Leptopilina japonica* Novković & Kimura (Hymenoptera: Figitidae). In South Korea, the parasitism rate of *D. suzukii* ranged from 0 to 17%, with the braconid species being the main control agent (Daane et al., 2016). In Yunnan province, southwest China, the two figitid parasitoids together achieved substantial parasitism rates of up to 63%. In this area, *G. brasiliensis* is the primary parasitoid of the pest, achieving maximum parasitism rates of 40% (Giorgini et al., 2019; Girod et al., 2018). In Japan *D. suzukii* populations are almost exclusively controlled by *G. brasiliensis*, with parasitism rates reaching 75.6% (Girod et al., 2018).

These field surveys, along with quarantine tests conducted on collected parasitoids (Biondi et al., 2021; Daane et al., 2021; Wang et al., 2020) set the basis for the classical biological control (CBC) of *D. suzukii*, identifying *G. brasiliensis* as the most suitable candidate for the CBC programs. Laboratory and semi-field studies proved that a specific genetic group of *G. brasiliensis*, namely G1 (Giorgini et al., 2019; Nomano et al., 2017) exhibits the highest host specificity, targeting exclusively L1 and L2 *D. suzukii* larvae within fruits (Girod et al., 2018; Seehausen et al., 2022). These findings led to governmental approvals for area-wide releases of *G. brasiliensis* G1 as Biological Control Agent (BCA) in Italy (Lisi et al., 2022) and in the U.S. (Beers et al., 2022). Initial release attempts in northern Italy have demonstrated *G. brasiliensis*'s ability to disperse, overwinter, and specifically parasitize *D. suzukii* (Fellin et al., 2023), encouraging the continuation of the CBC project and the integration of this biological control agent into current IPM programs against *D. suzukii*. To promote a synergistic combination between biological and chemical control, it is therefore crucial to assess the influence of insecticides on *G. brasiliensis*.

The acute toxicity and sublethal effects of insecticides have been evaluated for numerous non-target biocontrol arthropods species, including predators (Fernandes et al., 2016; Passos et al., 2022; Zanuzo Zanardi et al., 2017) and parasitoids (Costa et al., 2023; Jiang et al., 2019; Nozad-Bonab et al., 2021; Teder and Knapp, 2019). Over the past decades, a variety of agrochemical products have been tested and approved for *D. suzukii* control in invaded areas to suppress the pest and limit resistance development (Mishra et al., 2018). Chemical classes commonly utilized to suppress *D. suzukii* population include pyrethroids, carbamates, organophosphates, diamides, and, to a lesser extent, neonicotinoids (Shaw et al., 2019b; Shawer et al., 2018; Tait et al., 2021), while organic farming strongly relies on spinosyns (Gress and Zalom, 2019a). The latter, together with diamide and phosmet have been proven lethal to all life stages of *D. suzukii*, including egg and larval instars

(Mermer et al., 2021). Several chemical classes (neonicotinoids, organophosphates, pyrethroids and spinosyns) have been proven highly toxic to the pest's pupal parasitoids of the genera *Trichopria* (Hymenoptera; Diapriidae) and *Pachycrepoideus* (Hymenoptera; Pteromalidae) (Lisi et al., 2023; Schlesener et al., 2019). Spinosad-based insecticides utilized in organic farming have demonstrated particular toxicity to Hymenopterans (Biondi et al., 2012) and showed high mortality to *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) when exposed to contaminated host puparia (Cossentine and Ayyanath, 2017).

While evidence suggests that biological control with pupal parasitoids is incompatible with chemical application, there are currently no ecotoxicology studies investigating the effects on *D. suzukii* larval parasitoids, although they are considered the most suitable antagonists of the pest in its native environment (Lee et al., 2019). The application of insecticides has the potential to affect all beneficial arthropods, including parasitoids (Biondi et al., 2012; Fernandes et al., 2016; Nawaz et al., 2017; Stanley and Preetha, 2016; Zanuzo Zanardi et al., 2017). Such an impact poses a risk to the long-term crop protection they could provide. However, the presence of semi-natural habitats acting as reservoirs can mitigate such negative effects by facilitating field recolonization from the surrounding areas (Gillespie et al., 2016; Knoll et al., 2017; M. V. Rossi-Stacconi et al., 2019). In landscapes characterized by widespread land dedicated to specialized farming activities, the intensive use of insecticides discourages the dispersal of parasitoids, thereby reducing recolonization processes. This is particularly evident in sweet cherry cultivation, where the number of insecticide applications against *D. suzukii* can reach up to eight per season, depending on pest abundance, cultivar susceptibility, and environmental factors (Mori et al., 2019). Such a scenario is particularly unfavourable for inoculative CBC programs, especially at initial stages when chemical applications, combined with low density of the released population and the absence of natural reservoirs, could

undermine the success of the entire intervention. A rational management of insecticide treatments that integrates with biological control strategy can provide both short- and long-term pest control benefits. To attain this objective, it is essential to achieve a thorough comprehension of the selectivity of the available pesticides. This understanding is crucial for assessing risks to beneficial organisms and allowing the selection of insecticides that preserve important natural enemies (Torres and Bueno, 2018).

In this study, we provide a first step for this process, assessing the lethal and sublethal effects of topical applications of five different classes of insecticide on *G. brasiliensis* G1 adults. Additionally, we present the result of field trials, where three selected products have been tested to evaluate the lethal effects of residual contact on one of the most cultivated crops in Italy, grapevine (*Vitis vinifera* L.) (Amato and Valletta, 2017; Corinto and Pioletti, 2019) and one of the most affected by *D. suzukii* damage, sweet cherry (*Prunus avium* L.) (Knapp et al., 2021; Shaw et al., 2019b).

8.2 Material and method

8.2.1 Insects

The *D. suzukii* colony was established from multiple field collections of living adults occurring in 2020 and 2021 in Northeast Italy (Trento province). Insects were mass-reared in the laboratories of the Edmund Mach Foundation (FEM) in San Michele all'Adige (Trento, Italy) under controlled conditions with a photoperiod of 16: 8 (L:D) at 24 ± 2°C and 70 ± 10% relative humidity. Adult flies were placed in rearing cages (30 x 30 x 70 cm, Bugdorm BD4F3074, MegaView Science Co., Ltd, Taichung, Taiwan) and provided with water and an artificial cornmeal diet as substrate medium, which was replaced twice a week. The diet was prepared according to Dalton et al. (2011) with few modifications. Sugar (15g), soybean flour (10 g), yeast flakes (17 g), cornmeal (71 g), and agar powder

(6 g) were added to one liter of boiling water. After cooking for 30 minutes, the mixture was allowed to cool to 60°C. At this temperature propionic acid (10 mL) and vitamin fortification mixture (5 g) were added. The diet was then poured into sterile petri dishes (9 cm diameter) and refrigerated for later use.

The *G. brasiliensis* G1 colony was established from specimens received from CABI quarantine facilities (Delémont, Switzerland) in 2019, previously collected from their native environment (Tokyo, Japan) in field sampling that occurred from 2015 to 2017 (Girod et al., 2018). The insects were maintained in quarantine laboratories at FEM in controlled conditions and photoperiod of 16: 8 (L:D) at 24 ± 2°C and 70 ± 10% R.H. Parasitoids were reared according to the methodology described in Rossi-Stacconi et al. (2022). Adults' emersion was monitored daily to isolate insects of known age. In 2022, a sample of both flies and parasitoids were shipped to the laboratories of the University of Verona (Verona, Italy), where new colonies were established to conduct field trials. Rearing conditions and methodology were the same as described above.

8.2.2 Insecticides

Insecticides have been chosen to represent a wide range of different chemical groups currently utilized to suppress *D. suzukii*: neonicotinoids, diamides, pyrethroids, organophosphates and spinosyns (Del Fava et al., 2017; Shaw et al., 2019b; Shawer, 2021; Shawer et al., 2018). For the toxicological bioassays carried out in laboratory conditions in 2021-2022, commercial formulations of cyantraniliprole (Benevia®), acetamiprid (Epik®), (λ)-cyhalothrin (Karate Zeon ®), spinosad (Laser®) and phosmet (Spada®) were tested. For the field trials carried out in 2022-2023, commercial formulation of cyantraniliprole (Exirel®), deltamethrin (Meteor®) and spinosad (Laser®) were utilized. For field trials, the cyantraniliprole formulation, Exirel®, was changed compared to the laboratory experiments to follow national regulations that authorized Exirel® for both 2022 and 2023

season (Health Ministry, Registration n. 18383 of May 25, 2023). Although formulation was different, active ingredient (a.i.) and concentrations were the same as in the previously tested product (Benevia®). Phosmet has been banned in 2022 fruit growing season (European Union, 2022), thus no further tests were conducted in the field. Deltamethrin has been selected for the field trials due to its frequent use by local farmers as an affordable pyrethroid-based product (Del Fava et al., 2017). Label information of each pesticide's formulation are shown in Table 8.1 (IRAC, 2024).

Table 8.1: Label information on the pesticides tested in the laboratory toxicity bioassays and field trials. * Formulations: Liquid Solution (LS), Emulsifiable Concentrate (EC), Suspo-Emulsion (SE), Capsule Suspension (CS), Suspension Concentrate (SC). † Group code according to the Mode of Action (MoA) classification of the Insecticide Resistance Action Committee (IRAC) ‡ Laboratory bioassays (LAB) conducted in 2021-2022. § Field trials (FIE) conducted in 2022-2023

Active ingredient	**Trade name and formulation** *	**Chemical group** (Group code) †	**Maximum label dose** (mL hL^{-1} ; a.i. g L^{-1})	**Target crop**	**Target pest**	**LAB** ‡	**FIE** §
Acetamiprid	Epik® SL	Neonicotinoid (4A)	220 ; 50	Raspberry	*Drosophila. suzukii*	X	
Cyantraniliprole	Benevia® EC	Diamide (28)	75 ; 100	Strawberry	*Drosophila. suzukii*	X	
Cyantraniliprole	Exirel® SE	Diamide (28)	75 ; 100	Cherry	*Drosophila. suzukii*		X
λ-cyhalothrin	Karate Zeon® CS	Pyrethroid (3A)	20 ; 100	Stone fruit	*Drosophila. suzukii*	X	
Deltamethrin	Meteor® SC	Pyrethroid (3A)	80 ; 15.7	Cherry	*Drosophila. suzukii*		X
Phosmet	Spada 200® EC	Organophosphate (1B)	375 ; 200	Stone fruit	*Ceratitis capitata*	X	
Spinosad	Laser® SC	Spinosyn (5)	25 ; 480	Grapevine	*Drosophila. suzukii*	X	X

8.2.3 Baseline toxicity bioassays

Topical bioassays (direct spray) were conducted in the laboratories at FEM from December 2021 to May 2022. Insecticide exposure started immediately after the dilutions of the five commercial insecticides. For this, products were added to distilled water under a fume hood and stirred for 15 minutes to ensure homogenization. *Ganaspis brasiliensis* adults (5 ± 1 days-old) were placed in a vial

tube using a mouth aspirator and anaesthetized through cold exposure for 2 minutes. Using a soft brush, they were gently moved to sterile Petri dishes (Ø = 9 cm). Dishes were sprayed using 3 mL of insecticide solution at a constant pressure of 55 kPa using the Potter spray tower (Burkard Manufacturing Co. Ltd, Hertfordshire, England), allowing a standard deposit of 6-6.5 mg/cm2 of insecticide solution on the surface area. Control samples were sprayed with distilled water. Following spraying, the insects were immediately transferred to plastic Dutscher rearing tubes (Ø x h: 28.5 x 95 mm) containing a hydrated cellulose plug at the bottom soaked with 2 mL distilled water and honey applied to the inner face of the lid. Four concentrations per each insecticide were tested for cyantraniliprole (250, 350, 500, 1400 mL hL^{-1}), acetamiprid (22, 110, 220, 1100 mL hL-1), spinosad (0.25, 1, 1.75, 2.5 mL hL^{-1}), phosmet (10.78, 15, 15.85, 26.25 mL hL^{-1}) and λ-cyhalothrin (2.5, 4.43, 6.93, 25 mL hL^{-1}). Doses selection was based on a preliminary screening conducted at standard dilutions of the field rate (100%, 50%, 10%, 5%) followed by a preliminary probit analysis conducted with resulting data. Each concentration was replicated from 12 to 20 times (60 to 100 adults respectively). The tested adults were distributed in a 1:1 female to male ratio. Mortality of treated wasps was assessed at 48 hours after exposure. Moribund and dead wasps were combined and considered as dead. Moribund refers to parasitoids that were not able to hold on the bioassay vials, due to clear sign of toxicity such as leg twitching and partial paralysis (Blouquy et al., 2021; Stanley and Preetha, 2016).

8.2.4 Sublethal toxicity on longevity

Sublethal effects on longevity were tested on individuals survived to the exposure to the estimated lethal concentration 5% and 30 % (LC_5 and LC_{30}) resulting from the baseline toxicity assay. Spraying was conducted as described in the previous paragraph. Forty-eight hours after insecticide application, for every concentration tested (LC_5 and LC_{30}), 30 survived parasitoids per sex were

transferred individually into Dutscher rearing tubes (Ø x h: 28.5 x 95 mm). A cellulose plug moistened with 2 mL of distilled water was placed at the bottom of each tube. A second cellulose plug, coated with a drop of honey on the inner side, was used to seal the vial and provide a food source. Water was added weekly to ensure that the bottom plugs were maintaining an adequate moisture level. Parasitoid mortality was monitored every other day and day of death was recorded.

8.2.5 Toxicity of residual exposure in field trials

Field trials were conducted on grape in 2022 and on cherry in 2023. The tested insecticides were applied at maximum label dose (Table 8.1) using a motorized backpack sprayer (FOX MOTORI SRL, Poviglio, Italy) equipped with an air inclusion flat fan spray green nozzle AI110015VS (Teejet Technologies, Glendale Heights, U.S.A) with an application volume of 1000 L ha^{-1}. The first trial was performed in a pergola-trained vineyard (cv. Corvina) located in the San Pietro in Cariano municipality (Verona, Italy) (45°30'22.1"N 10°52'16.2"E) and insecticide application was carried out on September 12, 2022 (T_0). Treatment plots consisted of ten consecutive grapevines separated by untreated rows to prevent the effects of potential drift contamination. One hour after insecticide application, allowing time needed for vegetation to dry, parasitoids were placed in contact with the treated vegetation using a cylindrical net sleeve cage (diameter: 12 cm, length: 25 cm). Each cage, containing at least one treated leaf, housed ten 5 ± 2 days-old *G. brasiliensis* adults (5 females and 5 males). Four net sleeves were installed per each treatment plot and untreated control (4 replicates). Honey droplets and a water dispenser were inserted in the net sleeve to avoid insect death due to a lack of food/water resources. New clean net sleeves containing new insects were installed on plants 3, 7, 14, and 21 days after the application (September 15, 19, 26, and October 3, 2022). Seventy-two hours after each insect caging, caged vines were cut, brought to the laboratory, net sleeves removed, and the number of dead parasitoids was counted. Dataloggers (RC-51H, Elitech, London, U.K.) were

installed inside the net sleeve to measure temperature and relative humidity conditions experienced by the parasitoids during the trials.

The trial conducted on cherry trees took place in a Kym green bush-trained orchard (cv. Ferrovia) located in the Grezzana municipality (Verona, Italy) (45°33'06.1"N 11°02'46.7" E), with insecticide application occurring on June 27, 2023 (T_0). Treatment plots consisted of four consecutive cherry plants separated by an untreated row. In the net sleeves, in addition to leaves, five treated cherries were enclosed. Mortality estimation was carried out 3, 7, and 14 days (June 30, July 4 and 11, 2023) after application. The evaluation was not extended further as cherries were no longer naturally available on the plant. The insecticide application methodology was as described above for the vineyard trials.

8.2.6 Statistical analysis

The baseline toxicity of five tested insecticides on *G. brasiliensis* was assessed using a probit regression model through a logarithmic transformation of the data (Finney, 1971) using SPSS v12®(IBM) software.

Further analysis on the data from sublethal toxicity on longevity and residual exposure bioassays in field trials were conducted on software R (4.3.0) (R Core Team, 2020). Survival curves were generated through Kaplan–Meier model and Log-Rank pairwise comparisons were carried out using survival-package (Therneau, 2023; Therneau and Grambsch, 2000). To test the toxicity of residual exposure, generalized linear mixed models built with the “glmmTMB” package (Brooks et al., 2017) were used. The response variable, modelled with a binomial distribution, was represented by the ratio between death and total parasitoids. The categorical explanatory variables were treatment, time, and their interaction. Random effects were included to address potential correlations within experimental

plots. Models were validated by analyzing both observed and simulated residuals and conducting tests for autocorrelation. Post-hoc pairwise comparisons were conducted using the "emmeans" package (Lenth et al., 2020), applying Holm's adjustment for multiple comparisons. Field data were plotted using "ggplot2" package (Wickham, 2011).

8.3 Results

8.3.1 Baseline toxicity bioassays

The probit models were fitted to the observed data for all the treatments. No significant differences between observed and expected data were found ($P > 0.05$), thus the estimations of LC_{50}, LC_{30} and LC_5 were considered valid (Table 8.2). Comparing LC_{50} values, cyantraniliprole resulted the least toxic molecule, showing the highest concentration required to experimentally kill 50% of the treated wasps. On the contrary, the highest toxicity was recorded for spinosad followed by λ-cyhalothrin, phosmet and acetamiprid (Figure 8.1).

The ratio between lethal concentration and maximum field rate (FR) (LC/FR) shown in Table 8.2, confirmed the highest toxicity of spinosad, with a LC/FR ratio of 3.72×10^{-3} for LC_{50}. Cyantraniliprole was the least toxic molecule at field rate, with a LC/FR ratio of 10.53 for LC_{50}. LC/FR values at LC_{50} of the other products ranged from 4.94×10^{-3} for phosmet, 2.56×10^{-2} for λ-cyhalothrin and 7.65×10^{-2} for acetamiprid.

Table 8.2: Baseline toxicity of the five insecticides tested resulting from the Probit Analysis. * Maximum Field Rate according to insecticide label expressed in g a.i. L-1. † Slope and standard error of the concentration–mortality regression line. ‡ Number of tested insects excluding control. § Lethal concentrations and the 95% fiducial limits. ¶ Ratio between Lethal Concentration (LC) and Maximum Field Rate (FR).

Active ingredient	**Field Rate (**g a.i. L^{-1}) *	**Slope ± SE** †	χ^2 **(df)**	***N*** ‡	***P***	**LC (95% FL)** (g a.i. L^{-1}) §	**LC/FR** ¶
Acetamiprid	0.11	4.290 ± 0.490	3.953 (2)	240	0.139	LC_{50}= 8.42×10^{-3} (6.71×10^{-3} - 1.06×10^{-2})	7.65×10^{-2}

						LC_{30}= 4.7×10^{-3} (3.51×10^{-3} - 5.93×10^{-3})	4.27×10^{-2}
						LC_{5}= 1.35×10^{-3} (7.65×10^{-4} - 1.98×10^{-3})	1.22×10^{-2}
Cyantranilip role	0.075	0.224 ± 0,125	2.724 (2)	240	0.255	LC_{50}= 0.79 (0.651 – 1.03)	10.53
						LC_{30}= 0.45 (0.37 – 0.55)	6.07
						LC_{5}= 0.14 (0.08 – 0.20)	1.87
Phosmet	0.75	14.184 ± 1.735	5.897 (2)	298	0.052	LC_{50}= 3.70×10^{-3} (3.45×10^{-3} - 4.01×10^{-3})	4.94×10^{-3}
						LC_{30}= 3.01×10^{-3} (2.79×10^{-3} – $0.003.21\times10^{-3}$)	4.02×10^{-3}
						LC_{5}= 1.94×10^{-3} (1.63×10^{-3} - 2.17×10^{-3})	2.58×10^{-3}
λ-cyhalothrin	0.02	5.583 ± 0.657	0.083 (2)	240	0.959	LC_{50}= 5.13×10^{-4} (4.45×10^{-4} – 5.92×10^{-4})	2.56×10^{-2}
						LC_{30}= 3.12×10^{-4} (2.54×10^{-4} - 3.65×10^{-4})	1.56×10^{-2}
						LC_{5}= 1.08×10^{-4} (6.74×10^{-5} - 1.47×10^{-4})	5.40×10^{-3}
Spinosad	0.12	10.61 ± 1.164	1.107 (2)	298	0.575	LC_{50}= 4.46×10^{-4}(3.74×10^{-4} - 5.17×10^{-4})	3.72×10^{-3}
						LC_{30}= 3.04×10^{-4} (2.38×10^{-4} - 3.64×10^{-4})	2.53×10^{-3}
						LC_{5}= 1.35×10^{-4} (8.60×10^{-5} - 1.81×10^{-4})	1.13×10^{-3}

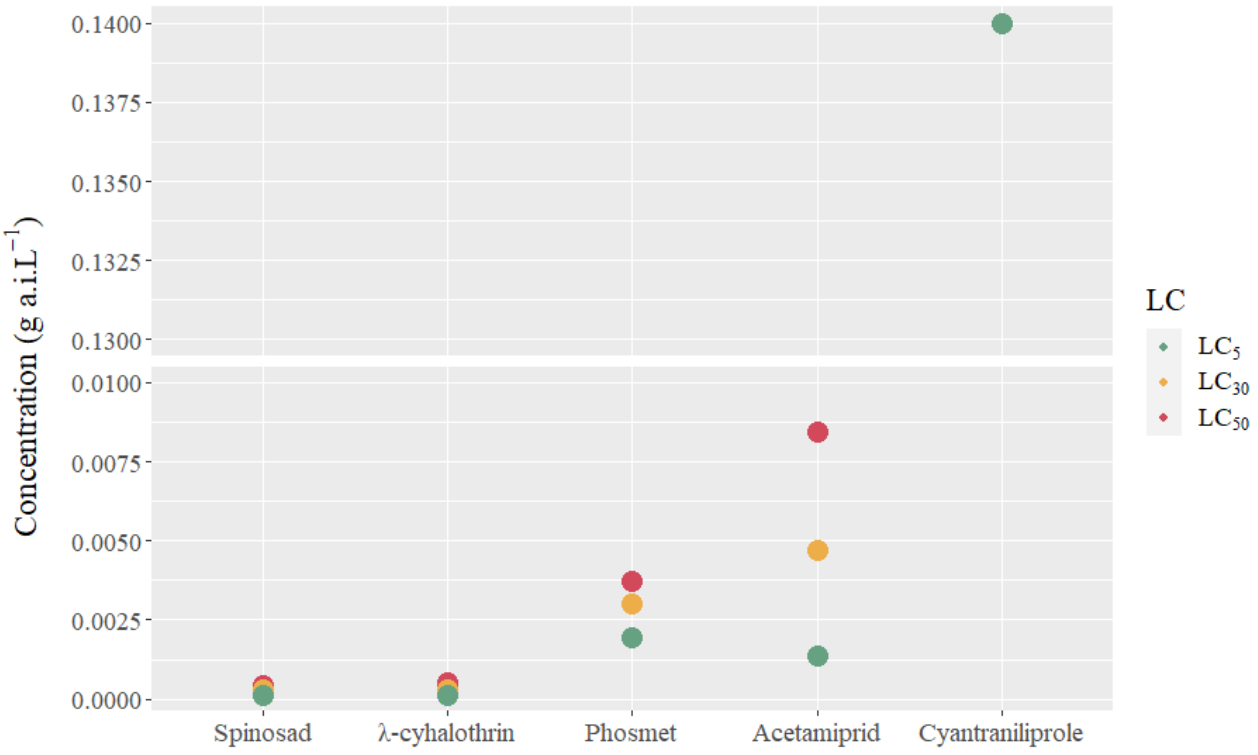

Figure 8.1: visual representation of lethal concentrations (g a.i. L^{-1}) of the five different products tested, ordered from the most toxic (left) to the least toxic (right) according to resulting LC_{50}. Cyantraniliprole LC_{50} and LC_{30} have been omitted to improve readability.

8.3.2 Sublethal toxicity on longevity

In the untreated control, wasps mean longevity ranged from 48 ± 1.7 to 83 ± 2.2 days for male (Figure 8.2) and female (Figure 8.3) respectively. Male insects treated with LC_5 of the different insecticides did not show sublethal effects on longevity except for cyantraniliprole, where longevity was reduced by 65%, to a mean lifespan of 17 ± 3.8 days ($P < 0.01$) (Figure 8.2). Female adults' longevity was not negatively affected by the application of LC_5 of any product ($P > 0.05$). LC_{30} of cyantraniliprole, spinosad and λ-cyhalothrin significantly reduced male longevity to 8 ± 1 ($P < 0.01$), 6 ± 3.3 ($P < 0.01$) and 33 ± 2.7 ($P < 0.01$) days respectively (Figure 8.2). This reduction corresponded to a life decrease of 84, 87 and 30%. LC_{30} of cyantraniliprole and λ-cyhalothrin also significantly

reduced female longevity to 45 ± 7.5 ($P < 0.01$) and 46 ± 7.5 ($P < 0.01$) days respectively (Figure 8.3). This reduction corresponded to a life decrease of 45 and 44%.

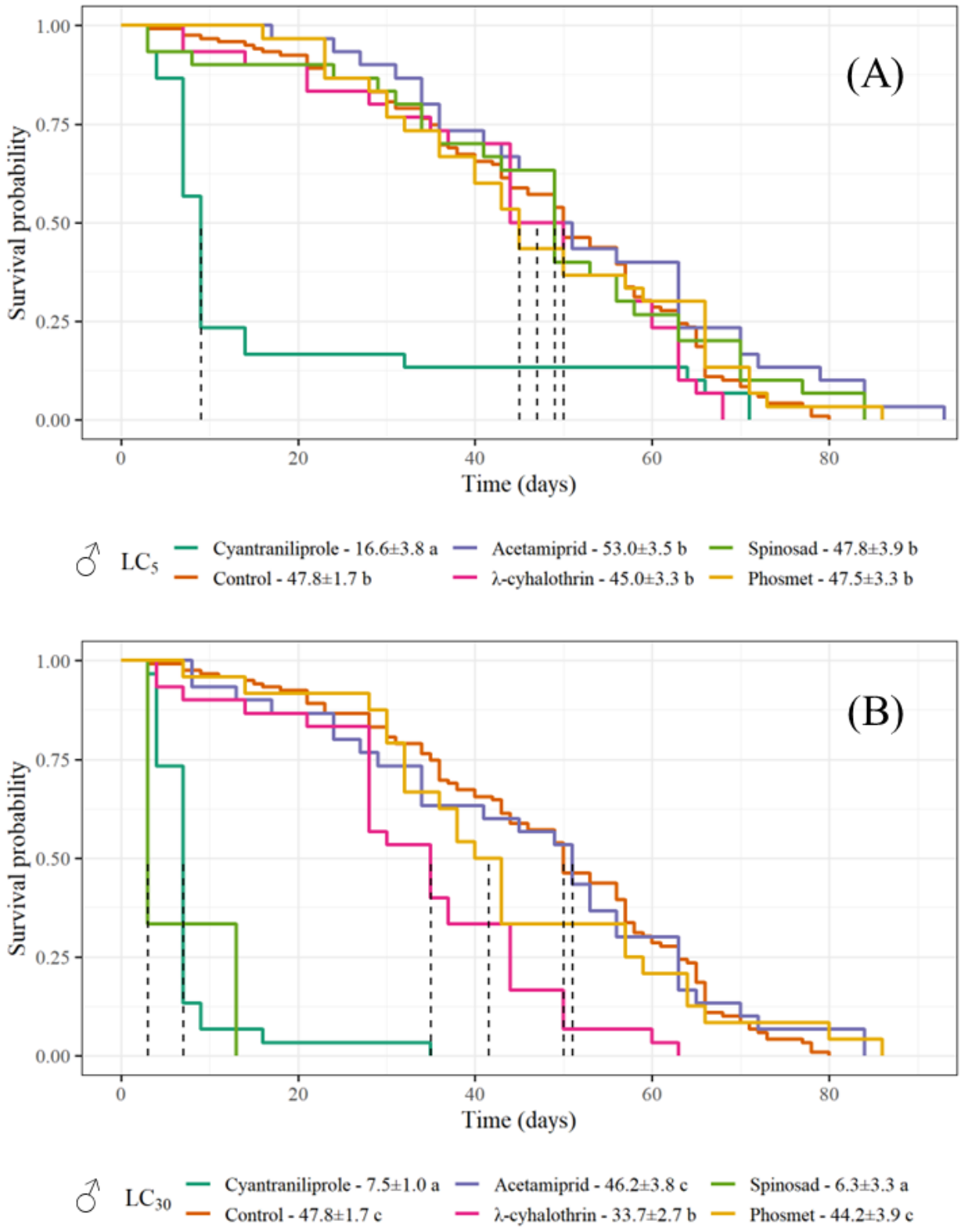

Figure 8.2: Survival curve of male adult *Ganaspis brasiliensis* exposed to LC_5 (**A**) and LC_{30} (**B**) of the five tested insecticides. The curves were generated through Kaplan–Meier estimators and compared in the Log-Rank test ($P < 0.05$). Different letters identify significant different curves. Numbers close to the product names are the mean average mortality expressed in days ± SEM. Vertical dotted lines show the median mortality.

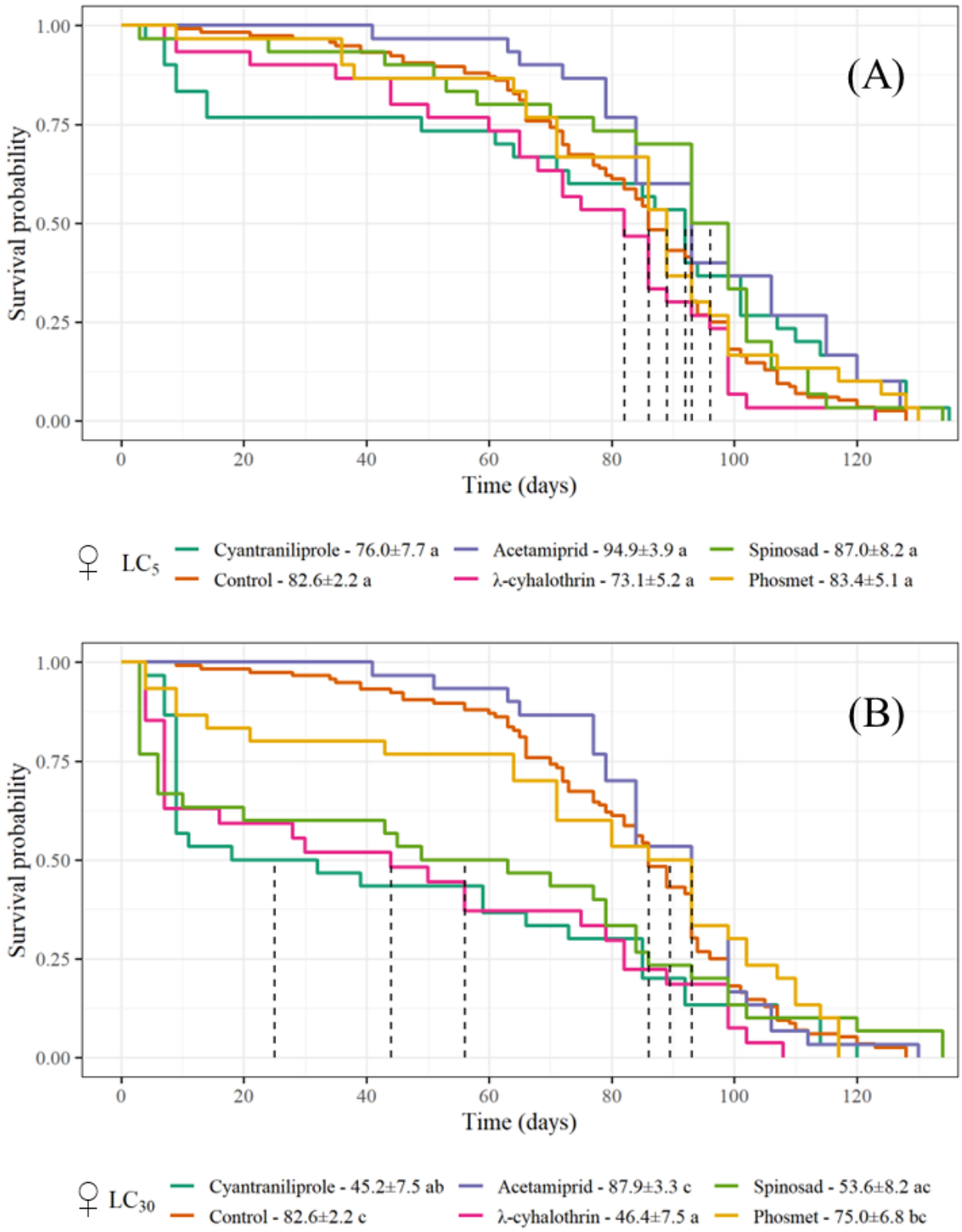

Figure 8.3: Survival curve of female adult *Ganaspis brasiliensis* exposed to LC_5 (**A**) and LC_{30} (**B**) of the five tested insecticides. The curves were generated through Kaplan–Meier estimators and compared in the Log-Rank test ($P < 0.05$). Different letters identify significant different curves. Numbers close to the product names are the mean average mortality expressed in days ± SEM. Vertical dotted lines show the median mortality.

8.3.3 Toxicity of residual exposure in field trials

During the entire field trial with grapevine, the control group showed a mortality from 2.5 ± 5% (SD) to 10 ± 0% throughout the different assessments (Figure 8.4). At T_0 and T_3, all treatments showed significant higher mortality compared to the control. At T_0 spinosad and deltamethrin were the most toxic active ingredients, causing 92.5 ± 5% and 92.5 ± 9.6% mortality respectively, while cyantraniliprole resulted in 52.5 ± 15% mortality. Three days after treatment, residues of spinosad exhibited higher mortality compared to cyantraniliprole (87.5 ± 9.6% vs. 37.5 ± 9.6%). One week after treatment, the residual effect of cyantraniliprole was not significantly different from the control, while spinosad and deltamethrin residues caused higher mortality of 40 ± 12% and 57.5 ± 9.6% respectively. Fourteen days after treatment, only deltamethrin residues showed higher mortality compared to control, resulting in 57.5 ± 9.6% mortality. Twenty-one days after treatment, none of the insecticides' residues had a significant effect on *G. brasiliensis* mortality.

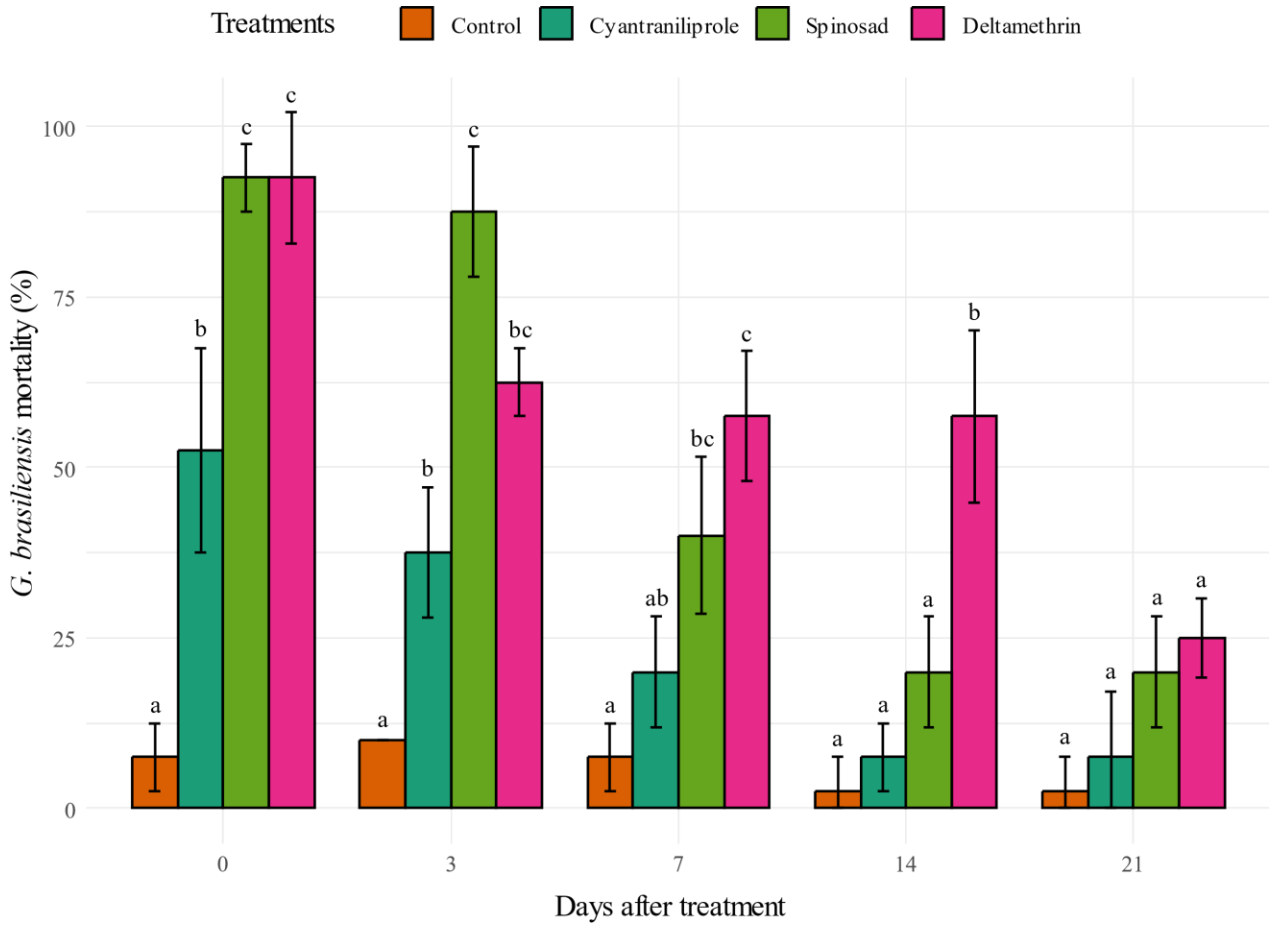

Figure 8.4: Residual effects of tested active ingredients on *Ganaspis brasiliensis* observed in the grapevine field trial 0, 3, 7, 14 and 21 days after insecticides application. Mortality (%) is reported as mean ± standard deviation represented by error bars. Treatments labelled with different letters are statistically different at $P < 0.05$.

During the field trial with cherry, the control group showed a mortality from 10 ± 8.2% to 20 ± 8.2% across the different assessments (Figure 8.5). At T_0, spinosad and deltamethrin residues showed the highest toxicity (92.5 ± 9.6%), while cyantraniliprole resulted in 47.5 ± 17.1% parasitoid mortality. Three days after treatment, the residual effects of all insecticides - spinosad, cyantraniliprole and deltamethrin - were statistically comparable, resulting in 40 ± 8.2%, 42.5 ± 9.6% and 62.5 ± 5% *G. brasiliensis* mortality. One week after treatment, cyantraniliprole was the only a.i. not significantly impacting parasitoid survival, whereas spinosad and deltamethrin resulted in higher mortality rates of

40 ± 14.1% and 47.5 ± 5%, respectively. At fourteen days after application only deltamethrin residues had a significant negative effect on parasitoids, causing a 52.5± 20.6% of mortality.

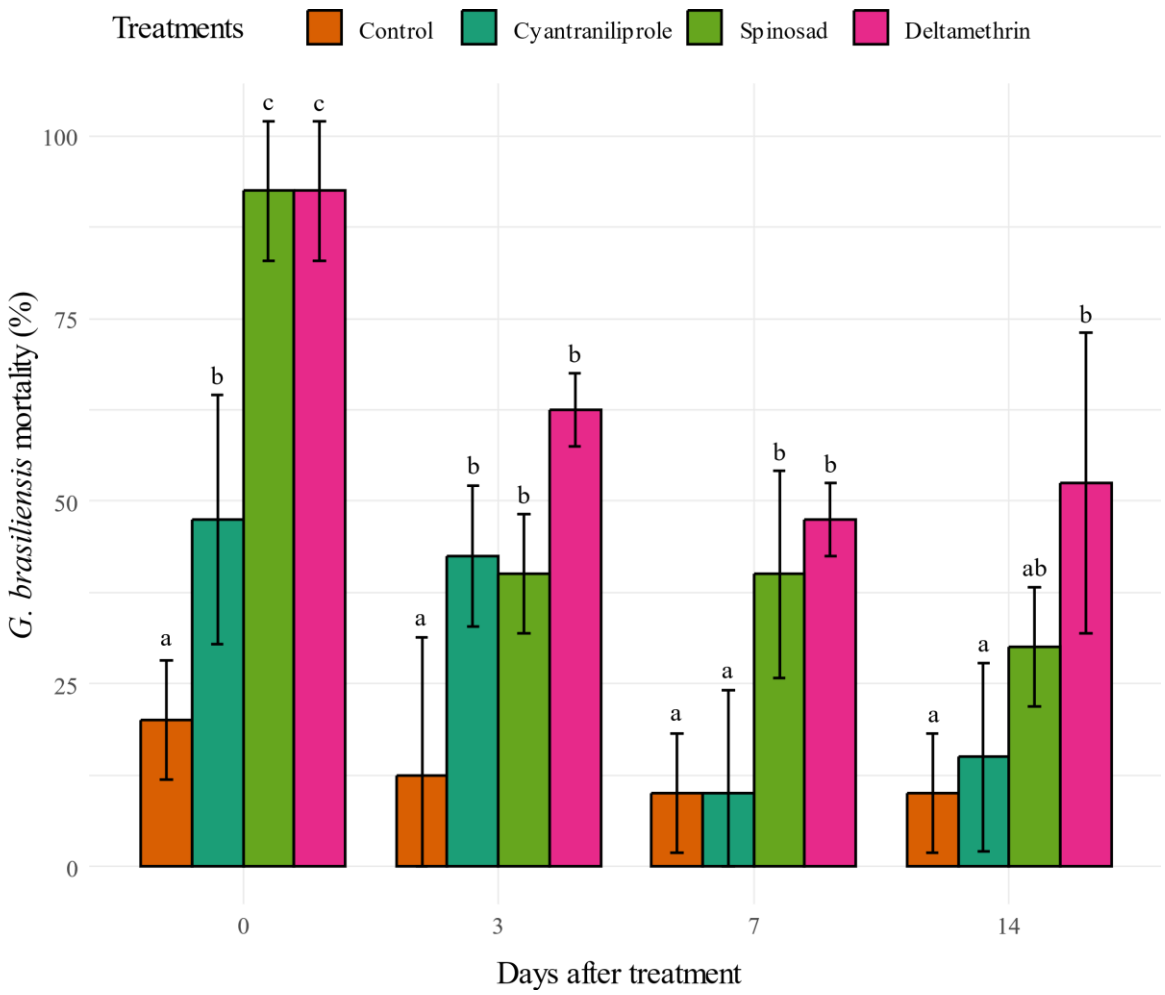

Figure 8.5: Residual effects of tested active ingredient on *Ganaspis brasiliensis* observed in the cherry field trial 0, 3, 7 and 14 days after insecticides application. Mortality (%) is reported as mean ± standard deviation represented by error bars. Treatments labelled with different letters are statistically different at $P < 0.05$.

8.4 Discussion

Ganaspis brasiliensis has been firstly released as BCA in Europe in 2021 (Fellin et al., 2023; Lisi et al., 2022), but currently there are no studies assessing its susceptibility toward pesticides

exploited to suppress its host, *D. suzukii*. The insecticide screening performed in this study allowed us to identify the most appropriate products to be used in a CBC programme perspective, reducing adverse effects on the beneficial non-target *G. brasiliensis*.

The insecticide evaluation revealed a high toxicity towards spinosad in both topical and residual exposures. This is in accordance with previous studies that classified the Hymenopteran as the most susceptible order to this molecule (Biondi et al., 2012). The aphid parasitoid *Aphidius colemani* (Viereck) (Hymenoptera: Braconidae) for instance was nearly 20 times more susceptible to the bio-insecticide spinosad than the conventional insecticides, imidacloprid and λ-cyhalothrin (D'Ávila et al., 2018). The pupal parasitoid *P. vindemiae*, was found to be highly susceptible to spinosad when it came into direct contact with low concentration of the product (10 mg a.i/L) (Cossentine and Ayyanath, 2017). Topical bioassays conducted with four insecticides revealed that spinetoram was the most harmful to a closer relative of our target species, the figitid wasp *Ganaspidium nigrimanus* (Kieffer) (Hymenoptera: Figitidae), larval-pupal parasitoid of the leaf miner fly *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae) (Hernández et al., 2011). Our topical experiments revealed higher susceptibility of male wasps towards sublethal concentrations (LC_{30}) of spinosad. Moreover, both field trials showed a significant higher mortality of male wasps compared to females (Data not shown). Higher male susceptibility to insecticide is known in *D. suzukii* (Blouquy et al., 2021; Van Timmeren et al., 2019), and previously reported for *G. nigrimanus* (Hernández et al., 2011). The cause of this difference has been explored in other studies, where it has been attributed to sexual dimorphism (Pluthero and Threlkeld, 1981; Roy and Prasad, 2018) or activity of detoxification enzymes (Navarro-Roldán et al., 2017).

In our residual assay conducted in the field, spinosad showed residual activity up to 7 days after application in both grapevine and cherries. This time range is consistent with previous literature,

although spynosyn's persistence varies greatly across different matrixes, with half-life ranging from 1.2 days on Chilli to over 16 days on Kiwi fruit (Mandal et al., 2013). Sunlight strongly contributes to spinosad dissipation, potentially explaining the gradual decrease in mortality recorded in the field (Adak and Mukherjee, 2016).

Spinosad has been proven effective to suppress *D. suzukii*, with LC_{90} at 48 hours at 60.08% ($7.21\times10\text{-}2$ g a.i. L^{-1}) of the recommended field rate (Shaw et al., 2019a). Our results suggest that, even at this lower concentration, spinosad would be extremely toxic to *G. brasiliensis* adults. Our findings show that spinosad has a high knockdown effect in both topical and residual assays, but also affects longevity when sprayed at lower concentration, specifically in males. This underscores the incompatibility of this active ingredient to biological control programmes involving the release of *G. brasiliensis* as a BCA.

The pyrethroids, λ-cyhalothrin and deltamethrin, were also highly toxic to *G. brasiliensis*. λ-cyhalothrin and spinosad showed similar lethal concentrations, indicating equal toxicity of their active ingredients. However, because of the recommended field rate, the LC/FR ratio for λ-cyhalothrin was higher than for spinosad, making its field use theoretically less toxic. The high knockdown effect caused by λ-cyhalothrin in the topical assays is likely due to the mode of action of this molecule, which quickly permeates the epidermis to reach insect nervous system (Bhardwaj et al., 2020; Gajendiran and Abraham, 2018). In the field trials, deltamethrin had the most persistent effect on insect mortality, showing toxicity up to 14 days after insecticide application. This is consistent with previous studies showing persistence of deltamethrin residues on tea leaves up to 14 days (Paramasivam and Chandrasekaran, 2014). The reduction in toxicity observed in our study from day 3 onward is in accordance with the product half-life of 3.04 days (Paramasivam and Chandrasekaran, 2014), but the high mortality rates 7 and 14 after treatment suggests that residues can also be highly

toxic. The residual impact of this insecticide group is also evident in the reduced longevity observed in both male and female wasps treated with LC_{30} of λ-cyhalothrin.

Similar to spinosad, pyrethroids demonstrated high toxicity in both topical and residual assays, suggesting that their field application could be detrimental to CBC programmes. Compared to spinosad, λ-cyhalothrin is more toxic towards *D. suzukii*, with LC_{90} at 48 hours estimated at 24.66% (4.93×10-3 g a.i. L^{-1}) of the field rate (Shaw et al., 2019a). According to our results, even at this lower concentration, *G. brasiliensis* would be strongly affected.

Cyantraniliprole was the least toxic active ingredient, in both topical and residual assays. This outcome is supported by the fact that often broad-spectrum insecticides affecting the nervous and respiratory systems are more toxic towards parasitoids compared to selective ones compromising insect growth (Teder and Knapp, 2019). Cyantraniliprole is a second-generation ryanodine receptor modulator that possess the capacity to bind and stimulate receptors within the insects' muscle cells, causing contraction, paralysis, and ultimately death (Lahm et al., 2009; Selby et al., 2013). This mode of action leads to a slower death, as it gradually impedes the insect's ability to move and feed. For instance, residual exposure of cyantraniliprole at field rate was found scarcely toxic to the adults of *Cydia pomonella* L. (Lepidoptera: Tortricidae), but negatively affected insect movement and mating (Knight and Flexner, 2007). Similarly, it disrupts feeding in white fly adults, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Cameron et al., 2014). In the baseline bioassay, cyantraniliprole showed low toxicity but caused high reduction of longevity in the sublethal assessments. This can be attributed to the slow knock-down effect of cyantraniliprole on the tested parasitoids and the 48-hour mortality assessment period selected for the bioassays. The high mortality recorded at LC_{30} starting a week after application suggests that mortality assessment for this active ingredient should be conducted later, as done in previous studies on stored product pests, where

mortality was checked at 7 and 28 days after pesticide application (Mantzoukas et al., 2022). A later assessment would have probably revealed higher baseline toxicity and lower LC values, resulting in increased adult longevity in the sublethal effect trial.

Cyantraniliprole formulation tested in the field trials has been proven effective in suppressing *D. suzukii* with a 90% mortality at 20.56% (equivalent to: 0.015 g a.i. L^{-1}) of the field rate (Shaw et al., 2019a). Considering our findings and the mortality recorded in the bioassays and field trials, cyantraniliprole can be considered the most selective insecticide tested. In fact, field rates that would cause > 90% mortality in *D. suzukii* only resulted in 52.5 and 47.5% *G. brasiliensis* mortality in vineyard and cherry orchard respectively.

Phosmet active ingredient was the third most toxic. But considering the high concentration applied at maximum field rate, it would have been the second most toxic product based on LC/FR ratio. Given the documented health hazard posed by phosmet to human and other organisms (Anastassiadou et al., 2021), and its withdrawal from use in Europe (European Union, 2022), the product has not been further evaluated.

One of the major concerns undermining control management efforts is the development of insecticide resistance (Van Timmeren et al., 2019; Whalon et al., 2008). Studies have shown that some *D. suzukii* population in America have developed resistance to spinosad (Disi and Sial, 2021; Ganjisaffar et al., 2022b; Gress and Zalom, 2019b) and pyrethroids (Ganjisaffar et al., 2022a). Selection bioassays conducted on Italian populations revealed that after eight generations, *D. suzukii* was less susceptible to deltamethrin and cyantraniliprole (LC_{50} values increased 25.0 and 2.2-fold respectively) (Civolani et al., 2021). For all the active ingredients tested in our field trials, resistance has been recorded, indicating a future need to increase insecticide concentrations to maintain effectiveness and the necessity to utilize multiple insecticides with different mode of actions (Bielza,

2016; Whalon et al., 2008). On the other hand, very few cases of pesticide resistance have been documented for parasitoids compared to pests (Bielza, 2016). This is likely due to the fewer generations per year they produce. In fact, in our temperate climate we estimate the occurrence of five generations of *G. brasiliensis* compared to the seven to fifteen estimated for *D. suzukii* (Cini et al., 2012). This might result in slower accumulation of genetic diversity within the population, including potential resistance-conferring mutations. If this is the case, the resistance gap to insecticides between *G. brasiliensis* and *D. suzukii* will likely increase. However, more studies are required to test this hypothesis, as the influence of generation time on resistance evolution cannot be generalized (Rosenheimi and Tabashnik, 1990).

The timing of pesticide application is crucial for effectively targeting pests while minimizing adverse effects on non-target species. This is challenging, as the close evolutionary bond between parasitoids and their hosts often results in their overlapping presence in the field (Stanley and Preetha, 2016) *Drosophila suzukii* can develop at lower temperatures, with a thermal threshold of 8.1°C (Ryan et al., 2016), while *G. brasiliensis* (G3 strain from South Korea) enters diapause at temperatures below 17.2°C (Hougardy et al., 2019). Although there is limited literature on the overwintering performance and diapause exit of *G. brasiliensis*, we can infer that in spring, there will be a period when *D. suzukii* is actively developing and colonizing new habitats while *G. brasiliensis* remains dormant. In temperate climates *D. suzukii* overwintering females with mature eggs can be collected as early as late February (Grassi et al., 2018). They move from forest habitat to cultivated orchards after the cold season (April-June) (Santoiemma et al., 2019), and *G. brasiliensis* might take longer to follow its dispersal due to dormancy and the longer development time (476.2 vs. 222.2 degree days) (Hougardy et al., 2019). Early season application of adulticides is estimated to be more effective in reducing *D. suzukii* population growth (Wiman et al., 2016) and might be the best option to minimize adverse

effects on *G. brasiliensis*. However, it is important to note that most commercial crops fruit in summer when both species are likely to coexist in the same agroecosystem. Therefore, choosing the most selective pesticides is essential. In this scenario, habitat management could be an effective strategy to prevent the depletion of *G. brasiliensis* population following pesticide application. Thanks to its ability to develop into numerous wild species (Kenis et al., 2016), *D. suzukii* heavily relies on wild habitat, such as surrounding vegetation (Klick et al., 2016) and forests (Santoiemma et al., 2019). Although marginal landscape complexity can be seen as counter-productive (Santoiemma et al., 2018), it also provides refugee for beneficials. A feasible option to support the *G. brasiliensis* population while avoiding an increase in pest damage is the adoption of augmentoria (M. V Rossi-Stacconi et al., 2019) in untreated portions of the field or along natural hedgerows. This technique allows for maintaining a safe reservoir of host juveniles to support parasitoid population year-round, and has already been proven effective to increase control pressure on *D. suzukii* (Rossi-Stacconi et al., 2018).

Both field trials yielded consistent results, although in the cherry trial, mortality was assessed only up to fourteen days after the treatment due to a shortage of cherries on the plants. Overall, higher temperatures were recorded during the cherry trials, although fluctuations occurred throughout the experimental period. The average mean temperature in cherry trial was 22.7 ± 2.7 °C, whereas in the grapevine trial it was 18.1 ± 2.9 °C (Supplementary Figure S7.1).

This study offers valuable guidance for integrating BCA within an IPM framework by presenting the results of topical and residual toxicity bioassays and field trials. While we assessed sublethal effects on adult longevity, further research should evaluate other physiological and behavioural responses to fully understand the toxicity of active ingredients toward *G. brasiliensis*. These might affect also immunology, fecundity, sex ratio, mobility, feeding, and oviposition (Desneux

et al., 2007). We performed an additional trial to test whether residual insecticide application affected *G. brasiliensis* offspring production in sweet cherry orchard. Results (available in the Supporting Material) show a reduction of offspring for all tested products compared to control (Supplementary Figure S7.2), in accordance with the bioassays. These preliminary results can be a starting point to further investigate if the reduction we observed is due to higher adult mortality, or if chemical application altered parasitization success or insect fecundity, as previously reported for *D. suzukii* pupal parasitoids (Lisi et al., 2023; Schlesener et al., 2019).

8.5 Conclusions

To author knowledge this is the first ecotoxicology screening performed on the non-target *G. brasiliensis*, and can provide basis for insecticide selection in an area subjected to a classical biological control programme. According to our results, spinosad is the most toxic among the product tested, with a high knock-out effect in both laboratory and field trials. λ-cyhalothrin was also highly toxic and its application at LC_{30} significantly reduced both male and female longevity. In field trial, deltamethrin showed the most prolonged residual effect, causing higher mortality up to 14 days after treatment. Cyantraniliprole was the least toxic active ingredient in both topical and residual bioassays, suggesting its potential as the most selective option among the tested insecticides. Given that the parasitoid complex of this pest is rapidly moving towards invaded territories (Beers et al., 2022; Nair and Peterson, 2023; Puppato et al., 2020), it is important for future studies to evaluate insecticide susceptibility of other major *D. suzukii* parasitoids, such as *Leptopilina japonica* (Novković and Kimura) (Hymenoptera: Figitidae).

8.6 Supplementary information

8.6.1 Climatic data

Climatic data regarding the two field trials are shown in the graphs below (Figure S8.1).

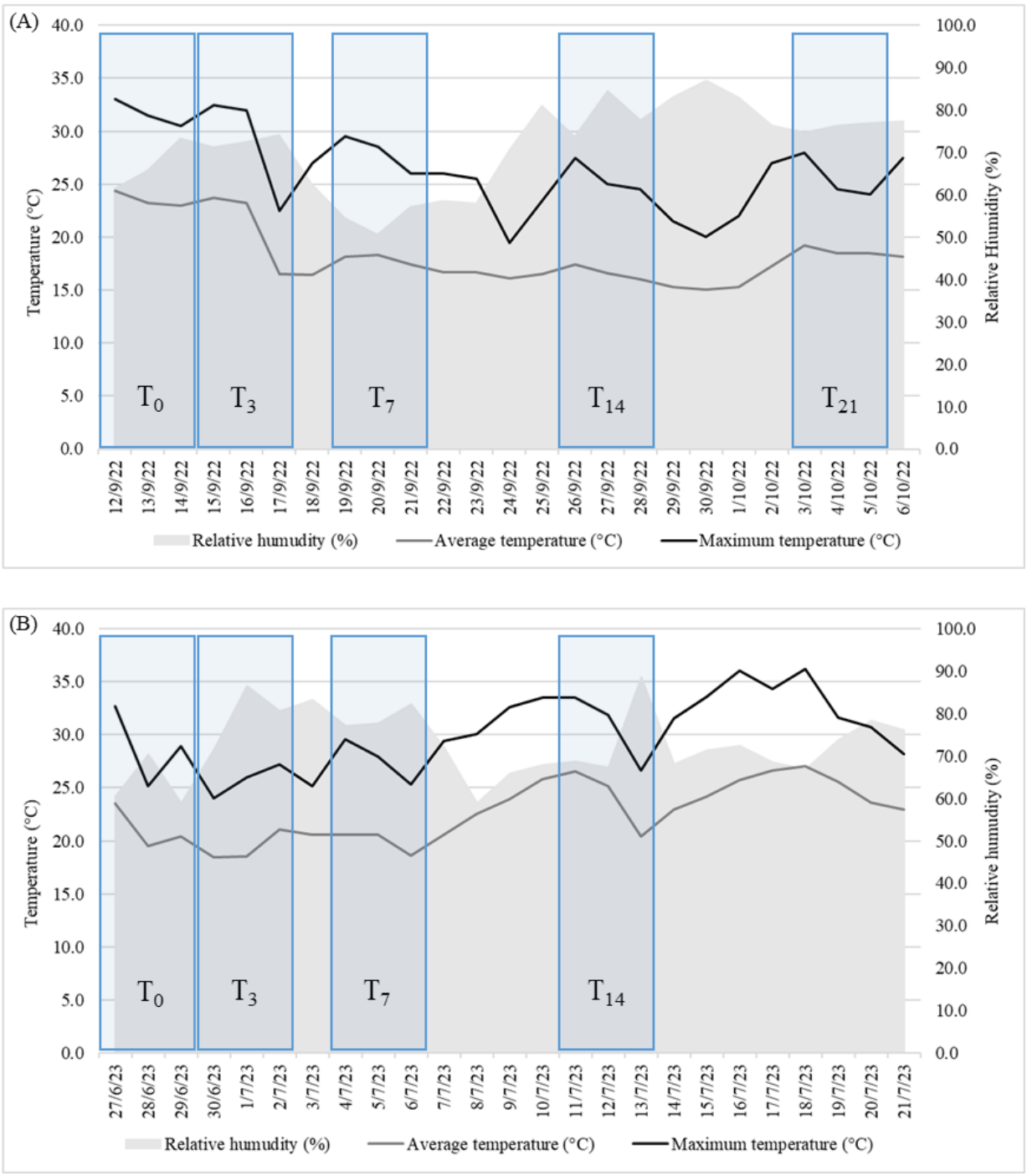

Figure S8.1: Climatic data recorded during the field trials on grapevine in 2022 (A), and in sweet cherry orchard in 2023 (B). The left y-axis represents the temperature (°C), while the right y-axis the relative humidity (%). Blue boxes highlight the 72-hour periods during which parasitoids were caged in the treated vegetation.

8.6.2 Residual exposure effects on offspring abundance

During sweet cherry field trials, we also performed a preliminary test to assess the effect of insecticides' residual exposure on *G. brasiliensis* offspring abundance.

8.6.2.1 Material and method

The ability of *G. brasiliensis* to parasitize and develop new offspring was tested counting the number of emerged adult parasitoids from treated and infested cherries. The day before parasitoids' caging at T3, T7 and T14, cherries were infested by 10 *D. suzukii* mated females of 6±2 days old. During each mortality sampling, cherries contained in the sleeves were removed and kept inside ventilated boxes and then stored in a climatic chamber at 22±2 °C and 70±10% relative humidity. The offspring's abundance was assessed counting the number of emerged parasitoids.

8.6.2.2 Statistical analysis

To test offspring abundance, generalized linear mixed models built with the "glmmTMB" package were used. The response variable, assumed to follow a Poisson distribution, was the number of emerged *G. brasiliensis*. The categorical explanatory variables were treatment, time, and their interaction. Random effects were included to address potential correlations within experimental plots. Models were validated by analyzing both observed and simulated residuals and conducting tests for autocorrelation. Post-hoc pairwise comparisons were conducted using the "emmeans" package, applying Holm's adjustment for multiple comparisons. Field data were plotted using "ggplot2" package.

8.6.2.3 Results

The analysis revealed that the treatment - time interaction was not significant (*P*: 0.9801, $\chi 2$(df): 1.13 (6)) so it was removed from the original model. Compared to control, all tested active ingredients had a significant negative impact on offspring abundance of *G. brasiliensis* as shown in Figure S8.2. From cherries in the control plots 3.6 ± 1.8 specimens of *G. brasiliensis* emerged, while cherries treated with cyantraniliprole, spinosad and deltamethrin, yielded respectively 1.3 ± 1, 0.4 ± 0.8 and 0.4 ± 0.7 parasitoids, resulting in a reduction of 63%, 88% and 88%.

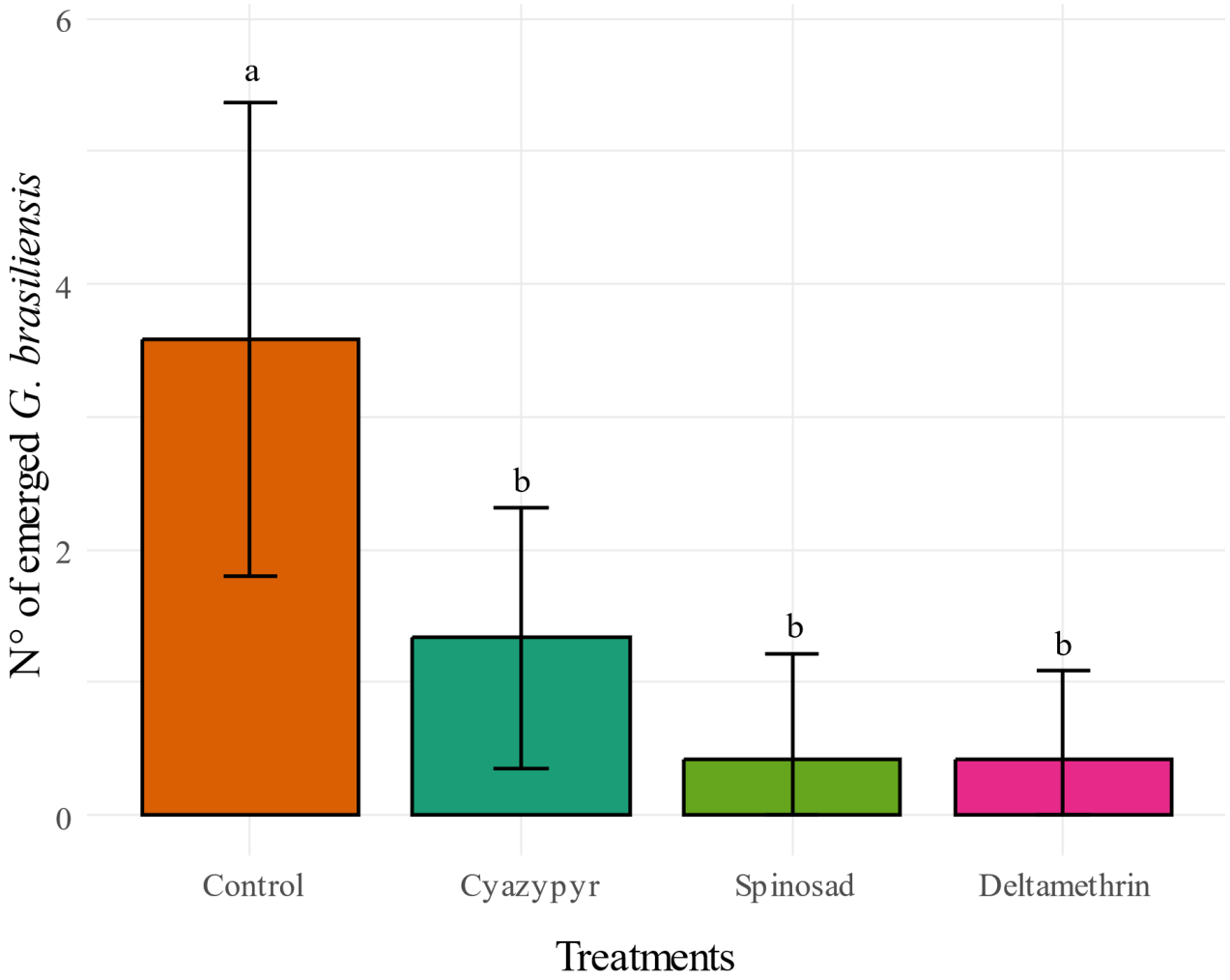

Figure S8.2: Offspring abundance of *Ganaspis brasiliensis* originating from treated cherries sampled during field trial, expressed as mean ± standard deviation represented by error bars. Treatments labelled with different letters are considered statistically distinct ($P < 0.05$).

8.7 References

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CHAPTER 9. GENERAL DISCUSSION

The selection of a BCA is a long and complex process, with risk assessment serving as a crucial step in classical biological control programs. While preliminary evaluations mainly focus on host specificity, other aspects of a BCA's biology, ecology and behavior often remain unknown until after its release. This thesis aimed to address these gaps by conducting post-release evaluations to assess the efficacy of *G. kimorum* as a BCA in its newly introduced environment in Northern Italy. The findings contribute to refining future risk assessments and enhancing ongoing classical biological control programs.

Ganaspis kimorum as biological control agent: effective, yet not without challenges.

The results of the first report (Chapter 2) are encouraging. Despite the limited number of released adults, *G. kimorum* successfully established in at least half of the release sites. Additionally, its high specificity toward *D. suzukii* was confirmed. Similar findings have been reported in North America, where adventive populations of *G. kimorum* are establishing exclusively on *D. suzukii* (Beers et al., 2022; Gariepy et al., 2024; Stahl et al., 2024). While its potential for establishment and host specificity has been confirmed, the extent of its control over *D. suzukii* depends on how much its population can grow to exert effective regulation. In our studies, we assessed various stressors that may limit *G. kimorum* population growth, including abiotic factors (climatic conditions and insecticide exposure), external biotic factors (competition), and internal biotic factors (host patch location and search mechanisms).

Many laboratory studies rely on parasitism rate as a key measure of parasitoid efficacy (Girod et al., 2018; Rossi-Stacconi et al., 2019; Seehausen et al., 2020; Wang et al., 2018). While this

approach is valuable, it does not account for additional challenges that parasitoids face in natural conditions, particularly competition at multiple levels (Harvey et al., 2013). Our field experiments (Chapter 3) indicate that interspecific competition led to a 62% reduction of *G. kimorum* potential emergence, likely due to both secondary infestation by *Drosophila* spp. and multiparasitism by *L. japonica*. Compared to *G. kimorum*, *L. japonica* appears to be a more efficient biological control agent due to its broader host range and competitive advantages in parasitization and development. Unlike *G. kimorum*, *L. japonica* can successfully parasitize a multiple range of *Drosophila* species (Chapter 2) and exploit hosts that have already been parasitized by other parasitoids, including *Ganaspis* spp. (Wang et al., 2019). Despite the fact that these ecologically risky characteristics have prevented *L. japonica* from being selected as a BCA for classical biological control, its lack of host discrimination allows it to exploit a larger pool of available hosts, a significant advantage in our study system, where its populations are already well established and more abundant. Additionally, its faster developmental rate enables *L. japonica* to outcompete *G. kimorum*, even when the latter oviposits earlier (Chapter 3). Given these dynamics, *G. kimorum* releases should be prioritized in areas where *L. japonica* is not yet dominant or, ideally, absent. This strategy could facilitate *G. kimorum*'s establishment and enhance the potential additive impact of both parasitoid species on *D. suzukii* suppression. In fact, specialists such as *G. kimorum* are more likely to establish and coexist if they enter the host life cycle before generalists (Hassell and May, 1986).

Climatic conditions such as temperature, humidity and seasonal variability, play a crucial role in the establishment and effectiveness of biological control agents, influencing their survival and reproduction (Colinet et al., 2015; Colinet and Boivin, 2011; Hance et al., 2007). CLIMEX models used to predict the potential geographical range of *G. kimorum* prior to its redescription (Wang et al., 2020) indicated that the southernmost European countries were suitable locations. This information

was integrated into risk assessments to support BCA releases. However, these models operate at a large spatial scale, whereas a more detailed spatiotemporal resolution is necessary to assess how climatic factors influence insect distribution and abundance in specific locations (Rebaudo et al., 2016). Our findings on *G. kimorum* overwintering have important practical implications. Classical biological control programs aim to maximize establishment success while minimizing costs, which can only be achieved through appropriate site selection. In our assessment, successful overwintering occurred between 216 and 660 m asl, yet emergence did not occur at all sites within this range. According to our experiments, sites with milder temperatures and fewer extreme climatic events (days with temperatures below 0°C or above 30°C) had a higher chance of successful emergence in spring. Specifically, sites where minimum temperatures remained above -6°C and where the number of cumulative days with an average temperature below 0°C was fewer than five exhibited the highest survival rates. However, even in this predator-free environment, survival rates remained low, ranging from 0.4% to 17.6%.

In light of our findings, it remains uncertain whether releasing 300 females per site per season is sufficient to ensure successful and stable establishment. Successful establishment occurs when summer-released parasitoids produce a sufficiently large overwintering population capable of sustaining itself in the following spring. In our studies we quantified two major stressors that significantly reduce field survival: a 62% competition rate occurring at larval stage (Chapter 3) and an overwintering survival rate ranging from 0.4 to 17.6% in favorable sites (Chapter 4), alongside a host searching activation rate of approximately 93% (Chapter 5). By integrating these findings with existing data on competition at the pupal stage (Ballman et al., 2017; Woltz et al., 2015) and survival/fertility estimates from laboratory studies (Wang et al., 2018) or field-oriented experiments (Seehausen et al., 2022), it would be possible to develop predictive models of *G. kimorum* population

dynamics. Such models could provide valuable insights to policymakers, helping to refine release strategies and improve the long-term success of *G. kimorum* as a biological control agent.

Another crucial aspect emerging from these findings is the importance of optimal release timing for BCA success. To establish a population capable of withstanding competition and surviving through winter, *G. kimorum* (like any other organism) requires sufficient time to reproduce and build up numbers. If releases occur too late in the summer (e.g., August), the parasitoid is unlikely to produce enough overwintering offspring to sustain the population in the following season. This could jeopardize the effectiveness of the classical biological control program or limit its geographical expansion.

Behavioral studies are crucial for explaining field observations. Our research on *G. kimorum* revealed that host patches are olfactory attractive for a relatively short time window (Chapter 5) and that vibrational cues likely play a role in host location (Chapter 7). These factors help explain its high specificity. While host specificity is a key criterion in BCA selection, it also comes with trade-offs. Generalist parasitoids can exploit a broader range of hosts, leading to more stable and potentially larger populations compared to specialists. This appears to be the case with *L. japonica*, which, despite its preference for *D. suzukii*, can parasitize other drosophilids as well (Chapter 2), giving it a competitive advantage and allowing it to thrive in diverse environments. The results of this thesis therefore suggest carefully considering these seemingly controversial aspects when selecting the most suitable BCA for classical biological control. While increased interspecific competition at the larval stage (Chapter 3) may slow *G. kimorum*'s establishment, coexistence between specialists and generalists remains possible (Hassell and May, 1986) and indeed it is observed in both native and invaded environments (Beers et al., 2022; Girod et al., 2018a). However, comparative studies (where both species are assessed simultaneously) could provide deeper insights into their host-searching

behaviors and how these influence their efficacy as BCAs. *Ganaspis* spp. rely on vibrotaxis, resulting in longer stationary periods compared to *Leptopilina* spp., which continue probing the substrate while walking (Vet and Bakker, 1985). While this behavioral difference may not significantly impact parasitism rates under laboratory conditions (Wang et al., 2018), it could lead to differences in host detection efficiency in the field. The morphological differences observed in ovipositor length (Chapter 2) may also be related to host searching behavior. The shorter ovipositor of *G. kimorum* might have evolved to discriminate larvae closer to the surface, as they are initially located through vibrational cues. In contrast, the longer ovipositor of *L. japonica* may result from its reliance on substrate probing for host location, which could also explain its ability to parasitize *D. suzukii* even on artificial diets (Girod et al., 2018).

Integrated pest management is regarded as the best approach to control *D. suzukii* (Mori et al., 2019; Tait et al., 2021), but its implementation can be challenging due to incompatibilities between different control methods. In particular, integrating biological and chemical control is often difficult, due to pesticide's effects on non-target beneficials (Biondi et al., 2012; Teder and Knapp, 2019). The final section of this thesis (Chapter 8) addresses this challenge and provides useful recommendations for using specific agrochemicals that are less harmful to *G. kimorum*. For example, spinosyns are highly toxic and, when possible, should be replaced with more target-specific products such as cyantraniliprole. The timing of pesticide application is also critical. By combining our ecotoxicology (Chapter 8) and overwintering (Chapter 4) findings, we can now offer better guidance on when to apply chemicals. The thermal threshold we developed allows for the estimation of when *G. kimorum* will emerge from diapause, helping to regulate pesticide application accordingly. Depending on local climatic conditions, the parasitoid will exit diapause between mid-April and June. This period is crucial for controlling *D. suzukii*, as it may coincide with the pest's colonization of cultivated fields

(Santoiemma et al., 2019). Thus, in early season pesticide application is crucial to limit toxicity towards the non-target *G. kimorum*, while being effective in controlling *D. suzukii*, especially at field margins (Klick et al., 2016; Wiman et al., 2016).

Last but not least, this comprehensive set of studies can provide valuable guidance for refining the drafting of preliminary risk assessments. While current risk assessments primarily focus on host specificity tests (Barratt et al., 2023; Heimpel and Mills, 2017), integrating ecological and environmental factors could offer crucial insights. A part from supporting the selection of the best BCA, these assessments could help determine the optimal release time and the number of insects to release (taking into account the survival reduction due to abiotic and biotic factors), as well as identify the most favourable sites for release (based on overwintering survival). Although these preliminary evaluations may be time consuming and costly, they would help concentrate future resources in areas with higher establishment potential, ultimately increasing the success of classical biological control programs.

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CHAPTER 10. SATELLITE PROJECT #1

Methods for rearing the parasitoid *Ganaspis brasiliensis*, a promising biological control agent for the invasive *Drosophila suzukii*

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Abstract

Native to East Asia, the spotted-wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), has established widely in the Americas, Europe, and parts of Africa over the last decade, becoming a devastating pest of various soft-skinned fruits in its invaded regions. Biological control, especially by means of self-perpetuating and specialized parasitoids, is expected to be a viable option for sustainable area-wide management of this highly mobile and polyphagous pest. *Ganaspis brasiliensis* Ihering (Hymenoptera: Figitidae) is a larval parasitoid that is widely distributed in East Asia, and has been found to be one of the most effective parasitoids of *D. suzukii*.

Following rigorous pre-introduction evaluations of its efficacy and potential non-target risks, one of the more host-specific genetic groups of this species (G1 *G. brasiliensis*) has been approved recently for introduction and field release in the United States and Italy. Another genetic group (G3 *G. brasiliensis*), which was also commonly found to attack *D. suzukii* in East Asia, may be considered for introduction in the near future. There is currently enormous interest in rearing *G. brasiliensis* for

research or in mass-production for field release against *D. suzukii*. This protocol and associated video article describe effective rearing methods for this parasitoid, both on a small scale for research and a large scale for mass-production and field release. These methods may benefit further long-term research and use of this Asian-native parasitoid as a promising biological control agent for this global invasive pest.

CHAPTER 11. SATELLITE PROJECT #2

Current status of Drosophila suzukii classical biological control in Italy

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Abstract

For over a decade, the invasive pest *Drosophila suzukii* (Matsumura) has threatened the soft-skinned fruit production worldwide, causing increased management costs and yield losses. Current integrated pest management (IPM) exploits different control tools but relies mainly on insecticides. The local natural enemy community mostly consists of generalist species, mainly parasitoids attacking the puparia of the fruit fly. These antagonists resulted unable to control the pest efficiently, regardless the adoption of conservative or augmentative approaches. By contrast, in the native area of *D. suzukii*, sympatric larval parasitoids have co-evolved with the pest and provide a stable control of its population. Foreign explorations and quarantine risk assessment studies for classical biological control programs have identified different species of parasitoids characterized by a variable level of

specificity. The Japanese G1 lineage of the larval endoparasitoid *Ganaspis brasiliensis* (Ihering) has proved to be much more selective and efficient than other larval parasitoids, including *Leptopilina japonica* Novković & Kimura recently reported in Europe. In this context, a voluntary partnership of Italian researchers imported a colony of the G1 lineage of *G. brasiliensis* into Italian quarantine facilities and proposed its release in Italian fields. A three-year working program has been set up in several locations of nine Italian regions/provinces. Field releases of laboratory-reared parasitoids have been planned. Pre- and post-release samplings of fresh and fallen fruits around the release points will be undertaken to assess the impact of the exotic *G. brasiliensis* on *D. suzukii* and its potential interactions with other non-target insects in the field. The possible establishment of this efficient and specific biological control agent would promote a long-lasting control of this invasive species less dependent on the use of chemicals, reducing the negative effects associated with them.

CHAPTER 12. SATELLITE PROJECT #3

Non-target effects of neurotoxic insecticides on *Ganaspis* cf. *brasiliensis*, a classical biological control agent of the spotted wing *Drosophila*

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Abstract

BACKGROUND. The spotted wing drosophila, *Drosophila suzukii*, is an invasive pest causing significant economic losses worldwide. Current pest control strategies mainly rely on insecticides, which negatively impact fruit marketability and the sustainability of integrated pest management (IPM) programs. In addition, pesticides can have dramatic consequences on non-target species when persisting in the environment at low concentrations after field applications. In this context, chemical control can strongly interfere with the releases of the G1 strain of the Asian larval parasitoid *Ganaspis* cf. *brasiliensis*, which is currently the adopted classical biological control agent to manage *D. suzukii* infestations worldwide.

METHODS. Probit analysis was used to assess the baseline toxicity of acetamiprid, cyazypyr, lambda-cyhalothrin, phosmet, and spinosad on G1 *G.* cf. *brasiliensis* adults through residual contact exposure in the laboratory. Then, adult parasitoids were exposed to insecticide low Lethal

Concentrations (LC_5 and LC_{30}) and their mortality was checked daily to assess the survival of treated wasps.

RESULTS. Lambda-cyhalothrin showed the highest toxicity on the parasitoid with a LC_{50} of 1.38 × 10–3 g active ingredient (a.i.) /L, while cyazypyr seemed the safer active ingredient with an estimated LC_{50} of 0.20 g a.i./L without affecting parasitoids at sublethal doses. Spinosad and phosmet significantly reduced wasp survival at both LC_{30} and LC_5, while lambda-cyhalothrin and acetamiprid affected parasitoid lifespan only at LC_{30}. Spinosad, lambda-cyhalothrin and phosmet LC_{30} caused the major survival reductions, followed by acetamiprid LC_{30}. The least significant reduction in parasitoid survival was 21.6% by spinosad LC_5.

CONCLUSION. Overall, this study highlighted the importance of carefully selecting insecticides to minimize adverse effects on non-target organisms. In particular, cyazypyr was the most promising candidate to integrate inoculative biological control with chemical treatments. By contrast, the application of phosmet, spinosad and lambda-cyhalothrin should be avoided alongside parasitoid field releases. Although acetamiprid is less used against *D. suzukii* in the field than the other tested molecules, it should be used with caution due to its sublethal toxicity on the parasitoid. These results provide the first evidence of *G.* cf. *brasiliensis* susceptibility to insecticides in order to promote sustainable and efficient pest management strategies.

CHAPTER 13. SATELLITE PROJECT #4

Adventively-established *Leptopilina japonica*: a new opportunity for augmentative biocontrol of *Drosophila suzukii*

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Abstract

The invasive spotted-wing drosophila, *Drosophila suzukii*, has emerged as a significant global pest over the past decade, threatening fruit production worldwide. The parasitoid *Leptopilina japonica*, presumed native to East Asia, has established adventive populations in Europe and North America and is increasingly recognized for its ability to parasitize substantial proportions of *D. suzukii* larvae across diverse habitats. Here, we provide a broad review of the biology, establishment, distribution, and potential impacts of *L. japonica*. Using field data from international monitoring programs, we document the seasonal dynamics of plant–host–parasitoid associations and assess evidence for *L. japonica*'s impact on *D. suzukii* and non-target organisms. Findings indicate that *L.*

japonica has successfully established in several areas where *D. suzukii* is present in Europe and North America, showing promise as a biological control agent to support sustainable pest management. Current data suggest it provides some suppression of *D. suzukii* populations with minimal non-target effects. However, long-term studies are necessary to clarify its food web interactions and efficacy as a biological control agent. In areas when *L. japonica* has established, we propose *L. japonica* for use in augmentative biological control programs to enhance its impacts in specific agricultural settings. Case-specific evaluations of its ecological effects and role in integrated pest management, supported by continued monitoring, are essential. The case for *L. japonica* illustrates the need for clear, research-informed policies to guide the use of adventively established non-indigenous natural enemies in pest management.

Keywords:

Adventive establishment, spotted-wing drosophila, Figitidae, biological control, invasive species