Advancing the biological control of the cosmopolitan pest *Drosophila suzukii* with parasitoid wasps

DISSERTATION

zur Erlangung des Doktorgrades der Naturwissenschaften

(Dr. rer. nat.)

der Fakultät für Biologie, Chemie und Geowissenschaften

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vorgelegt von

Benedikt Josef Maria Häußling

aus Bad Kreuznach

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"*In nature's economy the currency is not money, it is life*."

— **Vandana Shiva**

Content

Summary

Biological pest control has gained prominence as an essential component of sustainable food production. Its growing popularity is due to the advantages it offers over commonly used chemical pest control methods, such as reduced biodiversity loss.

A pest of global concern is the invasive fruit fly *Drosophila suzukii* (spotted wing drosophila) as it can severely damage fruit crops. There is, therefore, a strong demand for biological pest control of this particular pest. The pest's ability to lay eggs in ripe fruit makes biological control a more appealing alternative as growers are reluctant to use insecticides shortly before the harvest due to the risk of chemical residues on the fruit. Another drawback of most available insecticides is that they are non-selective and thus can cause harm to a wide range of non-target organisms, in turn reducing biodiversity in orchards. Overall, there is a need for alternative biological methods.

A commonly used and successful approach for biological pest control is to use parasitoids from the native range of the pest or resident parasitoids from the invaded regions to control the pest. Unfortunately, many of these parasitoids are unable to successfully parasitise the invasive pest. This inability is presumably due to a pest's strong immune response. However, pupal parasitoids indigenous to the invaded areas and larval parasitoids from the native range of *D. suzukii's* show potential in managing this pest.

To improve the utility of parasitoids for biological pest control of *D. suzukii*, I chose to study the pupal parasitoid *Trichopria drosophilae*

and the Asian larval parasitoid *Asobara japonica*. Specifically, I focused on the specificity of the pupal parasitoid for *D. suzukii* over the common fruit fly, *D. melanogaster*. This specificity remains consistent even with variations in host pupal size. When the parasitoid is released as a biocontrol method, its specificity for the pest should result in a reduced risk of parasitisation of non-target species in infested crops.

Interestingly, contrary to expectations, *D. suzukii* does not use the fruit as its primary pupation site but instead usually chooses the soil. However, I have found that the ability of *T. drosophilae* to parasitise in the soil is limited. This lack of soil parasitism can be exploited in the field, as a layer of sandy soil or plastic mulch around fruit plants can disrupt the movement of *D. suzukii* larvae, thereby increasing their susceptibility to desiccation, and exposing them to additional parasitoid threats.

A central aspect of my research focuses on the immunity of the two seasonal phenotypes of *D. suzukii*, the summer and winter phenotypes. In northern temperate regions, the winter phenotype typically predominates for the majority of the year. Unfortunately, the immune status of this phenotype, as well as its immune response to parasitisation, remains unknown. Given this gap in knowledge, my research primarily investigated the immunity of *D. suzukii*, examining differences in immune responses between the two phenotypes across their various life stages. The study of the pest's immunity showed variations between its winter and summer phenotypes throughout its life stages. However, the efficiency of the parasitoids remains virtually unaffected, despite the different levels of immunity observed in the two phenotypes.

A different result was obtained when examining the pupal stage, where the pupal parasitoid *T. drosophilae* was found to have an increased parasitisation success rate on pupae of winter phenotype of *D. suzukii*. This difference highlights that a favourable time to release the pupal parasitoid is in early spring, when the winter phenotype is still most prevalent in the northern temperate regions.

This dissertation provides a comprehensive strategy to improve the biological control of *D. suzukii* using parasitoids, particularly *T. drosophilae*. By merging insights into parasitoid preferences and immunity mechanisms, along with considering the seasonal variations and parasitisation of the pest in different soil types, I could formulate a more cohesive and effective approach to improving biological pest control efforts.

German Summary – Zusammenfassung

Die biologische Schädlingsbekämpfung gewinnt als wichtiges Element einer nachhaltigen Lebensmittelproduktion zunehmend an Bedeutung. Ihre wachsende Beliebtheit ist auf die Vorteile zurückzuführen, die sie im Vergleich zum herkömmlichen chemischen Pflanzenschutz bietet, wie etwa der geringere Verlust an biologischer Vielfalt.

Ein weltweit bedeutsamer Schädling ist die invasive Fruchtfliege *Drosophila suzukii* (Kirschessigfliege), die reifende Früchte massiv schädigen kann. Aus diesem Grund besteht eine hohe Nachfrage nach biologischer Schädlingsbekämpfung. Die Fähigkeit des Schädlings, seine Eier in reifende Früchten abzulegen, macht die biologische Schädlingsbekämpfung zu einer attraktiven Alternative, da Landwirte den Einsatz von Insektiziden kurz vor der Ernte aufgrund des Risikos von möglichen Rückständen auf den Früchten vermeiden wollen. Ein weiterer Nachteil vieler verfügbarer Insektizide besteht darin, dass sie nicht selektiv wirken und somit eine Vielzahl von Nichtzielorganismen beeinträchtigen können. Das wiederum kann zur Verringerung der Artenvielfalt führen, z.B. in Obstplantagen. Insgesamt besteht also dringender Bedarf an biologischen Methoden zur Schädlingsbekämpfung.

Ein erfolgversprechender und häufiger Ansatz zur biologischen Schädlingsbekämpfung ist der Einsatz von Parasitoiden aus dem natürlichen Verbreitungsgebiet des Schädlings oder von einheimischen Parasitoiden im invasiven Verbreitungsgebiet. Allerdings sind viele dieser Parasitoiden nicht in der Lage, den invasiven Schädling wirksam zu parasitieren, vermutlich aufgrund seiner starken Immunreaktion. Einheimische Puppenparasitoide aus dem invasiven Verbreitungsgebiet

und Larvenparasitoide aus dem heimischen Verbreitungsgebiet von *D. suzukii* zeigen jedoch Potenzial zur Bekämpfung dieses Schädlings.

Um den Nutzen von Parasitoiden in der biologischen Schädlingsbekämpfung zu optimieren, wurden der Puppenparasitoid *Trichopria drosophilae* und der asiatische Larvenparasitoid *Asobara japonica* untersucht. Insbesondere wurde die Spezifität des Puppenparasitoids für *D. suzukii* im Vergleich zur weit verbreiteten Fruchtfliege *D. melanogaster* untersucht. Es wurde festgestellt, dass eine klare Präferenz für *D. suzukii* besteht, welche auch bei der Variation der Größe der Wirtspuppen erhalten bleibt.

Interessanterweise nutzt *D. suzukii* entgegen der Erwartung die Frucht nicht als primären Verpuppungsort, sondern wählt stattdessen meistens den Boden aus. Die Ergebnisse diese Dissertation zeigen jedoch, dass die Fähigkeiten von *T. drosophilae*, im Boden zu parasitieren, sehr beschränkt sind. Das Wissen um diese Einschränkung kann jedoch in der Praxis genutzt werden, indem eine Schicht aus Sandboden oder Mulchfolie um die Pflanze gelegt wird. Dadurch wird die Fortbewegung der Larven von *D. suzukii* gestört, wodurch sie anfälliger für Austrocknung werden und zusätzlichen Parasitoiden ausgesetzt sind.

Ein zentraler Aspekt dieser Forschungsarbeit konzentriert sich auf die Immunität der beiden saisonalen Phänotypen von *D. suzukii*, nämlich des Sommer- und des Winterphänotyps. In den nördlichen gemäßigten Breiten dominiert üblicherweise, die meiste Zeit des Jahres, der Winterphänotyp. Allerdings ist der Immunstatus dieses Phänotyps und seine Immunantwort auf die Parasitierung nach wie vor unbekannt. Angesichts dieser Wissenslücke wurde in dieser Forschungsarbeit vor

allem die Immunität von *D. suzukii* untersucht, indem die Unterschiede in den Immunreaktionen der beiden Phänotypen in den verschiedenen Entwicklungsstadien der Fliege untersucht wurden. Diese Studie über die Immunität des Schädlings zeigt, dass es in allen Entwicklungsstadien Unterschiede zwischen den Winter- und Sommerphänotypen gibt. Der Parasitierungserfolg der Parasitoiden bleibt jedoch trotz der unterschiedlichen Immunität der beiden Phänotypen nahezu unverändert.

Ein anderes Ergebnis ergab sich aus der Untersuchung der Parasitierung von Puppen durch den Puppenparasitoid *T. drosophilae*. Dieser Parasitoid hatte eine höhere Erfolgsrate bei der Parasitierung von Puppen des Winterphänotyps von *D. suzukii*. Dies zeigt, dass ein günstiger Zeitpunkt für die Freisetzung des Puppenparasitoide das zeitige Frühjahr ist, wenn der Winterphänotyp in den nördlichen gemäßigten Regionen noch am weitesten verbreitet ist.

Zusammenfassend präsentiert diese Dissertation eine umfassende Strategie zur Verbesserung der biologischen Bekämpfung von *D. suzukii* mithilfe von Parasitoiden, insbesondere *T. drosophilae*. Durch die Verknüpfung von Erkenntnissen über die Präferenzen und Immunitätsmechanismen der Parasitoiden sowie der Berücksichtigung der saisonalen Variationen der Phänotypen und der Parasitierung des Schädlings in verschiedenen Bodentypen wurde ein kohärenter und wirksamer Ansatz zur Verbesserung der biologischen Schädlingsbekämpfung formuliert.

1 Introduction

1.1 Biological pest control – An overview

Crop production has been a challenge in the past, when the focus was mainly on food security. However, increasing biodiversity loss and climate change will make crop production even more challenging in the future. Sustainable crop production must therefore address all these challenges to ensure long-term food security. This also includes pest management, as about 18 % of agricultural yields are lost to pests before the crop harvest (Oerke 2006), which is expected to increase due to climate change (Deutsch et al. 2018).

Over the past 40 years, chemical pesticides have been one method of reducing yield losses. Their use has risen by a factor of 15 to 20 (Oerke 2006). The use of these chemicals has been considered controversial for years due to their potential to cause heart diseases, neurological and reproductive disorders, liver damage, stomach ulcers and cancer problems in humans (Hoppin et al. 2006, Donham 2016, Patel et al. 2018). In recent years, there has been a major scientific and public debate about the use of chemical pesticides, especially insecticides (particularly neonicotinoids) as one of the reasons for declining insect populations worldwide (Hallmann et al. 2017, Sánchez-Bayo & Wyckhuys 2019, van Klink et al. 2020, Wagner 2020). The need for alternatives to insecticides is therefore of great concern.

In this context, biological control is playing an increasingly important role. This is especially true as it has been estimated that natural enemies of pests are probably responsible for about 50 - 90 % of the pest control

in the field (Pimentel 2005). This illustrates the potential of biological control, where natural enemies are used to control agricultural pests in cropping systems.

A historically famous example of biological pest control is the use of ants to control populations of caterpillars in ancient China - probably one of the oldest known written records of the use of biological pest control. As early as 324 BC, farmers purchased and encouraged populations of the ant *Oecophylla smaradina* to control caterpillars in citrus trees. The ants build paper nests and feed on insects, thereby reducing the number and hence the damaging effects of the caterpillars (Offenberg et al. 2013). This practice continued in the Shan States of northern Burma until the 1950s (Liu et al. 2014).

As there are different ways in which biological control can be achieved, biological control is classified into different categories: Classical biocontrol, augmentative biocontrol (inundative and inoculative biological control) and conservative biocontrol.

Classical biocontrol is the permanent establishment of the biological control agent in a new area where natural enemies are absent. The classic example of classical control is the introduction of the vedalia beetle (*Rodolia cardinalis*) into California. In 1880, citrus orchards in California were severely damaged by a new pest, the cottony pillow bug (*Icerya purchasi*). This pest was introduced to California through citrus fruits imported from Australia. When the entomologist Albert Koebele discovered in Australia in 1888 that vedalia beetles were feeding on this cottony pillow bug, he collected them. Then he shipped them to California, where they were released. By 1890, all infestations of the pest

in California had been completely decimated. This famous example is considered the beginning of the classical biological control (Mallis 1971).

Augmentative biocontrol is the (usually repeated) release of the biological control agent. This is with the expectation that the agent will not establish and that only the released organisms themselves will control the pest; the agent can reproduce and control the pest for an extended period (Inoculative biocontrol), or short term with rapid control (Inundative biocontrol) but not permanently (Eilenberg et al. 2001). The first mass-reared natural enemy was the egg parasitoid of the genus *Trichogramma* in England in 1895. Widespread use of mass-reared antagonists did not occur until the 1970s, when they began to be widely used in greenhouses (Hajek 2012), which are a closed system compared to open fields. They were used in the greenhouse, because the released insects stay in the greenhouse for a longer period than in the field. In addition, the climatic conditions in greenhouses can be shifted to favour the natural enemy, which increases the success rate of this augmentative biocontrol method.

Conservative biocontrol differs from other biocontrol methods in that no natural enemies are released. Instead, the resident population of natural enemies is enhanced and conserved (Eilenberg et al. 2001, Hajek 2012). This method requires a thorough knowledge of the biology and ecology of the natural enemies. The aim is to create a habitat that provides shelter, food, hosts, and, as mentioned for greenhouses, favourable abiotic conditions for the natural enemy species. One way to improve the food supply for parasitoids is to increase the species

abundance and diversity of the floral fauna, which can be achieved by planting flower strips next to crop fields (Albrecht et al. 2020).

1.2 *Drosophila suzukii,* a major pest in soft fruits

The fruit fly *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), commonly known as the spotted wing drosophila, is a major pest of fruit crops. The fly can cause significant economic damage to a wide range of fruits, including berries, cherries, and grapes (Kanzawa 1939, Bolda & Goodhue 2010, Farnsworth et al. 2017, Mazzi et al. 2017, DiGiacomo et al. 2019). The increased scientific importance is also reflected in the rise of the total number of publications published annually over the last ten years, from 47 in 2012 to 1036 in 2022 (data from Dimensions AI 2023).

Unlike most other *Drosophila* species, *D. suzukii* can lay its eggs in ripe fruit. Other *Drosophila* species can only oviposit in overripe or previously damaged fruit. *D. suzukii* has this ability to penetrate intact fruit due to its enlarged and serrated ovipositor (Lee et al. 2011, Atallah et al. 2014) (Figure 1). The ovipositor allows the female fly to serrate small holes in soft-skinned fruit and lay its eggs inside. The serrated ovipositor of *D. suzukii* females allows the pest to exploit a new ecological niche: close ripe to ripe, undamaged fruit (Atallah et al. 2014). For most other *Drosophila* species, these ripe, undamaged fruits are inaccessible. One of the few limitations of *D. suzukii* is the fruit skin; if the fruit epidermis is too strong or thick to penetrate, the fly cannot oviposit (Lee et al. 2015a, Häussling 2016).

Figure 1: Morphology of *Drosophila suzukii*. Left: Male *D. suzukii* with one dark spot on each wing. Right: Female *D. suzukii* with a serrated ovipositor. © B. Häussling

The fruit fly *D. suzukii* originated from Southeast Asia and has now spread across the globe to North America (Hauser 2011), Europe in 2008 (Calabria et al. 2012), and to South America (Deprá et al. 2014) and Africa in 2013 (Anfora et al. 2020). One factor contributing to the spread of *D. suzukii* is its ability to survive in a wide range of environments. The fly can survive winter temperatures down to around -9 °C as a winter phenotype (Toxopeus et al. 2016, Winkler et al. 2021), in which it can hibernate through diapause (Zerulla et al. 2015) and its insensitivity to cold temperatures (Shearer et al. 2016).

Another factor contributing to the rapid and widespread expansion of *D. suzukii* is its ability to reproduce rapidly. *D. suzukii* females can lay up to 400 eggs in their lifetime, and the larvae can develop from eggs to adults in as little as ten days under optimal conditions (Tochen 2014, Hamby et al. 2016, Winkler et al. 2021). The temperature range for development starts at about 10 °C, has an optimum at about 28 °C and a maximum at about 30 °C (Tochen 2014, Winkler et al. 2021). Temperatures above 31 °C can reduce population growth (Tochen et al.

2014). The resulting high reproductive rate allows populations to grow rapidly. Due to the high reproductive rate, large populations can build up early in the season and then massively infest fruit orchards. On average, Minnesota growers have reported yield losses of 20 % in raspberry fields, but the maximum reported loss has been massive yield losses of up to 80% in these orchards (Bolda & Goodhue 2010, Goodhue et al. 2011, DiGiacomo et al. 2019).

The fly can also reproduce on a wide range of non-crop host plants that are available for reproduction throughout the year. With this wide range of host plants and their enormous reproductive output, they can establish high populations around orchard fields (Lee et al. 2015b, Poyet et al. 2015, Kenis et al. 2016). These populations can then mass invade the orchard when the fruit is ripe or close to the ripening (Urbaneja-Bernat et al. 2020).

1.3 Pest control of Drosophila suzukii

Cultural management practices are the first step in an integrated pest management programme. For *D. suzukii* control, these include: sanitation measures; mulching, especially under the crop plant; exclusion netting; the timing of harvest; pruning; and canopy management such as defoliation and shoot thinning (Schöneberg et al. 2020). These measures mainly affect the microclimate in the field. They can reduce the humidity and increase the temperature, thereby reducing *D. suzukii* oviposition, as oviposition is positively influenced by a high relative humidity (Tochen et al. 2016). Further benefits include the risk reduction

of oomycete infections, such as *Plasmopara viticola* or ascomycete infections, such as *Botrytis cinerea*.

Although previous studies have described and applied various cultural management practices, pest management of *D. suzukii* is still often based on chemical control. This is not least because many fruits have zero tolerance to pests such as *D. suzukii* (Haye et al. 2016). Studies in recent years have shown that the insecticides pyrethroids, organophosphates, carbamates, and spinosyns are effective against the fruit fly (Beers et al. 2011, Van Timmeren & Isaacs 2013, Tait et al. 2021). These insecticides predominantly target the adult stage of *D. suzukii,* as the larvae and eggs are enclosed in the fruit and larval contact with the insecticide can be limited (Wise et al. 2015).

Unfortunately, chemical pest control just before the harvest is limited by the pre-harvest interval. This interval means that harvesting has to be suspended for a certain period of time after the pesticide has been applied. As the efficiency of the chemical applications can be limited and, as mentioned above (1.1), can have a wide range of side effects, there is a strong demand for alternatives. One method of reducing these side effects is the attract-and-kill method. The lure attracts the fly to a location where an additional killing agent is applied. The success of this method depends on the pest coming into contact with or feeding on the killing agent (Cloonan et al. 2018). It is considered to be more selective than conventional insecticide spraying, as the agent is sprayed selectively on the plant, targeting only the fruit zone, and the attractant is specific for the target pest. Therefore, it has less impact on non-target organisms (El-Sayed et al. 2009). However, the attract-and-kill method is not yet

applicable and tested for wide-field situations, and research is ongoing (Babu et al. 2022, Jones et al. 2022, Rhodes et al. 2023).

Therefore, combining multiple methods is of great interest, especially focusing on the part of biological control treatments to control *D. suzukii* populations. The biological control treatments also include parasitoids, predators, entomopathogens (fungi, bacteria, viruses) and the endosymbiont *Wolbachia* (Tait et al. 2021). The use of predators is abundant in organic farming, but so far, the generalist predators have only a limited ability to control *D. suzukii (Lee et al. 2019)*. Predators that have been observed feeding on *D. suzukii* include earwigs, predatory bugs, as well as spiders and ants (Woltz et al. 2015, Woltz & Lee 2017, Wolf et al. 2018). The commercially available predators tested to date have shown limited efficacy in field trials and have often been compromised by high control costs (Woltz et al. 2015, Lee et al. 2019). In light of these facts, predator augmentation does not seem promising. Nevertheless, conservative biological control practices may be an alternative, especially for organic farmers where insecticide applications are less frequent (Lee et al. 2019). This strategy can include providing shelter and supplemental plant food to attract and encourage predator populations targeting *D. suzukii* (Landis et al. 2000).

Entomopathogenic fungi have also been tested for control of *D. suzukii*. The strains tested, such as *Beauveria bassiana* and *Metarhizium brunneum*, have been shown to be virulent to *D. suzukii* under laboratory conditions (Yousef et al. 2017, Ibouh et al. 2019). However, they have little effect when applied in the field or under field-like conditions (Woltz et al. 2015, Alnajjar et al. 2017). This may be due

to suboptimal field conditions that lead to rapid degradation of the spores (Alnajjar et al. 2017). One promising way to avoid this degradation is to use autoinoculation devices. Adult flies are attracted to the device by food odour, and once inside, they are dusted with a spore solution (Cossentine et al. 2016, Yousef et al. 2017).

Entomopathogenic bacteria have also been considered for the control of *D. suzukii*. Different strains of the widely used *Bacillus thuringiensis* have been tested (Biganski et al. 2018, Cahenzli et al. 2018, Cossentine et al. 2019). Similar to the entomopathogenic fungi, the *B. thuringiensis* stains performed promisingly in laboratory trials but did not show high fly mortality when placed on dried residues (Cahenzli et al. 2018) or even resulted in no increase in mortality in laboratory experiments (Biganski et al. 2018). Another *B. thuringiensis* strain tested produced a toxin harmful to vertebrates, disqualifying it as a biological agent (Cossentine et al. 2019).

Entomopathogenic viruses with an apparent effect on *D. suzukii* control are lacking (Schetelig et al. 2018, Lee et al. 2019, Tait et al. 2021). In contrast, thoracic injections of Drosophila A virus, La Jolla virus, Drosophila C virus, Cricket paralysis virus or Flock house virus have shown high mortality (Lee & Vilcinskas 2017, Carrau et al. 2018). Therefore, these entomopathogenic viruses could potentially control the pest if it is determined how they can be delivered and spread among *D. suzukii* under field conditions.

Another possible control method is the sterile insect technique (SIT), which is a species-specific method. It relies on the release of sterile insects that mate with wild insects and cause reproductive failure. The

pest insect is mass-reared and then sterilised by exposure to ionising radiation, followed by a sustained area-wide release at regular intervals (Lanouette et al. 2017, Kruger et al. 2018, Tait et al. 2021). The application of SIT is still under research. The effectiveness of this method is higher when only male sterile insects are released (Rendón et al. 2004). Although biotechnologically enhanced SIT can achieve this population control, it is not a viable option for organic farmers due to GMO regulations.

1.4 Parasitoids to control *Drosophila suzukii*

Parasitoids are a promising method to control *D. suzukii*. They are important in regulating the populations of some *Drosophila* species (Janssen et al. 1987, Fleury et al. 2009). Parasitoids are insects whose larvae develop by feeding on other arthropods' bodies, eventually killing their host. Adult female parasitoids lay their eggs on (ectoparasitoids) or in (endoparasitoids) the host eggs (egg parasitoids), host larvae (larval parasitoids), host pupae (pupal parasitoids) or host adult (adult parasitoids) of another arthropod (Godfray 1994). The parasitoid insect then emerges in place of the parasitised host. As the host is usually killed or reduced in fitness, parasitoids can reduce the population sizes of insect pest species and are their most effective natural enemies (Omkar 2023).

Research on parasitoids for the control of *D. suzukii* includes not only parasitoids from the native range of *D. suzukii*, i.e. Asian parasitoids, for their use in classical control (1.1), but also resident parasitoids for augmentative biological control (1.1, Wang et al. 2020b). Resident larval

parasitoids for augmentative biological control include the parasitoid wasp species *Asobara tabida*, *Leptoplina boulardi* or *Leptoplina heterotoma*. Unfortunately, all of these species failed to parasitise *D. suzukii* to an acceptable degree (Chabert et al. 2012, Kacsoh & Schlenke 2012, Knoll et al. 2017, Lee et al. 2019, González et al. 2020). This is most likely due to the high immunity of *D. suzukii* compared to other *Drosophila* species (Kacsoh & Schlenke 2012).

On the other hand, pupal parasitoids are very promising for augmentative biocontrol of *D. suzukii*. These species include *Trichopria drosophilae* (Figure 2) and *Pachycrepoideus vindemmiae* in North America and Europe and *Trichopria anastrephae* in South America. In laboratory tests, these species were effective against *D. suzukii*. The highest efficacy was observed for *T. drosophilae* (Wang et al. 2016b). Unfortunately, the few reported field trials showed low population suppression and further studies are needed to improve the augmentative release of *T. drosophilae* wasps (Falagiarda & Schmidt 2020, Gonzalez-Cabrera et al. 2021).

Classical biocontrol of *D. suzukii* with larval parasitoids from the pest's native range is also a very promising avenue to control *D. suzukii* populations. The wasp species that have been shown to be able to parasitise *D. suzukii* successfully are *Leptopilina japonica*, *Ganaspis brasiliensis* and *Asobara japonica*. These species differ in several aspects, such as their development time, with *L. japonica* grows the fastest (Wang et al. 2019), making this species particularly interesting as a biocontrol agent against *D. suzukii*. However, the parasitoid species with the highest potential to suppress *D. suzukii* populations is *A. japonica,* as its

parasitisation rates have been observed to be the highest (Wang et al. 2018a, Wang et al. 2020b), while the wasp species *G. brasiliensis* was shown to have the lowest impact on non-target organisms (Girod et al. 2018b, Girod et al. 2018a). Therefore, *G. brasiliensis* was used for the first classical biological control release in northern Italy in 2021 (Fellin et al. 2023). The first observations in 2022 are promising, as the released parasitoids were able to overwinter and emerge only on *D. suzukii,* mainly on fresh fruits. However, these are only first year results and as the evaluation of the release is ongoing, its success is still unpredictable.

Although the other two Asian parasitoid wasps of *D. suzukii* mentioned above have not been reported to have been used as biocontrol agents for *D. suzukii* in Europe or North America, they have recently been found in the wild and occur in the same invaded regions as *D. suzukii.* In small parts of North America, *L. japonica* and *G. brasiliensis* were first recorded in 2019 (Abram et al. 2020, Beers et al. 2022). In the same year, specimens of *L. japonica* were recorded in small parts of Europe (Italy) (Puppato et al. 2020), possibly due to unintentional introduction or simultaneous spread with *D. suzukii* eggs or larvae.

Figure 2: Pupal parasitoid *Trichopria drosophilae* parasitising *D. suzukii* pupae.
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1.5 Aims of this Thesis

The aim of this thesis was to contribute to the development of biological control methods for pests, specifically the control of the worldwide pest species *D. suzukii*. As mentioned above, this fruit fly is a major challenge for growers worldwide and an environmentally friendly control method for *D. suzukii* is strongly needed (Tait et al. 2021). I hereby contribute important knowledge to improve the control of *D. suzukii* in the field and to therefore reduce crop losses worldwide. In addition, the knowledge gained from my thesis can be applied to emerging pests, particularly in the *Drosophila* group, hopefully preventing or reducing harmful effects of future pest species.

For biological pest control, the parasitoid must prefer the pest species as a host in order to have a less negative impact on non-target organisms and a greater population-reducing impact on the pest (Nagaraja 2013). The parasitoid wasp *T. drosophilae* has been observed to prefer parasitising *D. suzukii* pupae over *D. melanogaster* pupae (Wang et al. 2016a, Boycheva Woltering et al. 2019, Yi et al. 2020). However, this preference was thought to be due to host size, as *D. suzukii* pupae are generally larger than *D. melanogaster* pupae (Wang et al. 2016a). Therefore, in **Publication 1,** I investigated whether this preference might indeed be species-specific. Species specificity would be very important for the use of *T. drosophilae* as a biocontrol agent against *D. suzukii*, as the pupal size of fruit flies can vary considerably under field conditions. It is not yet known whether *T. drosophilae* would still prefer *D. suzukii* pupae if, for example, they were smaller than those of *D. melanogaster*.

In addition, the parasitoid must have physical access to the pest in order to parasitise it. This is particularly important for biological control of *D. suzukii* flies, which pupate primarily in the soil (Ballman et al. 2017, Woltz & Lee 2017). However, physical access to pupae in the soil can be very limited (Tsitsipis & Papanicolaou 1979, Dimou et al. 2003). In addition, the kairomones used to locate the host may be less concentrated in the soil (Johnson & Gregory 2006). Therefore, in **Publication 2**, I investigated whether *T. drosophilae* is able to find and parasitise buried pupae.

Suppose further that the parasitoid is able to parasitise the host. In this case, the success of the parasitisation is still unknown, as the host's immune system may fight the parasitisation and attempt to encapsulate the parasitoid egg (Carton et al. 2008). This is particularly interesting for the *D. suzukii* pest, as several larval parasitoids of the *Drosophila* group fail to parasitise this species (Poyet et al. 2013, Lynch et al. 2016, Iacovone et al. 2018). *D. suzukii* has been found to have a strong immune system (high hemocyte load) (Kacsoh & Schlenke 2012, Poyet et al. 2013, Iacovone et al. 2018), but this has only been observed for the summer phenotype of the fly. However, in continental and temperate climates, the most commonly observed phenotype of *D. suzukii* throughout the year is the winter phenotype (Panel et al. 2018). Therefore, it is unknown how the immune system of the winter phenotype of *D. suzukii* responds to parasitism and whether parasitism is more or less successful in the winter phenotype compared to the summer phenotype (Figure 3).

This knowledge would be necessary for biological control of *D. suzukii*, as an augmentative release of its parasitoid species may be more effective early in the season when the pest population is still small, after the overwintering bottleneck. However, at that time, *D. suzukii* flies are still of the winter phenotype in temperate and continental climates (Rossi-Stacconi et al. 2016, Rossi Stacconi et al. 2018a). Therefore, I performed parasitisation experiments with winter and summer phenotypes of *D. suzukii* and *D. melanogaster* using the larval parasitoid *A. japonica* and the pupal parasitoid *T. drosophilae* (**Publication 3**). I also studied the immune status of adults to assess the immune status of winter and summer phenotypes throughout development from larvae to pupae to adults.

In summary, my aim in this thesis was to contribute important knowledge on the parasitism of *D. suzukii* to help reduce the negative impact of this pest on fruit growers worldwide. I investigated the parasitoid species specificity of the wasp *T. drosophilae* for *Drosophila* (**Publication 1**) and the wasp's ability to locate and parasitise *D. suzukii* pupae buried below the soil surface (**Publication 2**) to improve its use as a biocontrol agent against *D. suzukii*. Another aspect of my research was to determine the optimal time to release parasitoids against *D. suzukii* in continental and temperate climates, considering the impact of the flies' immune system with two seasonal phenotypes (**Publication 3**). Through my dissertation, I aimed to elucidate the pathways for releasing and using biological control agents to effectively reduce *D. suzukii* populations in orchards. In addition, the knowledge I gained

has potential applications in the management of emerging pest species within the *Drosophila* group.

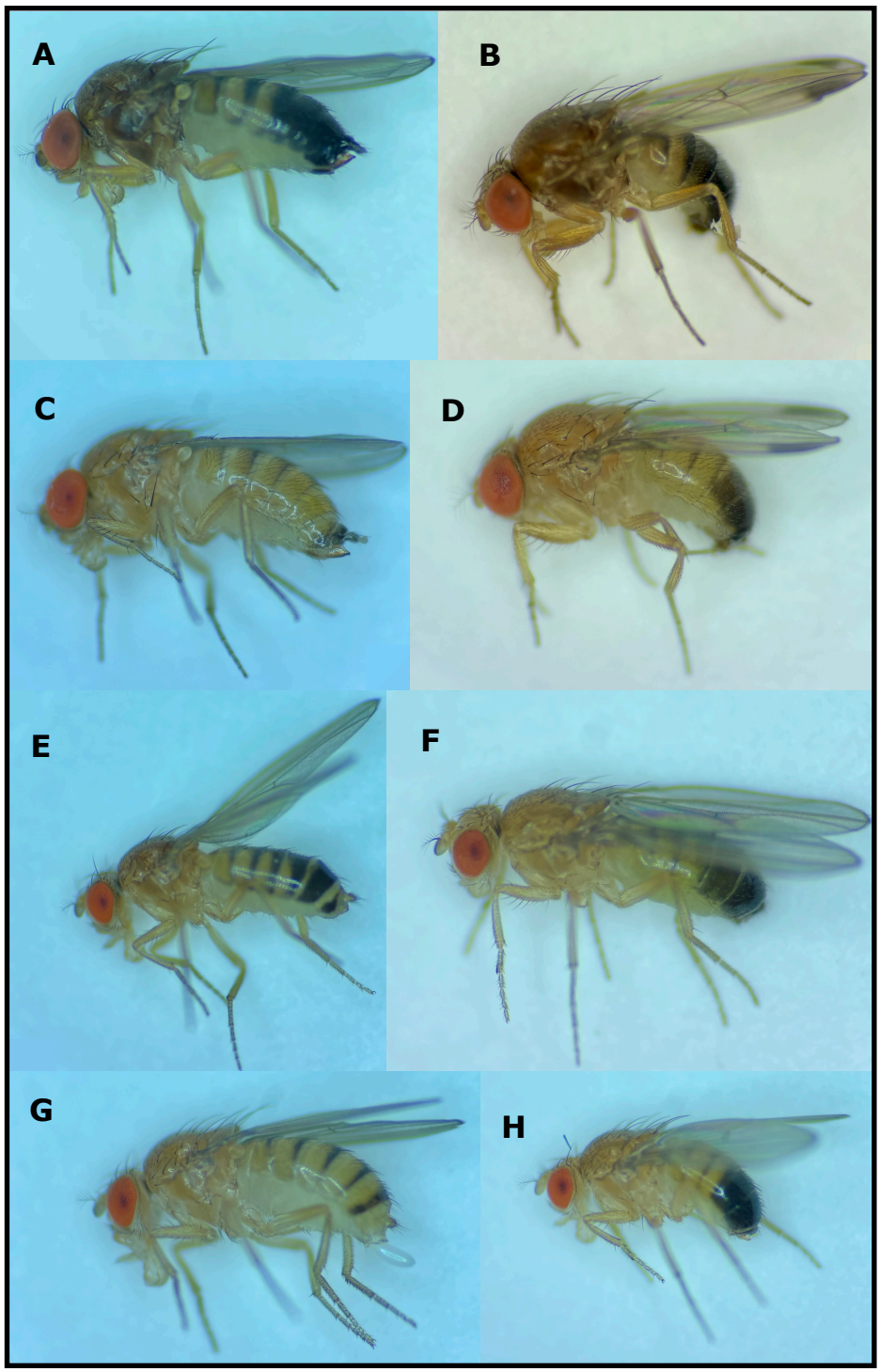

Figure 3: Phenotype plasticity in the colour of A: female *D. suzukii* winter phenotype; B: male *D. suzukii* winter phenotype; C: female *D. suzukii* summer phenotype; D: male *D. suzukii* summer phenotype; E: female *D. melanogaster* winter phenotype; F: male *D. melanogaster* winter phenotype; G: female *D. melanogaster* summer phenotype; H: male *D. melanogaster* summer phenotype. The winter phenotype of both species has more melanisation in the abdominal segments than the summer phenotype. © Benedikt Häußling

2 Synopsis: An Overview of the Publications of this Thesis

Publication 1

The preference of *Trichopria drosophilae* for pupae of *Drosophila suzukii* is independent of host size

Benedikt J. M. Häussling, Judith Lienenlüke and Johannes Stökl

Published in the Journal Scientific Reports (2021) 11, 995

The use of a parasitoid as a biocontrol method requires in-depth knowledge of the pest and the parasitoid. The biocontrol method must be effective in order to be successfully implemented in an Integrated Pest Management program (IPM). This can be quantified by quality parameters such as host recognition, specificity to the host, parasitism rate, and emergence rate (Nagaraja 2013). All this can help to select a possible candidate species or species population for biocontrol.

As mentioned in the introduction (1.4), the pupal parasitoid *Trichopria drosophilae* is a species that can be and already is being mass-reared (Mazzetto et al. 2016, Wang et al. 2016b). Crucially, the parasitoid can parasitise at lower temperatures than other pupal parasitoids (Rossi Stacconi et al. 2017, Wang et al. 2018b, Colombari et al. 2020). This is important because early release of the pest is essential in an IPM programme. The goal of the programme is to suppress the pest population below the acceptable pest level using the least intrusive

method. To achieve this goal, the pest population should not exceed the acceptable pest level. Therefore, the pest population should be relatively low before the fruit is close to ripening. This is particularly important for *D. suzukii* control because the canopy of the landscape surrounding to an orchard can strongly influence *D. suzukii* crop infestation (Häussling 2016, Haro-Barchin et al. 2018, Santoiemma et al. 2018, Drummond et al. 2019, Champagne-Cauchon et al. 2020). Thus, an early area-wide release may be necessary for successful population control below acceptable pest levels (Rossi-Stacconi et al. 2016, Wiman et al. 2016).

High specificity can reduce the potential impact on non-target organisms. When parasitoids are released, they should have a specificity for the host, in our case, for *D. suzukii.* This is important because a variety of *Drosophila* species may be present in particularly overripe fruit and do not need to be controlled (non-target organisms). If there is a lack of specificity, the non-target *Drosophila* species that may be present could be parasitised to greater extent than the pest species *D. suzukii*. Therefore, the parasitoid's preference for the host species over non-target organisms is essential (Nagaraja 2013). Furthermore, specificity for the pest will logically increase parasitisation of the pest, giving a higher probability of successful control.

The preference of *T. drosophilae* for *D. suzukii* over *D. melanogaster* has been observed in several studies (Wang et al. 2016a, Boycheva Woltering et al. 2019, Yi et al. 2020), although one study found no difference (Mazzetto et al. 2016). However, the proximate reason for this host preference remains unclear. Pupal size as a proximate cause would be supported by the findings of Wang et al. (2016a), who found that

D. suzukii pupae are larger than *D. melanogaster* pupae. To assess a factor for preference, we correlated pupal volume with pupal parasitism in **Publication 1**. This was made possible by video recording the parasitism and measuring pupal size. This direct observation had the advantage that the parasitism event and the success of each pupa could be observed, as well as the volume of each pupa could be measured (Figure 4). Furthermore, this approach allowed us to correlate the sex of the hatched parasitoids with the volume of the parasitised pupa.

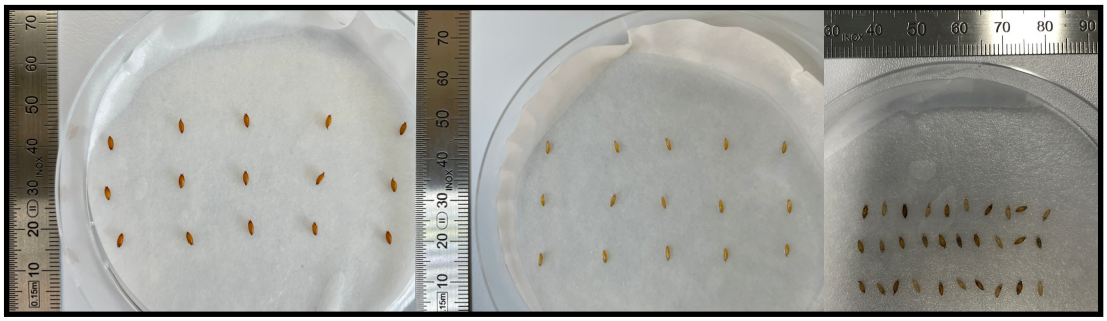

Figure 4: Experimental setup of A: *D. suzukii* pupae, B: *D. melanogaster* pupae and C: *D. suzukii* and *D. melanogaster* pupae alternately arranged for parasitisation by *T. drosophilae*. © Benedikt Häußling

We found that the preference of the parasitoid *T. drosophilae* for *D. suzukii* was independent of host size. Therefore, we can exclude host volume as a proximate cause of the preference for *D. suzukii*. This means that the reason for the preference remains unclear but is probably a combination of chemical and physical cues from the pupal anterior spiracles. As we observed during parasitism, the parasitoid decided to parasitise only after drumming with its antennae over the pupal respiratory spiracles. This behaviour of *T. drosophilae* was also observed by Romani et al. (2002).

In conclusion, these wasps can identify their preferred host species independent of the size of their host. This is important for implementation

in an IMP as this preference increases the likelihood of successful control of the pest with *T. drosophilae*, especially in cases where other hosts such as *D. melanogaster* are present. We also found that the wasps can recognise the pupal volume of their hosts. Our results show that pupal volume influences the sex ratio of hatched parasitoids. The probability of hatching a female wasp increased with increasing pupal volume (see Figure 6,7 in **Publication 1**). During oviposition, parasitoids determine the sex of the egg by fertilising it (diploid, female) or not fertilising it (haploid, male) (Aubert 1959). In several parasitoids, this shift in sex ratio has been observed to be more female-biased in larger hosts (Sandlan 1979, Charnov et al. 1981, King 1987). The question arises as to why the parasitoids shift the sex ratio in favour of females in larger hosts. Also, both sexes show an increase in fitness in larger hosts. One explanation could be the fact that the fitness increase of the female parasitoids is higher in voluminous hosts, and thus the increase is greater for females in a larger host than for males in a larger host (Charnov et al. 1981).

For mass rearing of *T. drosophilae* and probably other parasitoids, this volume-dependent sex shift can be used to improve the quality of the rearing process. A female-biased rearing population is likely to have higher control success when released, as females are responsible for parasitisation. This can be achieved by selecting species with high host volume and well-fed hosts, resulting in voluminous hosts.

To summarize, I found in this study (**Publication 1**), that the preference of *T. drosophilae* for *D. suzukii* over *D. melanogaster* is independent of the host size. In addition, the wasps prefer to lay fertilised

i.e. female eggs in larger host pupae, suggesting the need to select for larger pupal volumes during mass rearing of parasitoid wasps before releasing them as biocontrol agents against *D. suzukii*, as this will increase parasitisation success.

Publication 2

Below ground efficiency of a parasitic wasp for *Drosophila suzukii* biocontrol in different soil types

Benedikt J. M. Häussling, Melinda Mautner and Johannes Stökl

Published in the Journal Scientific Reports (2022) 12, 9130

A high parasitisation rate is crucial for successful biocontrol treatment of pest species with parasitoids. Therefore, in **Publication 1**, we investigated the specificity of parasitoid wasps for their hosts. Furthermore, it is also essential for the parasitoid to be able to locate and have physical access to the host. In the case of *T. drosophilae*, access to the pupae is necessary because this wasp species is a pupal parasitoid. Therefore, the parasitoids must locate the pupation site, which in the case of *D. suzukii* is typically buried in the soil beneath the fruit plant (Ballman et al. 2017, Woltz & Lee 2017, Buonocore Biancheri et al. 2023).

To achieve a high parasitisation rate, the parasitoid must be able to locate and parasitise these buried pupae, which can be challenging due to the different physical properties of the soil matrix compared to the fruit. Unfortunately, all parasitisation tests to date have been performed on fruit or in petri dishes (Mazzetto et al. 2016, Wang et al. 2016b, Rossi Stacconi et al. 2018b, Esteban-Santiago et al. 2021, Häussling et al. 2021, see Publication 1), where the parasitoid had relatively free access,

as opposed to an approach that mimics the most common pupation site, the soil.

In this study, we investigated the pupation behaviour of *D. suzukii* and the parasitisation rate of *T. drosophilae* in three different soil types: loamy sand, loam, and clay, since they're common soil types. Our aim was to predict the parasitisation success of the parasitoid wasps and the pupation behaviour of *D. suzukii* in different soil types.

Our findings indicated that *T. drosophilae* parasitised the pupae at a very low rate, with only 1.8 % to 5.1 % wasps hatching from the pupae. In contrast in the control group (petri dish), where the wasp had free access, the hatching was 20 to 7 times higher. These differences emphasize how important approaches are which emulate natural conditions.

These low numbers indicate that the physical properties of the soil matrix act as a barrier for the parasitoid, which hampers its ability to pass through the soil matrix (Guillén et al. 2002). In contrast, the pupal parasitoid *Coptera haywardi* was able to locate and parasitise fruit fly pupae up to a depth of 5 cm, possibly due to its hypognathous head morphology, which allows the wasp to locate pupae by digging (Sivinski et al. 1998, Guillén et al. 2002). In addition, the soil may not only act as a physical barrier, but due to its complexity, it may also reduce the diffusion of semiochemicals emitted by the pupae (Johnson & Gregory 2006). This could reduce the ability of the parasitoid to locate the pupae in the soil compared to those located in the fruit.

In conclusion, our results indicate that the soil matrix is a massive barrier for *T. drosophilae*, making soil parasitism almost impossible for

this parasitoid. This knowledge can be used to improve the biological pest control of *D. suzukii*. One possible approach could be to use plastic mulch or a layer of sandy soil underneath the fruit plant, as this could enhance the exposure of the pupae to antagonists, such as *T. drosophilae*. This could increase the chances of *T. drosophilae* successfully parasitising the pest, resulting in a reduced hatching rate of *D. suzukii*. Additionally, this sandy layer could also lead to higher desiccation rates of *D. suzukii* larvae. Simultaneous release of several different parasitoids may also be successful and applicable. A combination of the pupal parasitoid *Pachycrepoideus vindemmiae* together with *T. drosophilae* could be highly successful as *P. vindemmiae* has been observed to parasitise pupae on the soil surface (Guillén et al. 2002). Furthermore, it was observed that when *Trichopria anastrephae*, a relative of *T. drosophilae*, and *P. vindemmiae* were released together, there was an increase in the parasitisation rates in fruit (Buonocore Biancheri et al. 2023). This possibility of simultaneous release and the use of the different niches of the two parasitoids could improve the biological control of *D. suzukii*. (Buonocore Biancheri et al. 2023).

Publication 3

Does the seasonal phenotype of *Drosophila suzukii* influence cellular immunity and parasitisation?

Benedikt J. M. Häussling, Nathalie Rausch, Emely Klüsener and Johannes Stökl

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The success of parasitoids as biocontrol agents depends not only on their specificity for pest species (**Publication 1**) and their ability to access pupae (**Publication 2**) but also on the success of the parasitisation. Unfortunately, recent studies (Chabert et al. 2012, Kacsoh & Schlenke 2012, Biondi et al. 2020, Wang et al. 2020a) have shown that most larval parasitoids have low parasitisation success on *D. suzukii* larvae – especially those from regions where *D. suzukii* is not native, such as Europe and North America. For example, the larval parasitoid *Asobara japonica* from China has a parasitisation success rate on *D. suzukii* larvae of over 90 %, whereas *Asobara tabida* from France has a success rate of no more than 0 % (Chabert et al. 2012). It should also be noted that these rates vary for different strains. We used a different strain of *A. japonica* because the strain from China was not available. The strain we used had a much lower parasitisation success rate than the strain from China.

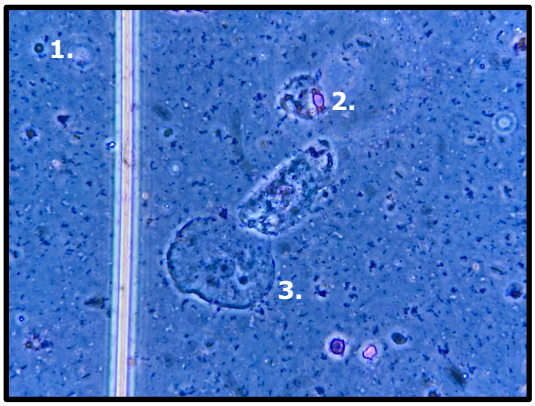

Figure 5: Hemocytes of *D. suzukii*: 1. Plasmatocyte, 2. Podocyte, 3. Lamellocyte. © Benedikt Häußling

So why do so many parasitoid species fail to parasitise the pest? Firstly, this resistance of *D. suzukii* could be due to its immunity, specifically the increased number of hemocytes in *D. suzukii* (Figure 5) compared to other *Drosophila* flies such as *D. melanogaster* is likely the reason (Kacsoh & Schlenke 2012, Poyet et al. 2013, Iacovone et al. 2018). These hemocytes are important for the resistance because they are responsible for encapsulating a foreign parasitoid egg in the larval body (Figure 6), and this encapsulation kills the parasitoid egg if the process is successful (Figure 6).

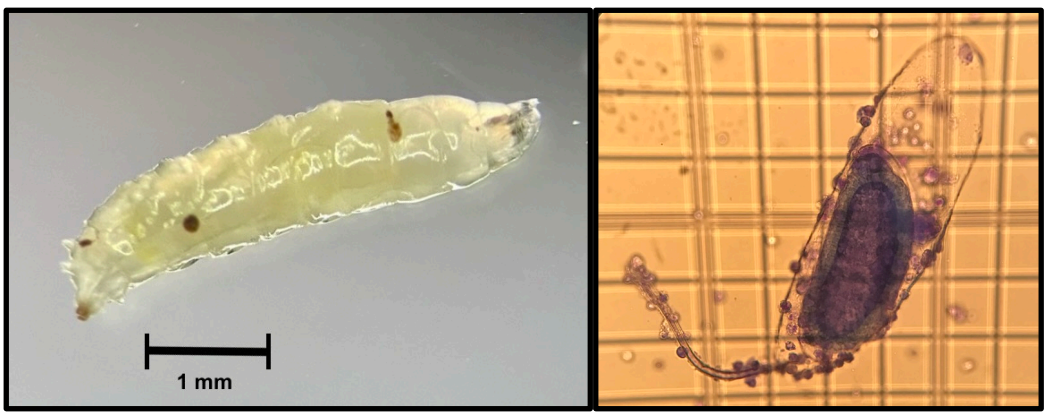

Figure 6: Left: *D. suzukii* larvae (winter phenotype) with encapsulations (dark spots) after parasitisation by the parasitoid *A. japonica*. Right: Parasitoid egg of *A. japonica* with hemocytes attached to the egg. © Benedikt Häußling

The observations of high invulnerability of *D. suzukii* were made in the summer phenotype of *D. suzukii.* However, *D. suzukii* has two seasonal phenotypes: a summer phenotype and a winter phenotype (also known as summer morph and winter morph). The winter phenotype of *D. suzukii* is morphologically and physiologically different from the summer morph. The winter phenotype has a larger and darker body, a longer lifespan at lower temperatures (Shearer et al. 2016, Wallingford & Loeb 2016), and enters a temperature-dependent reproductive diapause (Zerulla et al. 2015, Rossi-Stacconi et al. 2016, Toxopeus et al. 2016).

Throughout the year, the winter phenotype dominates in the population from September to June in northern Europe (Panel et al. 2018). This means that the winter phenotype is dominant in the spring. Biological pest control can be very effective in the spring due to the bottleneck in the pest population size after the winter. Therefore, the previously neglected immune response of the winter phenotype *D. suzukii* to parasitism is crucial.

To determine whether there is a difference in the immune response to parasitism between the two phenotypes, we performed parasitism experiments not only on the summer phenotype, but also on the winter phenotype of *D. suzukii*, and also on *D. melanogaster*. We used *A. japonica* as a larval parasitoid. We counted larval hemocytes from the hemolymph to assess the host immune response and measured the phenoloxidase activity (PO activity). Since hemocytes are part of the cellular immune response of insects through phagocytosis, encapsulation, and clotting (Lavine & Strand 2002), and phenoloxidase is a key component of the immune response of insects to the presence of foreign

objects (González-Santoyo & Córdoba-Aguilar 2012). Furthermore, we assessed the number of hatched flies and parasitoids to observe the effect of the parasitisation on the flies and the parasitoids.

We found differences in immune responses between the phenotypes and life stages, but these were inconsistent. At certain time points (0 h and 48 h after parasitisation), the summer phenotype of *D. suzukii* larvae had significantly higher hemocyte counts than the winter phenotype. However, these differences did not increase immune resistance and did not result in higher levels of infestation, success of parasitisation or encapsulation rates. Other studies have observed that the immune resistance of *Drosophila* larvae is associated with a high host hemocyte load (Kacsoh & Schlenke 2012, Poyet et al. 2013). In our study, the differences in hemocyte load did not lead to differences in resistance between phenotypes. One explanation could be that the difference between the phenotypes is too small to alter the observed immune resistance. Another possibility is that the immune process of encapsulation depends mainly on the hemocyte cell type lamellocytes (Eslin et al. 2009). Our results showed that the number of this cell type differed between the seasonal phenotypes at 24 h and 48 h after parasitisation. Consequently, the lack of an increase in immune resistance cannot be explained by a lack of difference in the number of lamellocytes in the hemolymph.

However, I would like to highlight one important factor that should not be underestimated: the fly strain. As we only used one fly strain, we cannot exclude that the hemocyte load between the summer and winter phenotype is different in other fly strains. An extreme example of immune

differences was observed between the summer phenotype of French and Japanese *D. suzukii* strains. The French strain had almost twice the hemocyte load of the Japanese strain (Poyet et al. 2013). Furthermore, the number of lamellocytes in the hemolymph was not significantly different between the two *D. suzukii* strains. This is similar to our study, where the number of lamellocytes did not differ between the two phenotypes. It appears that in this case the total number of hemocytes is a stronger indicator of the potential immune response of the fly than the number of lamellocytes alone, although the lamellocytes are important for the encapsulation process.

As the pupal stage is distinct from the larval stage, we continued the life cycle of the pest species *D. suzukii* and investigated the immune resistance of pupae of the two phenotypes to parasitisation. We used the same pupal parasitoid *T. drosophilae* as in **Publication 1** and **Publication 2**. As in the above publications, we offered the pupae to the parasitoid in a petri dish (Figure 4). Here we found differences in the parasitisation success of the two seasonal phenotypes of *D. suzukii*. The parasitoid had a significantly higher parasitisation success in the winter phenotype than in the summer phenotype (at 24 °C). This means that pupae of the winter phenotype are more susceptible to parasitism than those of the summer phenotype.

We observed a difference in parasitisation success between phenotypes in the pupal stage, but not in the larval stage, which raises the question of why there are different levels of immune resistance to parasitisation in two life stages. We expect that different parasitoids elicit different immune responses in the host. Especially if the parasitoids are

specialised on different life stages of the host, such as pupal and larval parasitoids. Parasitoids have evolved various strategies to overcome the immune response of flies, such as specialising on specific host stages, evading the immune system by sticking the egg into host tissue (Eslin & Prevost 2000), or injecting venom during oviposition to suppress the host's immune response (Schlenke et al. 2007, Moreau & Asgari 2015, Huang et al. 2021, Wertheim 2022). This virulence can be species-specific and often strain-specific (Cavigliasso et al. 2019). The comparison between the two life stages is therefore limited, as there is a complete morphological change during metamorphosis, including but not limited to the immune cells that are released at the onset of metamorphosis and the ingestion of doomed larval tissues (Lanot et al. 2001, Holz et al. 2003).

The adult life stage of *D. suzukii* is not of interest to parasitoids, as no parasitoids have been found to parasitise the adult stage. However, it is of interest to increase our knowledge of the adult immune system, as hemocytes and phenoloxidase are important not only for the response to parasitisation but also for infection with various pathogens. We are therefore completing the life cycle by analysing the number of hemocytes and the phenoloxidase activity in adult females. To my knowledge, this is the first time that these two immunity factors have been measured in adult female *D. suzukii* in general. Surprisingly, we found that the winter phenotype of adults of both fly species had significantly more hemocytes than the summer phenotype. In contrast, at the larval stage, the winter phenotype had similar or lower hemocyte counts stage than the summer phenotype. At the pupal stage, the winter phenotype was similar or less

susceptible to parasitisation than the summer phenotype. This marked difference between the phenotypes was unexpected because adult *D. melanogaster* hemocytes are derived from embryonic and larval development and consist of a mixture of embryonic and lymph gland-derived hemocytes (Holz et al. 2003). Therefore, the proportions of hemocytes found in larvae and pupae would be expected to be present in the adult stage.

A possible explanation for these contradictory results could be the influence of the lower temperature (15 °C) during the development of the winter phenotype on the total number of hemocytes. The summer phenotype was reared at a higher temperature (24 °C) than the winter phenotype, so temperature could influence the hemocyte load. Another possibility is a sex-dependent decrease of hemocytes with age (Sanchez Bosch et al. 2019, Boulet et al. 2021). A previous study observed a sex-dependent decrease of hemocytes with adult age in *Drosophila*, with adult female flies showing a lower decrease than males (Mackenzie et al. 2011). It is therefore possible that this sex-dependent decline is also phenotype dependent.

The other immune factor we measured, phenoloxidase, did not differ between the two phenotypes at either the larval or adult stage. This is consistent with the observed parasitisation success of the larvae, which was indifferent between the phenotypes. Furthermore, phenoloxidase is a key enzyme in the insect immune system, although it should be noted that the correlation between phenoloxidase activity and insect immunity is complex (González-Santoyo & Córdoba-Aguilar 2012).

In this study, we provide the first evidence that the immune system of the winter phenotype of *D. suzukii* is very similar to that of the summer phenotype, particularly during the larval stage. Consequently, the efficacy of biological pest control methods tested on *D. suzukii* summer phenotypes using different parasitoid species can be extended to *D. suzukii* winter phenotypes. Regarding the pupal stage, our results suggest that pupal parasitoids have greater success when targeting the winter seasonal phenotype. Based on our results, the most opportune time to release parasitoids into the field for *D. suzukii* population control would be early in the growing season. During this period, the winter phenotype dominates the *D. suzukii* population in the Northern Hemisphere, and parasitoid release then represents an effective treatment strategy, as *D. suzukii* populations remain relatively small following the winter bottleneck, in line with *D. suzukii* population dynamics. The smaller populations of winter phenotype *D. suzukii* can then be parasitised by releasing mainly female-biased populations of parasitoid wasps (see **Publication 2**).

3 Conclusion

In this thesis, I have contributed to the understanding of biological pest control using parasitoids to control the cosmopolitan pest *D. suzukii*. For this pest control, I used the promising pupal parasitoid *T. drosophila*, which is native to areas the pest has invaded. I investigated the preference of the pupal parasitoid *T. drosophilae* for two different *Drosophila* species for potential augmentative release (Häussling et al. 2021, see **Publication 1**), its parasitisation capabilities within the soil matrix (Häussling et al. 2022, see **Publication 2**) and the immune system and immune response of the two seasonal phenotypes of *D. suzukii* to a parasitisation by the larval parasitoid *A. japonica* (see **Publication 3**). *A. japonica* is a candidate for classical biocontrol. Furthermore, I also studied the immune status of adult flies. These findings contribute to predicting the success of *A. japonica* and *T. drosophilae* and other parasitoids as a biocontrol method against the pest *D. suzukii*.

For a parasitoid to be effective in an augmentative release as a biocontrol method, the parasitoid must meet various key quality parameters: host detection and identification, specificity to the pest and adequate parasitisation, emergence and female sex ratio rates for effective pest suppression (Nagaraja 2013). I address these quality parameters in my thesis. For the pupal parasitoid, *T. drosophilae*, a preference was found for the pest *D. suzukii* over the common *Drosophila* fly *D. melanogaster* (Häussling et al. 2021, see **Publication 1**). This preference is independent of the host pupal size, which ensures pest

specificity and reduces potential harm to non-target organisms such as *D. melanogaster* when the pupal sizes vary under field conditions.

After determining the parasitoids specificity, another challenge is locating the pest's primary pupation site. This pupation site is different from what one would expect: *D. suzukii* does not primarily pupate in the fruit, where the larvae feed and where the parasitoid would have easy access. Instead, the most common pupation site is the soil (Ballman et al. 2017, Woltz & Lee 2017). I found that the parasitisation of *D. suzukii* pupae by *T. drosophilae* is unlikely to be successful in the soil (Häussling et al. 2022, see **Publication 2**). The fact that the parasitisation is an exception applies to all three soil types studied: loamy sand, loam, and clay. The reason for the low parasitisation rate in the soil is probably due to the physical properties of the soil, which hamper the ability of the parasitoid to move within the soil matrix.

These findings can refine the augmentative release of *T. drosophilae* and potentially improve the biocontrol by adding a layer of sandy soil or a plastic mulch around the fruit plants. This layer of sand or mulch could increase the desiccation of the larvae searching for pupation sites in the soil, as the sandy soil strongly hampers the larval movement (Ballman & Drummond 2019, Häussling et al. 2022, see **Publication 2**) and exposes the pupae and larvae to several antagonists such as *T. drosophilae* and other pupal and larval parasitoids.

When a larval or pupal parasitoid finally parasitises these larvae or pupae, the outcome of this parasitisation remains uncertain due to the strong cellular immune system of *D. suzukii* compared to other *Drosophila* (Kacsoh & Schlenke 2012, Poyet et al. 2013, Iacovone et al.

2018). The invasive populations in North America and Europe exhibit an even more robust cellular immune system than the native Japanese population (Poyet et al. 2013). This high level of immunity has resulted in parasitism failure for numerous parasitoids, particularly endemic larval parasitoids from *D. suzukii* invasion regions (Chabert et al. 2012, Kacsoh & Schlenke 2012, Girod et al. 2018a, Matsuura et al. 2018).

Similar invasions with a failure of endemic parasitoids have been observed for other pests such as the diamondback moth (*Plutella xylostella*), one of the most destructive cosmopolitan pests of *Brassica* crops (Sarfraz et al. 2007). Several parasitoids have been introduced in different countries to control the pest, often because native parasitoids were unable to parasitise the host, e.g. in New Zealand the parasitoid wasps *Diadegma semiclausum* and *Diadromus collaris* the diamondback moth (Hardy 1938, Talekar & Shelton 1993).

A more recent example is the invasive brown marmorated stink bug (*Halyomorpha halys*). Native European egg parasitoids have mostly failed or have had less success in parasitising this stink bug (Haye et al. 2015, Herlihy et al. 2016, Dieckhoff et al. 2017). Similar to *D. suzukii,* the strong immune response of the stink bug has been postulated to be responsible for the failure of native parasitoids (Haye et al. 2015, Herlihy et al. 2016). In general, invasive herbivore species tend to be less attacked by native parasitoids than in their native region, especially when the invasion was recent and local parasitoids are not efficiently adapted to the herbivore (Cornell & Hawkins 1993).

In recent years, studies on the immunity of *D. suzukii* have focused on the summer seasonal phenotype. However, as mentioned in Chapter

1.5, the most commonly observed seasonal phenotype throughout the year in continental and temperate climates is the winter phenotype (Panel et al. 2018).

The immune response to parasitism varies between the winter and the summer phenotypes and is also inconsistent between larval, pupal, and adult life stages (see **Publication 3**). The larval stage is particularly important for biocontrol as the majority of parasitoids used for parasitisation tests of *D. suzukii* are larval parasitoids. The differences in immunity between the two phenotypes do not affect the success of a parasitoid in parasitising the larval stage (see **Publication 3**). In other words, the observed differences in immunity between the two phenotypes do not lead to differences in the success of the larval parasitoid. For biocontrol proposes, this finding means that the observed parasitisation rates of the summer phenotypes can be expected to be similar to those of the winter phenotypes when parasitised by a larval parasitoid. However, we did not test different larval parasitoid species, so some uncertainty remains for other parasitoid species. This knowledge of immune plasticity also provides a better understanding of pest invasion success, as plasticity is the key to that success (Little et al. 2020).

Seasonal phenotypic plasticity is known in *D. suzukii* and other species, such as *D. melanogaster*. Although closely related to *D. suzukii*, other species in this genus, such as *Drosophila simulans*, do not exhibit distinct seasonal phenotypes (Behrman et al. 2015). Notably, our study (see **Publication 3**) represents the first investigation of immunity between seasonal phenotypes of *D. melanogaster*.

Another prominent example of distinct seasonal phenotypes is the honey bee (*Apis mellifera* L.), where both summer and winter bees occur during the year. The complexity of phenotypic differences in honey bees parallels that of *D. suzukii*. Interestingly, comparisons between the two honey bee phenotypes revealed no differences in hemocyte counts (Kunc et al. 2019, Dostálková et al. 2020). However, in contrast to our observation in *D. suzukii* larvae (see **Publication 3**), winter bees show a more intense response to bacterial infection compared to their summer counterparts (Dostálková et al. 2020). These comparisons underline the importance of our investigations on seasonal phenotypic variation of *D. suzukii* and their immune responses.

Moreover, our research has direct implications for the augmentative release of the pupal parasitoid *T. drosophilae*. We have observed that *T. drosophilae* is more successful in parasitising winter phenotype pupae than summer phenotype pupae of *D. suzukii* (see **Publication 3**). This observation is particularly important for the augmentative release in early spring when the winter phenotype of the pest is dominant. The potential efficacy of this biocontrol approach on the winter phenotype could be increased by exploiting the higher success rate of the parasitoid on these pupae.

In conclusion, early parasitoid release during the fruit growing season (see **Publication 3**), combined with soil coverage strategies (Häussling et al. 2022, see **Publication 2**) and the specificity of the parasitoid *T. drosophilae* for *D. suzukii* (Häussling et al. 2021, see **Publication 1**), can enhance the success of biological pest control using parasitoids.

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5 Publications and Manuscripts

5.1 Publication 1: The preference of *Trichopria drosophilae* for pupae of *Drosophila suzukii* is independent of host size

The preference of *Trichopria drosophilae* for pupae of *Drosophila suzukii* is independent of host size

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Own contribution: Concept and study design: 75%, data acquisition 60%, data analysis and figures 90%, interpretation of results 80%, manuscript writing 85%.

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The preference of *Trichopria drosophilae* for pupae of *Drosophila suzukii* is independent of host size

Benedikt J. M. Häussling[1]✉, Judith Lienenlüke[2] & Johannes Stökl[1]

Controlling the cosmopolitan pest *Drosophila suzukii* (spotted wing drosophila) is a challenge for fruit growers. A promising agent for biological control of that pest are parasitoid wasps. Especially the widespread pupal parasitoid *Trichopria drosophilae* had shown the ability to parasitise the pest fly. However, as a biocontrol agent, parasitoids can only be effective when they prefer the pest to other insects. Until now studies have been inconsistent concerning the preference of *T. drosophilae* for *D. suzukii* and whether the preference depends on pupal volume. To clarify this inconsistency, we used video recordings of parasitisation experiments with a set up to observe the direct host preference of the parasitoid. Additionally, the volume of each host pupa was measured. We found significant preference of *T. drosophilae* for *D. suzukii* pupae independent of the pupal size and of the host species the wasps were reared on. The article also discusses the sex ratio and the success of the parasitoid in the different pupae characteristics.

The range and speed of the distribution of invasive insect pest species are increasing with globalisation across all agricultural ecosystems. These insects can bring considerable negative impacts along with potential massive economic losses for farmers[1,2]. An example par excellence is the invasive pest *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), also called the spotted wing drosophila (SWD). SWD is endemic in south-east Asia and, in the last few years, has become a severe pest to fruit growers in North and South America and Europe[3].

In contrast to most of the other fruit flies in the invaded regions, females of SWD have a serrated ovipositor[4], enabling them to lay eggs in healthy and undamaged fruits[5]. *D. suzukii* can reproduce on a broad range of wild and cultivated soft-skinned fruit crops and can have an extremely high reproduction rate[6]. Therefore, enormous populations can be build up quickly and infest fruit crops, where they cause massive economic damage[7].

The control of this *Drosophila* is still firmly based on the use of insecticides due to the lack of effective alternatives. Here, biological options, such as predators, parasitoids, nematodes, bacteria, fungi and viruses, could be a possible part of an Integrated Pest Management strategy (IPM)[8,9] and interest in them has been growing.

Parasitoids are, in particular, a promising option because, in natural systems, parasitisation rates of *Drosophila* can reach up to 50%[10]. Parasitoid wasps from the native range of SWD show high efficacy and specialisation on the pest flies[11–15]. Whereas some of these species were under consideration to be introduced to North America and Europe, first specimens were already discovered in North America[16] and Europe[17]. However, the larval parasitoids native on these continents are not able to successfully reproduce on *D. suzukii*[18,19]. The most promising native parasitoids in Europe and North America that can successfully reproduce on *D. suzukii,* are the pupal parasitoids *Pachycrepoideus vindemmiae* Rondani (Hymenoptera: Pteromalidae) and *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae)[19]. Ideally, these wasps should be implemented in an IPM approach.

For an augmentative release, knowledge of the species' quality parameters, such as host identification, specificity to the host, the ratio of parasitism, the ratio of emergence (e.g., ≥ 90% for trichogrammatids) and the ratio of females (≥ 50%) is essential[20]. Furthermore, the release of parasitic wasps should happen as early as possible in the growing season, when the population size of *Drosophila* is still small[21,22].

Trichopria drosophilae is the most promising candidate for augmentative biocontrol of SWD and is already available on the market[23,24]. This wasp species has a high foraging efficiency on *D. suzukii* pupae and a high load of mature eggs[19,23–29]. Furthermore, *T. drosophilae* can parasitise at lower temperatures (8–25 °C) than the species *P. vindemmiae*[30–32]. This early parasitisation is an advantage when implementing an IPM program as it can parasitise the first generations of the pest early in the year. *T. drosophilae* can parasitise a broad host range of many Drosophilidae species[33], including the widespread fly *D. melanogaster* Meigen (Diptera: Drosophilidae).

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This fly is not a pest of healthy fruits and consequently not a target of the IPM approach, similar to most other Drosophilidae species. For an efficient IPM, it is therefore essential to study the host specificity and the host identification mechanism of *T. drosophilae*.

A preference of *T. drosophilae* for *D. suzukii* over *D. melanogaster* has been observed in several studies[34–36], although one study has found no differences in parasitisation between the two species[23]. However, *D. suzukii* pupae are larger than pupae of *D. melanogaster*, at least under optimal food supply. Therefore, it is uncertain if this observed preference of *T. drosophilae* for *D. suzukii* is due to the larger host pupal size or to the host itself. Furthermore, the pupal size is an unsteady factor for host selection as varying food supply under natural conditions can lead to a high variation in pupal size.

Until today studies have used indirect measures for the oviposition preference of wasps on SWD, such as the number of emerged parasitoid wasps or the degree of infestation (DI)[34–36]. These measurements have uncertainties, primarily due to the immune response of the fly species, which can kill the wasp eggs oviposited into the larvae or pupae[37]. Therefore, the number of emerged wasps is usually smaller than the number of deposited eggs. To accurately study the host and oviposition preference, the oviposition events must be observed directly.

In this work, we studied the host preference of *T. drosophilae* for *D. suzukii* taking the size of the pupae and the immune response of the fly into account. To correct for pupal size, we measured the size of each pupa, and by direct observation of each oviposition, we determined the real oviposition preference.

This way, we provide evidence for the host-choice of *T. drosophilae* and contribute essential new knowledge about its behaviour during oviposition as well as about offspring sex-ratios of this promising biological control agent. With these new results, effective use of *T. drosophilae* wasps becomes more feasible, and the negative impacts of the invasive *D. suzukii* flies on crops could be decreased.

Material and methods

Insects. The fly species *D. suzukii and D. melanogaster* were used for oviposition preference tests of *T. drosophilae*. The strain of *D. suzukii* was caught in the state of Hesse, Germany, in 2016 and was refreshed in 2017. The strain of *D. melanogaster* is an established lab strain for multiple generations. *D. melanogaster* was reared on an artificial Drosophila diet (ingredients: 1 l water, 50 g cornmeal, 50 g wheat germ, 50 g sugar, 40 g baker's yeast, 8 g agar, 5 ml propionic acid, 20 ml methylparaben (10%)) in Drosophila vials.

Adult *D. suzukii* flies were kept in a BugDorm cage (MegaView Science Co., Taichung, Taiwan), where 10% of sugar water was provided ad libitum. The flies were allowed to lay eggs in Drosophila vials with the same artificial diet as *D. melanogaster*. Then the vials were removed from the cages. The fly development took place in these vials until the flies emerged. After some days, they were released into the cage. Variation in pupae volume was created by rearing both fly species with a higher and lower density of larvae per amount of diet.

The parasitoid wasp *T. drosophilae* was provided by the company "Bioplanet" in Cesena, Italy. In the lab, two different populations were reared for more than two years (approx. > 40 generations) in Drosophila vials on pupae of either *D. melanogaster* or *D. suzukii*, henceforth referred to as *T. drosophilae* < melanogaster > and *T. drosophilae* < suzukii > , respectively. After the wasps' emergence, they were fed with a 10% honey-water solution. The parasitoid females used for the experiments were 4–6 days old and were held together with males. All insects were kept in a climate- and light-controlled chamber at 24 °C and 70% to 80% RH with a 16:8 h day to night rhythm.

Host preference experiments. In a choice experiment, we wanted to test whether females of *T. drosophilae* prefer to oviposit in pupae of *D. suzukii* over *D. melanogaster*. For this, 15 pupae of each *D. suzukii* and *D. melanogaster* were arranged alternately (*D. suzukii* pupae next to *D. melanogaster* pupae and so on) on a disk of moist filter paper which was placed in a Petri dish (9.5 cm diameter). We increased the variation in the size of the pupae by rearing both fly species (*D. melanogaster* and *D. suzukii*) with a higher or lower amount of food per larvae. To accurately measure the size of the pupae, the Petri dish was photographed (Canon Eos M100) next to a precision ruler for scale. The length and width of each pupa were measured with the software ImageJ[38] from the photos, and the volume was calculated using the formula[39]:

$$V = \frac{4}{3}\pi \cdot \frac{l}{2} \cdot \left(\frac{w}{2}\right)^2 \quad (1)$$

where V is the volume, l the length and w the width of the pupae.

One female of *T. drosophilae* was added to each Petri dish, and the wasp oviposition was recorded using a digital video recorder (Lupustec LE 800 4 K, LupusElectronics GmbH, Landau, Germany) for six hours. To reduce possible self-superparasitisation, we used a shorter exposition time of the wasp to SWD pupae than in previously conducted studies[23,34–36]. The added female of *T. drosophilae* was either reared on *D. melanogaster* (*T. drosophilae* < melanogaster >) or *D. suzukii* (*T. drosophilae* < suzukii >). The oviposition events (host species and duration) were analysed using the event logging software BORIS[40]. An oviposition event was logged when the wasp pierced a pupa and did not move during that behaviour for a minimum of 30 s.

Eight Petri dishes were prepared for each wasp treatment. As a control, no wasps were added to eight Petri dishes with fly pupae. After the potential oviposition, the pupae were transferred individually to 96-well plates and the species (*D. melanogaster*, *D. suzukii*, or *T. drosophilae*) and the sex of the emerged insects were recorded. When only male wasps hatched from a repetition, it was assumed that the female wasp was unmated, and the repetition was excluded from the analysis. All experiments were conducted in the same climate-controlled chamber, under the same conditions as for the insects rearing (see "Insects").

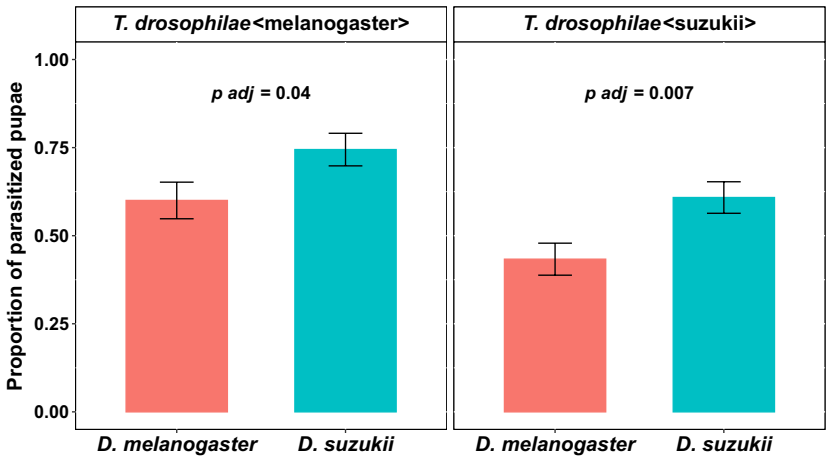

Figure 1. Proportion of parasitised pupae of *D. suzukii* (blue) and *D. melanogaster* (red) by the wasp *T. drosophilae*. The wasp was reared on either *D. melanogaster* pupae (left side) or *D. suzukii* pupae (right side) (Wilcoxon rank-sum test).

Sex ratio of emerged parasitoids. Previous studies observed a female-biased offspring sex ratio for wasps emerging from *D. suzukii* compared to *D. melanogaster*[34–36]. However, the size of the individual pupae was not measured in those studies. To confirm that the sex ratio of *T. drosophilae* is pupal-size dependent, we recorded in both experiments the sex of the emerged parasitoids. The measured size of each pupa was then used to determine this dependency.

Statistical analysis. The effect of the *Drosophila* species *D. suzukii* and *D. melanogaster* and of the pupal size on the number of parasitised pupae and the number of successful parasitisations by *T. drosophilae* females was analysed using a binomial generalised linear mixed model (GLMMs) in the R package lme4[41]. The model was used for the effect of pupal size on the sex of emerged wasps. As the interaction between wasp type and host species in the GLMM was not significant, it was excluded from further analyses. Separate GLMMs for each wasp type were performed. Female wasps without observed parasitisation or with only male offspring were excluded from testing. The parasitised pupae and number of successful parasitisation events for each wasp treatment and host species were compared using the Wilcoxon rank-sum test. Data were analysed in R 3.6.1[42].

Results

Observed oviposition preference. When the *T. drosophilae* females from the two populations (reared either on *D. melanogaster* or on *D. suzukii*) had the choice between *D. suzukii* and *D. melanogaster* as a host, significantly more pupae of *D. suzukii* were parasitised (*T. drosophilae* < melanogaster > W = 3465, *p adj* = 0.04; *T. drosophilae* < suzukii > W = 5940, *p adj* = 0.007, Fig. 1). Independent of the host species, *T. drosophilae* < melanogaster > parasitised significantly more pupae than *T. drosophilae* < suzukii > (Wilcoxon rank-sum test; W = 24,870, $p = 0.002$).

The preference of *T. drosophilae* < melanogaster > was not influenced by the pupal size of *D. suzukii* ($p = 0.89$) and *D. melanogaster* ($p = 0.44$) (Fig. 2). The *T. drosophilae* < suzukii > preference was also not influenced by the pupal size of *D. suzukii* ($p = 0.89$). It was, however, influenced significantly by the size of the *D. melanogaster* pupae ($p = 0.003$; Fig. 2, Table S1).

Parasitisation success. The number of emerged wasps out of previously parasitised pupae is given by the parasitisation success. *T. drosophilae* tend to have a higher parasitisation success in pupae of *D. melanogaster* compared to those of *D. suzukii*, although the difference is not significant (W = 2089, *p adj* = 0.16 and W = 2124, *p adj* = 0.18, Fig. 3). Independent of the pupae species, the wasp strain reared previously on *D. melanogaster* (*T. drosophilae* < *melanogaster* >) had a significantly higher parasitisation success than *T. drosophilae* reared on *D. suzukii* (*T. drosophilae* < suzukii >; Wilcoxon rank-sum test; W = 9410.5, $p < 0.001$).

The parasitisation success of *T. drosophilae* < melanogaster > was negatively influenced by the pupal volume of *D. melanogaster* pupae (Fig. 4, Table S2). This means that the probability of successful development of a wasp decreases with increasing pupal size of flies. Also, with an increasing pupal size of *D. suzukii* pupae, a visible tendency was observed for a decrease in the successful parasitisation of *T. drosophilae* < melanogaster > . The parasitisation success of *T. drosophilae* < suzukii > was not influenced by the pupal size of *D. melanogaster* pupae. However, in *D. suzukii* pupae, a slight tendency is observed that the successful parasitisation decreases with increasing pupal volume.

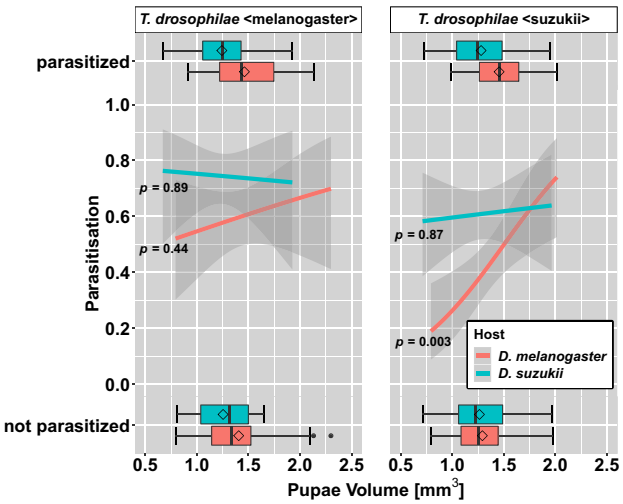

Figure 2. Proportions with a 95% confidence interval of parasitised *D. suzukii* (blue) and *D. melanogaster* (red) pupae in relation to pupae volume. The wasp *T. drosophilae* was reared either on *D. melanogaster* (left side) or *D. suzukii* pupae (right side). The variation of the volume of parasitised and not parasitised pupae volume is given in the box plots on top and bottom (for GLMMs see Table S1).

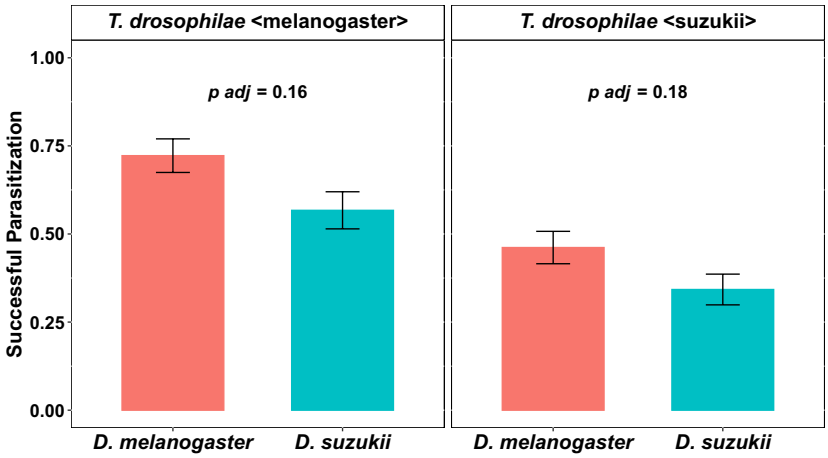

Figure 3. Mean (± SEM) proportion of successful parasitisation of pupae of *D. suzukii* (blue) and *D. melanogaster* (red) by the wasp *T. drosophilae*. The wasp was reared on *D. melanogaster* (left side) pupae and *D. suzukii* pupae (right side). No significant differences were observed between the successful parasitisation of pupae of *D. melanogaster* and *D. suzukii* with females of *T. drosophilae* reared on *D. melanogaster* or *D. suzukii* (Wilcoxon rank-sum test).

Emergence of *Drosophila* from parasitised pupae. To evaluate whether the high parasitisation success of the parasitoid, as described in "Parasitisation success", also means a lower probability that flies emerged out of the parasitised pupae, we calculated the proportion of emerged flies out of the parasitised pupae. On average, 20–30% flies emerged from the *D. melanogaster* pupae parasitised from the two different reared wasp strains. For the parasitised *D. suzukii* pupae, in both wasp strains, significantly fewer flies emerged (*T. drosophilae* < *melanogaster* > : W = 2184, *p adj* = < 0.001; *T. drosophilae* < suzukii > : W = 2414, *p adj* = < 0.001, on average 1–5% (Fig. 5).

Sex of emerged wasps depending on host pupal volume. To see if the sex ratio of emerged *T. drosophilae* can be potentially modified, we plotted the sex ratio of the wasps to the volume of the pupae, out of which the wasps emerged. The pupae volume of *D. suzukii* and *D. melanogaster* in a no-choice situation had a significant increasing effect on the female-biased sex ratio (Fig. 6). However, when the wasps had the choice between

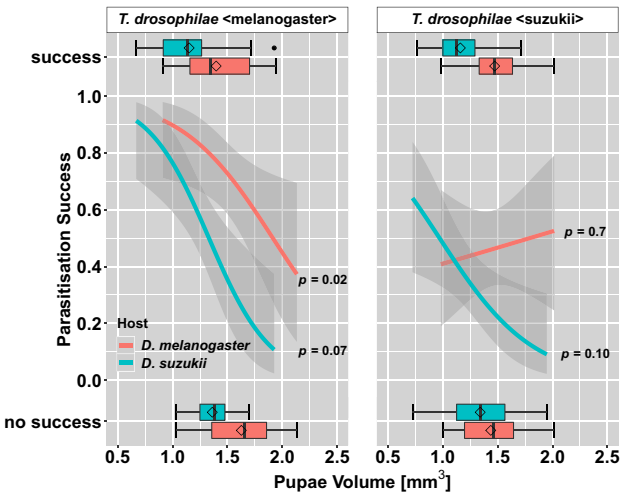

Figure 4. Proportions with a 95% confidence interval of parasitisation success on *D. suzukii* (blue) and *D. melanogaster* (red) pupae in relation to pupal volume. The wasp *T. drosophilae* was reared on *D. melanogaster* pupae (left side) and *D. suzukii* pupae (right side). The variation of success and no success of parasitisation to the volume of parasitised pupae is given in the box plots on top and bottom (for GLMMs see Table S2).

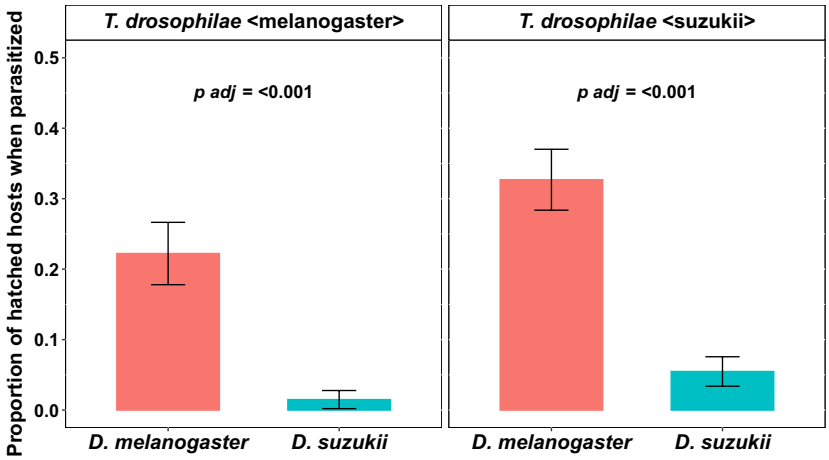

Figure 5. Mean (± SEM) Proportions of emerged hosts from parasitised hosts. The hosts were pupae of *D. suzukii* (blue) and *D. melanogaster* (red), parasitised from the wasp *T. drosophilae*. The wasp was reared on *D. melanogaster* (left side) pupae and *D. suzukii* (right side) pupae. In both wasp populations, *D. melanogaster* had a significantly higher proportion of emerged flies than *D. suzukii* (Wilcoxon rank-sum test).

the pupae of both *Drosophila* species (*D. melanogaster* and *D. suzukii*), the pupal size did not affect the sex ratio (Fig. 7). Furthermore, the sex ratios of the emerged wasps in the two *Drosophila* species were not significantly different, under the choice test set up (post hoc Tukey test: $p = 0.178$).

Discussion

Our results show that the parasitoid wasp *T. drosophilae* has an oviposition preference for pupae of the invasive pest *D. suzukii* over those of the widespread fly *D. melanogaster*. The preference for the invasive pest was regardless of the host species on which the wasps were reared. Furthermore, we can exclude the pupal volume as the reason for that species preference because the pupal size did not affect the oviposition preference, except for *D. melanogaster* pupae parasitised by *T. drosophilae* reared on *D. suzukii*. Even when the pupae of the two *Drosophila* species were adjusted to be similar in size, there was still a significant preference for the *D. suzukii* pupae. In total, we can conclude that the choice of *T. drosophilae* wasps for *D. suzukii* is a real preference for the species and not a preference for larger pupae as concluded in some studies[34,36].

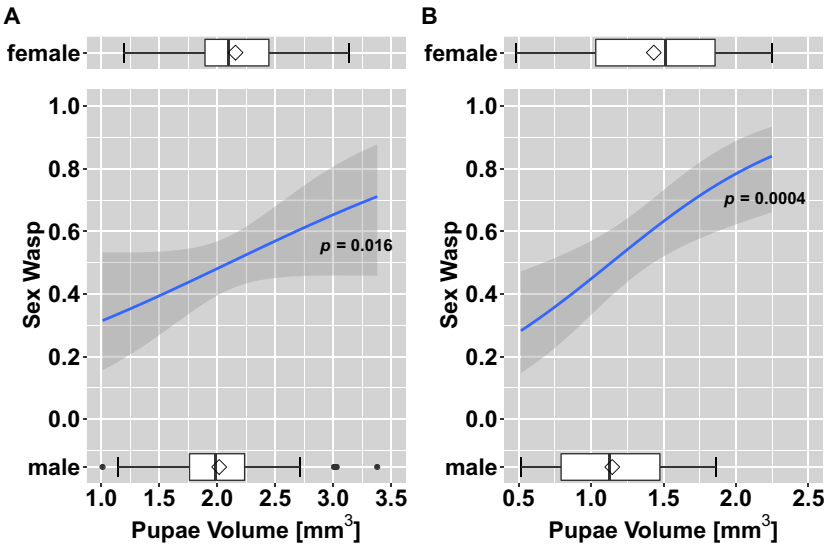

Figure 6. Sex of emerged *T. drosophilae* from a no choice test situation depending on the host pupal volume of A: *D. suzukii* and B: *D. melanogaster*. Boxplots give the variation in size for the pupae from which male and female wasps emerged. The curve is an estimated proportion of the sex as a function of pupal volume with a 95% confidence interval (binomial GLMM, Table S3).

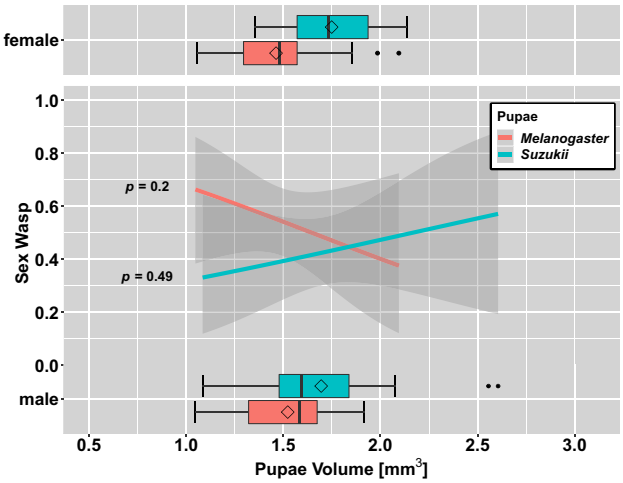

Figure 7. Sex of emerged *T. drosophilae* from a choice test situation depending on the host pupal volume of *D melanogaster* (red) and *D. suzukii* (blue). Boxplots give the variation in size for the pupae from which male and female wasps emerged. Curves are estimated proportions of the sex as a function of pupal volume with a 95% confidence interval (binomial GLMM, Table S3).

A preference of *T. drosophilae* for *D. suzukii* was reported in previous studies[34–36]. However, those studies did not measure the pupal size, and the preference was based on the number of emerged wasps and flies, not on direct observation of oviposition. This difference is important because our data show that direct observations are more accurate than the traditionally used measures: the degree of infestation (DI), which measures the proportion of successfully parasitised hosts, and the success of parasitism (SP), which measures the proportion of emerged wasps out of parasitised pupae. The DI was always higher than the here observed oviposition, and the SP was always lower than the here observed parasitisation success (Figure S1, Figure S2). DI and SP are consequently less adequate when evaluating wasp parasitisation success. One of the reasons is that defining the preference of a parasitoid by the number of emerged parasitoids does not take into account that a wasp larva has different

success rates in different *Drosophila* hosts. Direct observation of the parasitoid's preference using video recordings should thus be the preferred method for an accurate analysis of parasitisation of fly pupae.

One possibility of how parasitoids wasps can distinguish between pupae of different host species is that they could use species-specific chemical cues of the pupae[43]. Romani, et al.[44] observed for *T. drosophilae* that the host's chemical cues of the anterior spiracles of *D. melanogaster* are probably the most important cue for host recognition. In *D. suzukii* pupae, these anterior spiracles have seven to eight radially arranged branches[6] and are thus more structured than they are in *D. melanogaster*. The anterior respiratory spiracles are especially crucial because the anterior part of the pupae of *D. suzukii* is orientated outside of fruit or soil, and the soil is the most common pupation location of SWD[45]. Therefore, this is the only part of the pupae the wasp antenna has physical contact to during the searching process and, consequently, it may be crucial for the pupae recognition and perhaps pupae species recognition.

Furthermore, we noticed during observation of the parasitisation that *T. drosophila* wasps seem to make their decision to parasitise predominantly after drumming with their antennae over respiratory spiracles of the pupae. This *T. drosophilae* behaviour was also observed by Romani, et al.[44] and is in accordance with other studies stating that, during direct contact of the parasitoid with the host, the host's form and texture are essential for host selection and acceptance[46,47]. So, we can assume that the preference for *D. suzukii* is probably due to a combination of chemical and physical cues of the pupa's anterior spiracles, which mediates the parasitoid's recognition of a host.

The lower parasitisation success rates of the *T. drosophilae* wasp in *D. suzukii* compared to those in *D. melanogaster* could be due to a different immune resistance of the two fly species. Kacsoh and Schlenke[18] and Poyet, et al.[48] found a higher haemocyte load and lower encapsulation rates in *D. suzukii* larvae than in *D. melanogaster* larvae when they were parasitised with larval parasitoids. Furthermore, at least in *D. melanogaster,* the immune system of the pupae is different from the larval immune system, for example, the haemocytes undergo morphological changes in the pupal stage[49]. The immune system of *D. suzukii* pupae has not yet been studied, but we observed these morphological changes of the haemocytes also in *D. suzukii* pupae (unpublished results).

The lower success rate of *T. drosophilae* in pupae of *D. suzukii* does not benefit the host. It was exceptional for an adult fly to emerge out of a parasitised *D. suzukii* pupa. Such high mortality of flies during their development was not observed for *D. melanogaster.* This lack of survival advantage for *D. suzukii* was also observed by Kacsoh and Schlenke[18] and Iacovone, et al.[50]. The reason that nearly no parasitised pest fly emerged could be a hyperactive immune system in *D. suzukii*, as a hyper-activation of the JAK/STAT signalling pathway was observed to trigger self-encapsulation in *D. melanogaster* larvae[51].

However, self-encapsulation was not observed until now in pupae of *Drosophila* and is unlikely because the key haemocytes for encapsulation, the lamellocytes, are no longer present in the pupae and cannot be induced by injury[49]. Although the larval immune system of *D. suzukii* is known to resist parasitoids strongly, this effect has not yet been studied for its pupae. Further research is needed to determine whether, in general, the pupae of *D. suzukii* also have in comparison to other *Drosophila* a stronger resistance (low parasitisation success) against pupal parasitoids. It remains unclear whether the survival disadvantage of the pest fly is due to a hyperactive immune system or possibly due to the venom of the pupal parasitoid injected during parasitisation, which affects the *D. suzukii* pupae.

In parasitisation tests of *T. drosophilae* on pupae of different volume of either *D. melanogaster* or *D. suzukii* (no-choice tests), the sex of the emerged wasp depends on the pupal size: Out of larger hosts, female parasitoids predominately emerged, whereas male wasps predominantly emerged out of smaller hosts. So, the probability that a diploid (fertilised) egg was oviposited by a parasitoid female increased with increasing host pupal volume (Fig. 6). In several parasitoid systems, it was observed that the sex ratio shifts in larger hosts to be more female-biased[52–54]. The larger hosts give increased fitness for both sexes of the parasitoid; however, this increase is greater for female wasps than for male wasps[52]. Consequently, an ovipositing female should lay female offspring in larger hosts, which is what we observed for *T. drosophilae*.

However, in choice tests where wasps could decide between differently sized pupae of the two host species, we found no effect of the host size on the sex of the parasitoids offspring. This could be due to the decision to oviposit in different species being dominant over the sex ratio adjustment of a female *T. drosophilae*. Therefore, the effect of host size on sex ratio is missing in the species choice test. Boycheva Woltering, et al.[35] also found a higher female-biased sex ratio for *T. drosophilae* emerging from *D. suzukii* pupae than from those of *D. melanogaster* or *D. immigrans* under choice situations. In a no-choice situation, the sex ratios were similar for all three hosts. However, in those tests, they did not adjust the host size or measured the variance in host size. Therefore, in their study, the effect of host size on the sex ratio of the parasitoid offspring remains unclear.

For a mass-rearing, a high female-biased sex ratio is beneficial, especially for the last wasp generation, which will be released into the field. An adjustment to a higher female-biased sex ratio appears to be achievable by rearing the wasps on hosts with larger pupal sizes. Furthermore, the species for mass rearing can be *D. melanogaster* which is more accessible and the host species does not appear to negatively influence the parasitoid's preference for *D. suzukii*.

Conclusion

In the last years, the pest species *D. suzukii* causes massive agricultural losses worldwide. An effective control agent for *D. suzukii* in an IPM approach could be the parasitoid wasp *T. drosophilae*.

Here, a preference of *T. drosophilae* for the pest is essential for the success of a parasitoid release under field conditions. We could show that *T. drosophilae* has a significant preference for *D. suzukii* and that this preference is independent of the pupae size and the fly species on which the wasps were reared. Also, the probability of a successful parasitisation of *T. drosophilae* is not affected by the previous hosts. We, therefore, conclude that, for

mass rearing of the wasps, there is no benefit from using *D. suzukii* as a host. Instead, *D. melanogaster*, which is easier to handle and to rear, can be used to mass-rear the wasps.

The efficiency of a parasitoid wasp as a biocontrol agent also depends on the ratio of female wasps. Here we show that large-sized *D. melanogaster* pupae can be used to increase the proportion of female *T. drosophilae*, reared either on *D. suzukii* or *D. melanogaster*.

Overall, our study showed that *T. drosophilae* is a generalist parasitoid with a preference for *D. suzukii* over the very common fly *D. melanogaster*. This preference makes this parasitoid an even more promising candidate as a biocontrol agent in an IPM for *D. suzukii*.

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B.J.M.H. and J.S. designed the research plan. J.L. and B.J.M.H. conducted the experiments. B.J.M.H. analyzed the data and wrote the first manuscript. J.L. and J.S. edited the manuscript. All authors read and approved the manuscript.

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5.2 Publication 2: Below ground efficiency of a parasitic wasp for *Drosophila suzukii* biocontrol in different soil types

Below ground efficiency of a parasitic wasp for *Drosophila suzukii* biocontrol in different soil types

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B.J.M.H. and J.S. designed the research plan. M.M. and B.J.M.H. conducted the experiments. B.J.M.H. analysed the data and wrote the first draft of the manuscript. M.M. and J.S. edited the manuscript. All authors read and approved the manuscript.

Own contribution: Concept and study design: 85%, data acquisition 50%, data analysis and figures 90%, interpretation of results 85%, manuscript writing 90%.

Supplementary: Information 1 (R Notebook), Information 2 (Data), Supplementary Tables

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Below ground efficiency of a parasitic wasp for *Drosophila suzukii* biocontrol in different soil types

Benedikt J. M. Häussling✉, Melinda Mautner & Johannes Stökl

The parasitoid wasp *Trichopria drosophilae* is promising as a biocontrol agent for controlling the ubiquitous pest *Drosophila suzukii* (Matsumura). Crucial for the successful implementation of any biocontrol agent is a high parasitisation rate by the parasitoid. Most studies investigating the parasitisation rate of *D. suzukii* pupae have focused on parasitisation in the fruit or in a petri dish. However, the predominant pupation site of *D. suzukii* in the field is the soil. Unfortunately, little is known on how well parasitoid wasps can detect and parasitise pupae of *D. suzukii* buried in the soil. Therefore, we conducted soil parasitisation experiments of *T. drosophilae* on *D. suzukii* pupae using two pupation depths in three different soil types (loamy sand, loam, and clay). In all three soil types, we found generally low *D. suzukii* pupae parasitisation rate by *T. drosophilae*, independent of the pupation depth. The pupation behaviour of *D. suzukii* and the parasitisation behaviour of *T. drosophilae* are discussed in detail. For pest control in most soil types, our results mean that the number of *D. suzukii* larvae pupating in the soil should be reduced, e.g., by adding a layer of sandy soil or covering the soil with plastic mulch. This might increase the probability of success when using *T. drosophilae* as a biocontrol agent.

The cosmopolitan fruit pest *Drosophila suzukii*, also called spotted wing drosophila, is still a major challenge for farmers worldwide. Especially in years with favourable conditions for the pest, the risk of total yield losses can be high[1,2]. Therefore, functional integrated pest management methods are essential to control the pest[3,4]. Consequently, extensive knowledge is required for a broad range of different possible control methods. One promising candidate is larval and pupal parasitoids[5–9]. Parasitoids, mostly wasps, lay their eggs in or on a host, for example in the larvae or pupae of *D. suzukii*. The larvae of the parasitoid then feed on the host and eventually kill it.

One advantage of parasitoids as pest control is that they can be mass-reared and released at a certain date. Thus, population growth can be controlled if release is early in the growing season[9]. Especially for *D. suzukii*, early control is necessary because its population can be high in the surrounding habitats and the insects thus mass invade the fruits when they are nearly ripe[10]. In field and laboratory studies, naturally occurring parasitoids, such as the pupal parasitoids *Pachycrepoideus vindemiae* and, especially, *Trichopria drosophilae*, have proved promising results in controlling *D. suzukii*[8,11–15]. The pupal parasitoid *T. drosophilae* can parasitise the pupae of *D. suzukii* during the entire pupal development time[16]. A crucial ability of the parasitoid during parasitisation is locating the host pupae. The location of the pupae of *D. suzukii* can be directly in the fruit[12], but especially in the field, the large majority of the larvae pupate in the soil underneath the fruit plant[17,18]. This location means that the parasitoid needs to be able to locate the pupae in or near the fruit and in the soil matrix.

Guillén, et al.[19] found that *P. vindemiae* could only locate pupae of the Mexican fruit fly *Anastrepha ludens* when the pupae were on the soil surface. They could not locate them when they were in the soil. In contrast, another study found that *P. vindemiae* and *T. drosophilae* can parasite *D. suzukii* pupae in the soil[12]. In their study, Wang, et al.[12] studied the parasitisation rate of *D. suzukii* pupae in fruits and the soil. However, it is unclear whether the pupae were actually buried in the soil or lay accessible on the soil surface. Furthermore, neither Guillén, et al.[19] nor Wang, et al.[12] studied the parasitisation rate in different soil types. Therefore, it is still unclear in what soil type and to which soil depth *T. drosophilae* is capable of finding and parasitising the pupae of *D. suzukii*.

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To answer this question, we investigated the pupation behaviour of *D. suzukii* and the parasitisation rate of *T. drosophilae* in three different standardised soil types (loamy sand, loam, and clay) with the same soil moisture and at two soil depths (0–6 mm and 7–12 mm). Furthermore, the hatching rate of *D. suzukii* was assessed under these soil conditions.

Material and methods

Insects. *Drosophila suzukii* were caught in the state of Hesse, Germany, in 2016. The parasitoid wasps *T. drosophilae* were provided by Bioplanet s.r.l. (Cesena, Italy). *D. melanogaster* was the host for the *T. drosophilae*. The *D. suzukii*, *D. melanogaster* and *T. drosophilae* were reared and kept under the conditions as described in Häussling, et al.[15].

Standard soil types. Three different standard soils were used. The soils were chosen to be distinctly different in particle size distribution. According to the United States Department of Agriculture (USDA) classification, the soil types used for these experiments were: loamy sand, loam, and clay (Supplementary Table 3). Using these soil types ensured different physical properties for the fly larvae when they pupated and for the wasp when they searched and parasitised pupae in the soil. Soils were obtained from the "Landwirtschaftliche Untersuchungs- und Forschungsanstalt" (LUFA) in Speyer, Germany.

The Water Holding Capacity (WHC) of all soil types were measured (dried at 105 °C), and then the soils were then adjusted to 40% of the maximal WHC for each soil type. This percentage was the optimal soil moisture for finding the pupae in the soil after the parasitisation exposure. In soil with higher soil moistures, finding the pupae in the soil was challenging.

Experimental set-up. Plastic boxes (135 mm × 80 mm × 120 mm) were used as arenas for the parasitisation. The bottom of the box was filled with soil to 3 cm and covered with a plastic net with 3 mm mesh size. This layer was included to decrease temperature effects from the bottom of the boxes. Above the net, a layer of 6 mm of one of the three standard soils was added, then one more plastic net and 6 mm of the top layer of the same standard soil. The box was closed with a fine mesh net secured by a rubber band on the top of the plastic box. The top had a hole in the middle to allow airflow while the mesh prevented the escape of the flies and wasps.

We tested the parasitisation and pupation rates in the three soil types by providing each experimental box with 50 *D. suzukii* larvae of the 3rd instar that could decide freely in which of the two soil depths they pupated. To test whether the time of the parasitisation influenced the parasitisation rate, we either directly added five female wasps to the larvae or waited for 24 h before releasing the wasps, allowing the larvae to pupate first. The wasps had 24 h to parasitise the pupae in the soil. We did n = 57 replicates, 19 for each soil type, with 50 *D. suzukii* each. To determine the pupation rates without parasitisation, we performed a negative control treatment (n = 39, 13 for each soil type, with 50 *D. suzukii* each). Here, no wasps were released in the boxes. As a positive control, we also analysed the parasitisation rate when all pupae were easily accessible and not buried in the soil. For this positive control (n = 5), 30 pupae of *D. suzukii* were offered on a wet filter paper in a Petri dish to three female *T. drosophilae*. In all treatments, the wasps were provided with a drop of diluted honey and the boxes were placed in a greenhouse. The temperature and the humidity were logged during the investigation.

The pupae of each soil depth and type were collected separately after 24 h of parasitisation time. This time was observed to be sufficient for a successful parasitisation of *D. suzukii*[11,15,16,20,21]. The pupae were photographed next to a precise ruler for scale to determine the pupal size. The length and width of each pupa were measured with the software ImageJ, and the volume was calculated with the formulae from Otto and Mackauer[22]. Afterwards, the pupae were stored in a 96-well-plate in a light-controlled chamber at 24 °C and 70% to 80% RH with a 16:8 h day to night rhythm. The sex of the emerged *D. suzukii* and *T. drosophilae* was recorded.

Statistical analysis. The effect of the soil type, the soil depth and the presence of a wasp on the proportion of hatched *D. suzukii* was analysed using a binomial generalised linear mixed model (GLMMs) in the R package 'lme4'[23]. The GLMMs were also used to analyse the effect of soil type and pupation depth on the proportion of hatched *T. drosophilae* and of soil type on *D. suzukii* larvae pupated in the upper soil layer. In both analyses, we added the pupae from each box as a nested random effect. As the interactions among the predictors in the GLMMs were not significant, they were excluded from further analyses. We also found no influence of the time of wasp release and therefore did not differentiate between the two time points in our analyses (see Supplementary Tables 1, 2). Data were analysed in R 3.6.1[24].

Results

Pupation depth of *Drosophila suzukii* larvae. Pupation on the soil surface was an exception, so include them in the pupation depth 0–6 mm. The larvae of *D. suzukii* differed in their pupation depth depending on the soil types ($p = < 0.001$, Table 1, Fig. 1). In sandy soils, nearly all (median: 96%) pupae pupated in the upper soil layer; in loam soils, the median was 72%, and in clay, the median in the upper layer was 58%. The pupation depth in the sandy soil was significantly different from that in loam and clay soil types. However, the result in the latter two did not differ (Table 2). Additionally, we found that the pupae volume did not affect the pupation depth ($p = 0.75$, Table 1).

Hatching rate of *Trichopria drosophilae*. The proportion of emerged *T. drosophilae* was low for all soil types and pupation depths (Fig. 2). The soil type ($p = 0.72$) and the pupation depth ($p = 0.11$) had no effect on the proportion of emerged wasps (Table 3). In loamy sand, wasps hatched, on average, out of 1.8% of the pupae,

Predictor	χ^2	*df*	*p*-value
Soil type	61.06	2	**< 0.001**
Pupal volume	0.11	1	0.75

Table 1. Pupation depth—generalised linear mixed effect model (family = binomial, link = logit, random factors: "Box/pupae volume", "mean temperature") examining the effect of soil type and pupal volume on the proportion of larvae (*D. suzukii*) pupating in the upper soil layer. Significant values are in bold.

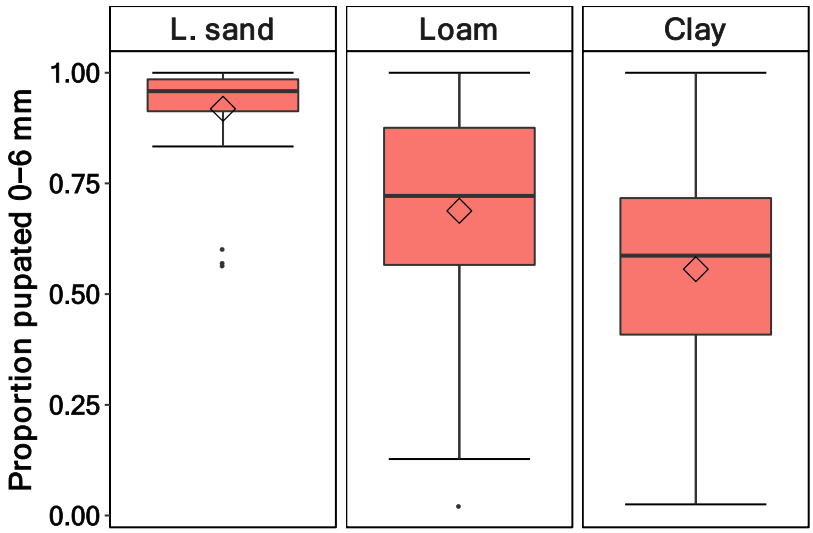

Figure 1. Proportion of *Drosophila suzukii* pupae that pupated in the upper layer (0–6 mm) in relation to the lower layer (7–12 mm). The larvae pupated in the three different soil types: loamy sand, loam and clay (n = 96).

Multiple comparison	Estimate	SE	*Z*	*p*-value
l. sand–loam	2.23	0.39	5.70	**< 0.001**
clay–loam	− 0.76	0.36	− 2.08	0.10
clay–l. sand	− 2.98	0.39	− 7.63	**< 0.001**

Table 2. Multiple comparison between soil types of the pupated pupae. Test: Tukey Honest Significant Difference. Significant values are in bold.

followed by clay with 4.5% and loam with 5.1% (Fig. 2). In contrast, in the positive control, where the wasps had free access to the pupae in a petri dish, the mean hatching rate was much higher (36%). The wasp hatching rate of the two pupation depths was similar for loamy sand (0–6 mm 1.5%; 7–12 mm 2.2%). In loam (0–6 mm 8.3%; 7–12 mm 1.9%) and clay soil (0–6 mm 6.5%; 7–12 mm 2.5%, Fig. 3), the difference was more distinct but also not statistically significant.

Hatching rate of *Drosophila suzukii*. There was no difference in the hatching rates of *D. suzukii* between the negative control and the wasp treatment ($p = 0.11$, Table 4). Furthermore, neither the proportion of emerged *T. drosophilae* nor the soil type ($p = 0.08$) affected the emergence rates of *D. suzukii*. Only the pupation depth affected the proportion of hatched flies ($p = 0.005$), with more flies hatching out in the deeper soil layer ($p = 0.02$, Table 5, Fig. 4). The median of the hatching rate was between 34.2 and 51.4% (Fig. 5). In contrast, in the positive control, in which the wasps had free access to the pupae, the hatching rate of the flies was much lower, with a median of 6.7%.

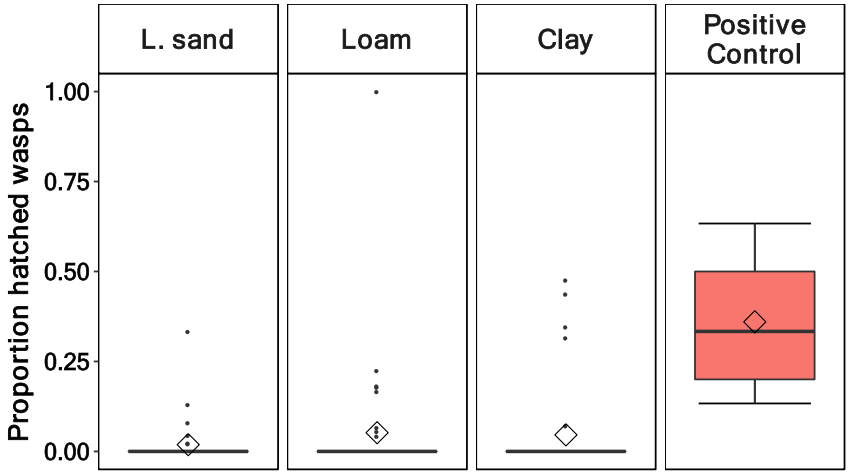

Figure 2. Proportion of hatched *Trichopria drosophilae* wasps out of pupae of *Drosophila suzukii* in the three soil types (loamy sand, loam, and clay) and the positive control where the wasp had free access to the pupae in a petri dish. (Soil types: n = 57, positive control: n = 5).

Predictor	χ^2	*df*	*p*-value
Soil type	0.68	2	0.71
Pupation depth	2.53	1	0.11

Table 3. Hatched wasps—generalised linear mixed effect model (family = binomial, link = logit, random factors: "Box/pupae number", "Percent Pupated") examining the effects of pupation depth and soil type on the hatching rate of the wasp *T. drosophilae* on *D. suzukii* pupae.

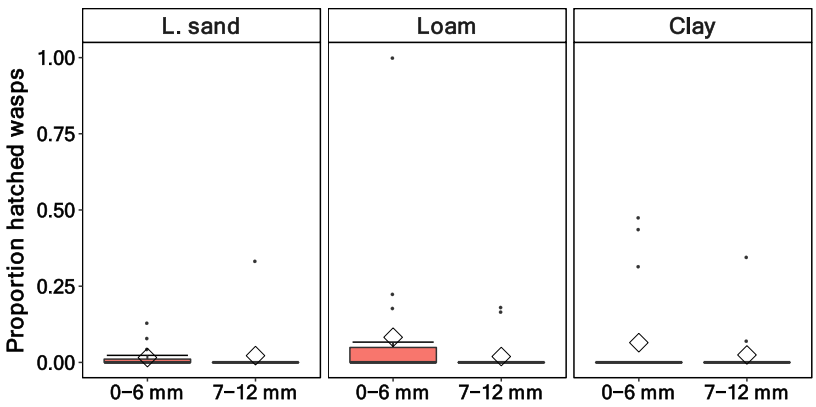

Figure 3. Proportion of hatched *Trichopria drosophilae* wasps between two different pupation depths (0–6 mm and 7–12 mm) in the soil types loamy sand, loam and clay (n = 57).

Discussion

The pupae of *D. suzukii* buried in soils were rarely parasitised by the wasp *T. drosophilae* in all three soil types, with wasps emerging from only 1.8% to 5.1% of the fly pupae. This result clearly demonstrates that the parasitisation of host pupae in soils through *T. drosophilae* is an exception. The few hatched wasps were mainly from the upper soil layer. When the wasp had free access in the positive control, the percentage of emerged wasps raised to 36%. This range was also observed from Chabert, et al.[7].

Predictor	χ^2	*df*	*p*-value
Soil type	5.10	2	0.08
Pupation depth	7.87	1	**0.005**
Treatment	2.51	1	0.11

Table 4. Hatched flies—generalised linear mixed effect model (family = binomial, link = logit, random factors: "Box/Pupae number", "Percent pupated", "Mean Temperature") output quantifying the effect of pupation depth, soil type and the treatment (with wasp/without wasp) on the hatching rate of the fly *D. suzukii*. Significant values are in bold.

Multiple comparison	Estimate	SE	Z	*p*-value
7-12–0-6 mm	0.35	0.13	2.81	**0.02**

Table 5. Multiple comparison between pupation depth of the hatched flies. Test: Tukey Honest Significant Difference. Significant values are in bold.

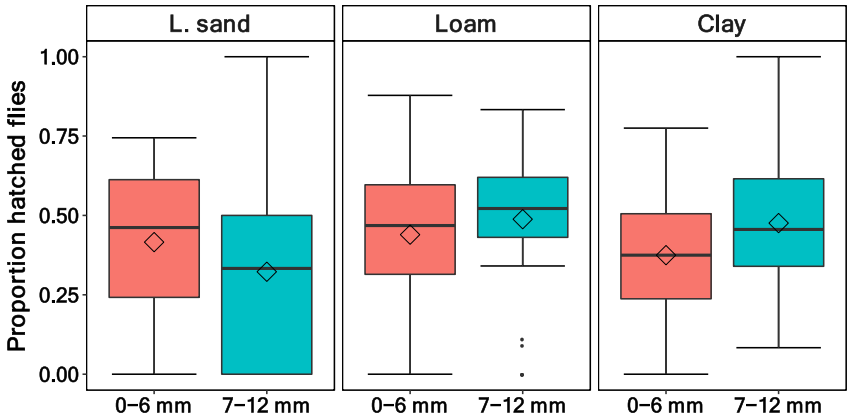

Figure 4. Proportion of hatched *Drosophila suzukii* flies between the two pupation depths 0–6 mm and 7–12 mm and the three soil types (loamy sand, loam and clay; n = 96 as waps and control treatments showed no difference).

These findings are consistent with the earlier results of Guillén, et al.[19], who observed very low parasitisation of pupae of the Mexican fruit fly *Anastrepha ludens* by the pupal parasitoid *Pachycrepoideus vindemiae* in the soil. In fact, in their study, the parasitisation of pupae only happened on the soil's surface. In our experiments, we had nearly no pupation on the top of the soil. Therefore, the few parasitisations in our study mainly happened in the soil. In contrast, Wang, et al.[12] found high parasitisation rates of *D suzukii* pupae in soils by *T. drosophilae.* (approx. 55%) and *P. vindemiae* (approx. 30%), but they did not differentiate between soil types or pupation depths. Perhaps the pupae were easily accessible on the soil surface in their study.

The low proportion of hatched wasps in our study is probably due to the physical properties of the soil, which hamper the wasps' ability to pass the soil matrix and parasitise the host pupae. Another possibility is, although it has yet to be verified, that *T. drosophilae* probably recognises its host through kairomones, and the kairomones are reduced when the host pupae are buried in the soil. This reduction is could be caused by the complex medium soil, where semiochemicals do not diffuse to outside the soil[25].

The number of hatched flies did not differ between the control treatment and the wasp treatment in soils (Fig. 5). In contrast, the number of emerged *T. drosophilae* wasps was seven times higher in the positive control without soil than in the treatment with soil (Fig. 2). Therefore, we conclude that the low number of hatched wasps is due to low parasitisation rates, rather than a difference in the flies' immune responses between soil treamtents and the control.

Previous studies have shown that the majority of *D. suzukii* larvae pupate in the soil and less often near or in the fruit[17,18]. It seems that the choice of the larvae's pupation site depends on the interspecific competition of the fly larvae: more larvae pupate outside of the fruit with increasing competition[26]. In our experiment, we found that most larvae, which had only the choice of pupating in the soil, pupated in the upper 0–6 mm soil layer (only a few on the top). We did not expect high interspecific competition in our experiment due to the relatively low

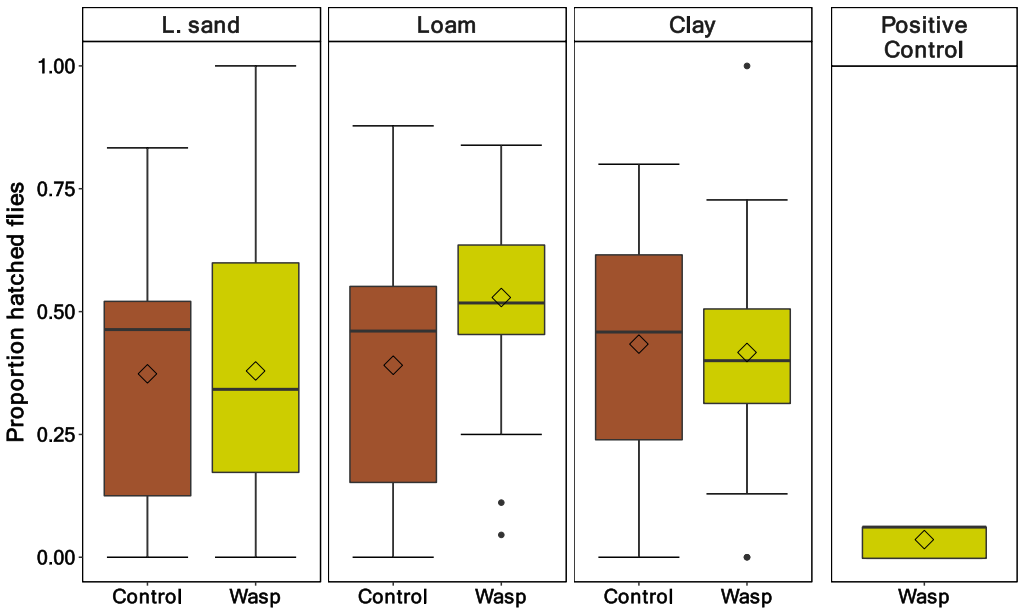

Figure 5. Proportion of hatched *Drosophila suzukii* flies from the three soil types (loamy sand, loam, and clay). Pupae were either exposed to the parasitoid wasp (Wasp) or not (Control). The fourth panel displays the number of hatched *D. suzukii* in the positive control, where the wasps had free access to the pupae in a petri dish (wasp: n = 57, control: n = 39, positive control: n = 5).

number of larvae. The larvae appear to choose their pupation depth differently depending on the soil type. In sandy soils, they pupate almost exclusively in the upper soil layer; in clay and loam soil, pupation also happened in the lower soil layers. Renkema and Devkota[27] also found that, in the field, the majority of larvae pupate in the upper soil layer, especially in saturated sandy soils. In contrast, in dry sandy soils, most larvae either desiccated or pupated on the soil surface. The pupation behaviour of *D. suzukii* larvae is affected by the soil type. In soils with smaller particles sizes (e.g., clay soil), *D. suzukii* larvae had a deeper pupation depth than in soils with larger particle sizes (Fig. 1, e.g., sandy loam, Supplementary Table 3). A possible explanation could be that the presence of predominantly larger soil particles hampers larvae movement. We observed that, particularly in sandy soils, several soil particles were attached to the pupae, possibly decreasing the movement ability of the larvae. This hypothesis is supported by the fact that in *Bactrocera oleae*, increasing soil particle size was found to reduce pupation depth[28,29].

Genetic analyses confirmed that a single gene can explain the pupation behaviour and preference for habitat differences (fruit or soil) in *D. melanogaster*[30,31]. The habitat choice of *D. melanogaster* is influenced not only by the soil water content but also by the air temperature and the fly strain. The larvae tend to choose the best suitable pupation site for emergence, which is high soil water content and has optimal temperature of 25 °C[32]. As for *D. melanogaster*, our results show the same effect for *D. suzukii* in which pupation in wet soils (40% WHC) also occurs in the upper soil layer. Furthermore, it is also possible that *D. suzukii* have a genetic polymorphism in their population that determines the pupation site similar to that of *D. melanogaster*. Interestingly, we found a significantly higher hatching rate for *D. suzukii* adults in the deeper soil layer. The higher hatching rates might arise from more favourable temperature conditions in deeper soil depth and/or, that only the physically fitter larvae can move to deeper soil layers.

In conclusion, this study shows that the soil is a massive barrier for the parasitoid *T. drosophilae* when parasitising its host.

Our results can be implemented in an integrated pest management method by adding a layer of sandy soil or a plastic mulch[33] on top of the ground under the fruit plants. This layer could decrease the hatching rate of *D. suzukii* due to the desiccation of the larvae and would expose the pupae to a range of antagonists, including *T. drosophilae*[18]. The area covered with the sandy soil can be minimal because *D. suzukii* larvae have a limited movement ability of less than 7.5 cm[34].

Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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Author contributions

B.J.M.H. and J.S. designed the research plan. M.M. and B.J.M.H. conducted the experiments. B.J.M.H. analysed the data and wrote the first draft of the manuscript. M.M. and J.S. edited the manuscript. All authors read and approved the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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5.3 Publication 3: Does the seasonal phenotype of *Drosophila suzukii* influence cellular immunity and parasitisation?

Does the seasonal phenotype of *Drosophila suzukii* influence cellular immunity and parasitisation?

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The study was initiated and designed by J.S. and B.J.M.H. N.R., E.K.K. and B.J.M.H. conducted the experiments. B.J.M.H. and N.R. analysed the data, and B.J.M.H. wrote the first manuscript. B.J.M.H. and J.S. edited the manuscript. All authors read and approved the manuscript.

Own contribution: Concept and study design: 85%, data acquisition 50%, data analysis and figures 90%, interpretation of results 90%, manuscript writing 90%.

Supplementary: Information (R Notebook and Data), Supplementary Tables

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ORIGINAL ARTICLE

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Does the seasonal phenotype of *Drosophila suzukii* influence cellular immunity and parasitisation?

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Abstract

Controlling the worldwide invasive pest *Drosophila suzukii* remains a challenge. One promising biological method for managing this pest is the use of larval and pupal parasitoids. Unfortunately, most of the larval parasitoids fail to successfully parasitise *D. suzukii* larvae in laboratory experiments due to the high immunity of the pest. So far, only the summer phenotype (summer morph) of *D. suzukii* has been tested for parasitisation. However, the winter phenotype (winter morph) is the dominant form of *D. suzukii* throughout the year in the northern hemisphere. Therefore, this study investigates the immunity during parasitisation for both phenotypes using the larval parasitoid *Asobara japonica* and the pupal parasitoid *Trichopria drosophilae*. It is the first to compare across all life stages the immunity of the winter phenotype to the summer phenotype of not only *D. suzukii* but also *D. melanogaster*. Our results indicate differences in the immunity between the two phenotypes for larvae, pupae, and adults. However, the degree and direction of these differences were inconsistent across the different life stages of *D. suzukii*. The findings have important implications for the integrated pest management (IPM) of *D. suzukii*.

KEYWORDS
biological pest control, haemocytes, immunity, phenoloxidase, spotted wing drosophila, winter morph

1 | INTRODUCTION

Pest management is an ongoing global challenge that requires effective and sustainable solutions (Omkar, 2016). One possible solution is the use of parasitoid wasps, which have been successfully employed in numerous biocontrol systems. A biocontrol method that can provide timely control is augmentative release, where the natural enemy is mass-reared and released periodically (Lenteren, 2003).

To implement a similar system, research has focused on parasitoids to control the cosmopolitan pest *Drosophila suzukii* in an augmentative biological control approach (Häussling et al., 2022; Knoll et al., 2017; Rossi Stacconi et al., 2015; Wang et al., 2016b, 2021). In contrast to *Drosophila melanogaster*, which aims at rotten fruits, the pest *D. suzukii* can cause quality and high yield losses in ripening and ripe thin-skinned fruits (Bolda & Goodhue, 2010; DiGiacomo et al., 2019; Farnsworth et al., 2017; Mazzi et al., 2017). The cosmopolitan pest is endemic to Southeast Asia and spread to Europe in 2008 (Calabria et al., 2012), North America in 2008 (Hauser, 2011), South America in 2013 (Deprá et al., 2014) and Africa in 2013 (Anfora et al., 2020).

Unfortunately, studies in recent years have shown low parasitisation success of parasitoids on *D. suzukii* larvae compared to

D. melanogaster larvae (Chabert et al., 2012; Kacsoh & Schlenke, 2012). In particular, larval parasitoids from the non-native regions, Europe and America, often have lower parasitisation success than those from native regions, such as China and Japan (Chabert et al., 2012; Girod, Borowiec, et al., 2018; Matsuura et al., 2018). For example, *Asobara japonica* from the native region of *D. suzukii* has a success rate of parasitism (SP) of over 90%, but *Asobara tabida* from France has a SP of 0% (Chabert et al., 2012).

One explanation for the high invulnerability of the *D. suzukii* larvae is probably the high haemocyte load of *D. suzukii* larvae compared to *D. melanogaster* larvae (Iacovone et al., 2018; Kacsoh & Schlenke, 2012; Poyet et al., 2013). Circulating haemocytes are blood cells that are responsible for detecting foreign organisms (e.g. parasitoid eggs) and rapidly forming multilayered capsules (encapsulation, lamellocytes), together with the phenoloxidase-mediated melanogenesis, they are part of the innate immune system (Carton et al., 2008). Interestingly, the invasive strains of *D. suzukii* in Europe and America have an even higher haemocyte load than those in the original distribution area in Asia (Poyet et al., 2013), probably due a bottleneck during the invasion event effect (Facon et al., 2011; Lee, 2002). This high load may enable the immune system of *D. suzukii* to resist parasitisation, particularly by parasitoids from regions outside the native range of *D. suzukii* (Kacsoh & Schlenke, 2012). Surprisingly, despite this high haemocyte load, encapsulation is delayed in *D. suzukii* compared to *D. melanogaster* (Iacovone et al., 2018).

The pest *D. suzukii* has two seasonal phenotypes: A summer and a winter phenotype, also known as summer and winter morphs. Adults of the winter phenotype have a longer lifespan at lower temperatures, have larger and darker bodies (Shearer et al., 2016; Wallingford & Loeb, 2016) and enter a temperature-dependent reproductive diapause (Rossi-Stacconi et al., 2016; Toxopeus et al., 2016; Zerulla et al., 2015). Furthermore, comparisons of the two phenotypes showed that the winter phenotype has an overexpression of detoxification genes in response to insecticides (Seong et al., 2022) and the summer morphs have a higher attraction to food odours (Schwanitz et al., 2022). The winter phenotype is adapted to colder conditions and is, therefore, the dominant phenotype at lower temperatures. In the Netherlands, for example, the winter phenotype is dominant from September to June (Panel et al., 2018). Therefore, the winter phenotype is likely to be the dominant phenotype when population control of *D. suzukii* is required for early spring in continental and oceanic climates. Population density control is more effective in early spring because the population is at a bottleneck after the winter (Rossi Stacconi et al., 2018; Rossi-Stacconi et al., 2016; Wiman et al., 2014). Furthermore, *D. melanogaster* also shows these seasonal phenotypes under the influence of the developmental temperature (Ayrinhac et al., 2004; Bouletreau-Merle et al., 1986; Gibert et al., 2000).

Consequently, for successful biocontrol in the given situation, parasitoids should be able to parasitise the winter phenotype. As mentioned above, only a few parasitoid species can overcome the larval immune response of the *Drosophila* larvae and successfully develop (Chabert et al., 2012; Gabarra et al., 2014; Girod, Borowiec, et al., 2018; Iacovone et al., 2018; Kacsoh & Schlenke, 2012; Wang et al., 2020). The most promising larval parasitoids are *Asobara japonica* (Biondi et al., 2020; Wang et al., 2021) *Leptopilina japonica* (Daane et al., 2016; Girod, Rossignaud, et al., 2018) and *Ganaspis brasiliensis* (Fellin et al., 2023; Wang et al., 2020) as well as the pupal parasitoids *Trichopria drosophilae* (Falagiarda & Schmidt, 2020; Hougardy et al., 2022) and *Pachycrepoideus vindemiae* (Knoll et al., 2017; Wang et al., 2016a). Unfortunately, so far, all parasitisation tests for *D. suzukii* have been performed on the summer phenotype. It remains unknown whether the winter phenotype larvae or pupae have the same immune response to parasitisation as the summer phenotype.

We tested the immune system response to parasitism in winter and summer phenotypes of *D. suzukii* and *D. melanogaster* using the larval parasitoid *A. japonica* and the pupal parasitoid *T. drosophilae*, both potential biocontrol agents (Colombari et al., 2020; Falagiarda & Schmidt, 2020; Girod, Lierhmann, et al., 2018; Häussling et al., 2021; Herz et al., 2021; Matsuura et al., 2018; Wang et al., 2020, 2021). Additionally, we determined the phenoloxidase activity of the larval haemolymph of the two phenotypes of *D. suzukii* and *D. melanogaster* and counted all four different types of haemocytes. The haemocyte counts and the phenoloxidase activity provides an overview of the innate immune system of both phenotypes. Furthermore, we measured the phenoloxidase activity of the haemolymph and counted haemocytes from adult females. This should provide a deeper understanding of adult immunity and allow conclusions about the life cycle of haemocytes.

2 | MATERIALS AND METHODS

2.1 | Insects

Drosophila suzukii were caught in the state of Hesse, Germany, in 2016. The parasitoid wasps *Trichopria drosophilae* were provided by the company 'Bioplanet' in Cesena, Italy. *D. suzukii*, *D. melanogaster*, and *T. drosophilae* were reared on *D. melanogaster* and kept under the conditions described in Häussling et al. (2021). *Asobara japonica* were received from the Department of Evolutionary Animal Ecology at the University of Groningen. They were reared on *D. melanogaster* larvae. The parasitoid wasps were fed with a 50% honey–water solution on filter paper, and both sexes were kept together. The parasitoid wasps were, as usual, 4–6 days old when used in the experiment, so they were mostly mated. The summer phenotypes of *D. melanogaster* and *D. suzukii* and the parasitoids were kept in a climate- and light-controlled chamber at 24°C and 70%–80% RH with a 16:8 h day/night rhythm. The winter phenotypes were kept for the hole development in a climate- and light-controlled chamber at 15°C and 70%–80% RH with an 8:16 h day/night rhythm.

2.2 | Parasitisation experiment

In the parasitisation experiment, we tested the parasitisation rate of *A. japonica* on second instar larvae and the parasitisation rate of *T. drosophilae* on pupae of both fly species. Second instar larvae of the two seasonal phenotypes of *D. suzukii* and *D. melanogaster* were used to test larval parasitisation. The larvae were flushed out of a Drosophila vial onto a fine sieve (300 µm aperture), and 740 medium-sized second instar (L2) larvae were collected with a fine paintbrush under a stereomicroscope and placed in Drosophila Ringer solution to free them from residues (per 1000 mL water: 0.33 g $CaCl_2 \cdot 2H_2O$, 13.6 g KCl, 1.21 g Tris base; pH adjusted to 7.2 with 1 N HCl). After the collection (max. 30 min), the larvae were dried on a precision wipe. For each treatment, 30 dried larvae were transferred to a piece of Drosophila diet in a Petri dish. For the parasitisation treatment, three female *A. japonica* wasps were placed on the Petri dish for 6 h at 24°C. We used three wasps to increase the likelihood that the larvae would be parasitised. The piercing was performed as described in the larval immunity experiment in Section 3.3. For each treatment, nine Petri dishes were prepared. After pupation, each pupa was transferred to each well of a 96-well plate. The plate was covered with a gas-permeable sealing membrane (Breath-Easy® sealing membrane, Sigma-Aldrich) and stored at 24°C. After hatching, the number of emerged wasps, flies and unhatched pupae were counted. The degree of infestation (DI) (Boulétreau & Fouillet, 1982; Boulétreau & Wajnberg, 1986) was estimated using the equation:

$$\mathrm{DI} = \frac{(D_C - D_T)}{D_C} \quad (1)$$

where D_C describes the number of emerged flies in the control group and D_T the number of flies that emerged from the treatment groups (parasitisation, pierced). $(D_C - D_T) < 0$, $(D_C - D_T)$ was set = 0. We included piercing for the DI to differentiate between wounding reactions and immune reactions.

In the pupal parasitisation experiment, 15 pupae of either the summer or the winter phenotype of *D. suzukii* or *D. melanogaster* were placed on moist filter paper. The pupae were tanned pupae of similar age. Tanning was visually similar between the phenotypes. This filter paper was in a Petri dish (9.5 cm diameter) and offered to one female parasitoid *T. drosophilae*. The temperature of the parasitisation and storage temperature was either the rearing temperature of the summer phenotype (24°C) or the winter phenotype (15°C) (see Section 3.1), but both phenotypes were observed at both temperatures. The oviposition behaviour of the wasp was recorded for 6 h using a video recorder (Lupustec LE 800 4K, Lupus-Electronics GmbH, Landau, Germany), as described in Häussling et al. (2021), and the number of oviposition events were counted. Multiple repetitions were observed simultaneously, each with its own camera. This method was used for *T. drosophilae* parasitism because, unlike in the *A. japonica* parasitism experiment, the pupae are unable to move, allowing the oviposition of each pupa to be observed. We counted an event as oviposition when the wasp pierced a pupa and did not move for at least 30 s. For *D. suzukii* we had sample sizes of 22 Petri dishes for the winter phenotype (9 at 24°C and 13 at 15°C) and 16 Petri dishes for the summer phenotype (8 at 24°C and 8 at 15°C). For *D. melanogaster*, we had sample sizes of 31 Petri dishes for the winter phenotype (22 at 24°C and 9 at 15°C) and 30 Petri dishes for the summer phenotype (25 at 24°C and 5 at 15°C). The numbers are not equal, as in some of the Petri dishes the parasitisation or the observation failed. As a control, no wasps were added to the Petri dishes (sample size of 9 for the winter and 9 for the summer phenotype of *D. suzukii* and 15 for the winter and 13 for the summer phenotype of *D. melanogaster*). All these pupae were then individually transferred to 96-well plates. These plates were sealed with a gas-permeable sealing membrane (Breath-Easy® sealing membrane, Sigma-Aldrich). Once the insect emerged, we identified whether it was a fly or a wasp and recorded its sex. The parasitisation success was measured by the proportion of infested hosts that give rise to an adult parasitoid (Boulétreau & Wajnberg, 1986).

2.3 | Larval immunity experiment

We counted all immune cells in the larval immunity experiment and measured the phenoloxidase activity after the larvae were parasitised. First, the larvae were collected from the rearing vials in the same way as for the parasitisation experiment (Section 3.2). One hundred of the collected larvae were used directly to extract the hemolymph at time point 0 h (no parasitisation). For each parasitisation and control treatment, 200 larvae per replicate were transferred to a Petri dish with a Drosophila diet. More larvae were transferred to compensate for potential losses due to parasitisation and development.

For the parasitisation treatment, 20 female *A. japonica* were added to the Petri dish for 6 h. The control group received no further treatment until hemolymph extraction. For the pierced treatment, 240 larvae were transferred to a Petri dish. More larvae were transferred for the pierced treatment to compensate for the potentially higher mortality due to piercing. Before and during the piercing, the larvae were transferred to an ice-cooled porcelain plate to reduce larval movement for the piercing treatment. Piercing was performed using a flame-sterilised insect needle in the posterior cuticles without harming the internal organs. The piercing treatment was included in the experiment to test the haemocyte induction for a potential effect of piercing during oviposition in the absence of wasp venom, similar to Kacsoh and Schlenke (2012), as the piercing can induce the production of lamellocytes (Markus et al., 2005). Pierced larvae were transferred to a new Petri dish with a Drosophila diet. The petri dishes of all treatments were closed with parafilm tape and placed in the climate chamber at 24°C under long-day (16:8 h day/night rhythm) conditions until haemolymph extraction.

We extracted the haemolymph from 60 larvae from all three treatment groups at 24 and 48 h. Prior to haemolymph extraction, larvae were collected and washed in Drosophila Ringer's solution. They were then dried on a precision wipe and, in groups of 20 larvae,

transferred to a microscope slide. 1 µL of 5 mM MOPS buffer containing protease inhibitor cocktail (Roche cOmplete, CAS 30827997, one tablet solved in 50 mL of 5 mM MOPS buffer) was added per larvae. The buffer compensates for evaporation and inhibits the protease. For the parasitisation treatment, the number of visual encapsulations were counted. The larvae were then cut below the mandibles with dissection scissors and left in the buffer to bleed for 1 min. The buffer containing the haemolymph from the 60 larvae was collected into a 1.5 mL Eppendorf tube and kept on ice. The tubes were frozen in liquid nitrogen and stored at −80°C. Prior to freezing, 5 µL of the haemolymph was mixed with 15 µL N-Phenylthiourea (0.01% in 1× PBS, Thermo Fisher Scientific, CAS 103-85-5) and 2.6 µL Giemsa-Stain (Sigma, CAS 51811-82-6) and loaded onto a haemocytometer (Neubauer improved, NanoEntek, C-Chip DHC-N01) for the haemocyte counting. The haemocytes were counted and discriminated in 16 diagonally arranged grids of the haemocytometer using a phase-contrast microscope (Zeiss, Axio Lab.A1) at 63× magnification. The counted haemocytes were plasmatocytes, podocytes, crystal cells, and lamellocytes. The counting took place directly after the load of the haemocytometer to avoid the loss of crystals in the crystal cells (Kacsoh & Schlenke, 2012).

We used the ratio of phenoloxidase to the total protein content as a measurement for the phenoloxidase. This ratio was used in order to be independent of the haemolymph concentration in each sample due to the different amounts of larval bleeding. The maximum linear rate of colour change (V_{max}) of the substrate L-DOPA converted by PO was used for the phenoloxidase. Total protein was measured using the Pierce™ Rapid Gold Protein Assay Kit (A53225, Thermo-Scientific™). This kit was used in line with the user guide with 5 mM MOPS buffer as blank. Absorbance was measured using a BioTek® Synergy H1 microplate reader.

We measured the phenoloxidase activity using a 10 mM L-DOPA solution and added 34.4 mg of L-DOPA (Sigma, CAS 59927) to 20 mL 5 mM MOPS buffer. This solution was vortexed for 15 min, followed by 3 min in an ultrasonic tub. To measure the samples, we used a microplate reader and kept the 96-well plate on ice. Samples were defrosted on ice, vortexed and centrifuged briefly on a benchtop centrifuge. 10 µL of 5 mM MOPS buffer was added to each well. This step was followed by adding 10 µL of sample haemolymph or, as a control, adding 10 µL of deionised water. The last 180 µL of ultrafiltered 10 mM L-DOPA solution was added and mixed. The set temperature of the microplate reader was 30°C. The samples were mixed orbitally, and the absorbance at 490 nm was measured every minute for 75 min.

2.4 | Adult immunity assay

For the adult immunity assay, we counted the haemocytes and measured the phenoloxidase for both phenotypes. For this purpose, the haemolymph of the female adult *D. suzukii* or *D. melanogaster* were collected by piercing the thorax of the adult flies. The wings were also removed with forceps. 15 female flies were then transferred to a 0.5 mL Eppendorf tube with a fine cut from a razor blade at the bottom. This tube was then inserted in a 2 mL Eppendorf tube containing 5 µL of MOPS buffer with protease inhibitor so that the haemolymph could drip down into the 1.5 mL tube during centrifugation and immediately mix with the protease inhibitor. The tubes were centrifuged at 4025 *g* rpm for 8 min at 4°C. The haemolymph sample from five tubes was pooled so that one sample contained the haemolymph of 75 female flies. 5 µL was used directly for counting circulating haemocytes. The remainder was frozen in liquid nitrogen and stored at −80°C until the phenoloxidase activity, and the total protein was measured as described in Section 3.3.

2.5 | Statistical analysis

The effect of the seasonal phenotype of *D. suzukii* and *D. melanogaster* on the phenoloxidase activity and the haemocyte counts were analysed using a binomial generalised linear mixed model (GLMMs) using the R package 'lme4' (Bates et al., 2015).

As a random factor for each GLMM, we used the repetition equivalent to one Petri dish. We tested the models for overdispersion. If there was overdispersion, we used a negative binomial model. The degree of infestation of the flies' larvae and pupae, the encapsulation rate and the haemocyte counts and female phenoloxidase activity were compared between the two seasonal phenotypes using the Wilcoxon rank-sum test.

All data were analysed in R 4.1.3 (R Development Core Team, 2008).

3 | RESULTS

3.1 | Larvae

3.1.1 | Degree of infestation of *Asobara japonica*

We found no significant difference in the degree of infestation between the winter and summer phenotypes of *D. melanogaster* and *D. suzukii*, neither in larvae parasitised by *Asobara japonica* nor in pierced larvae (Figure 1 and Figure S1).

3.1.2 | Encapsulation rate

The encapsulation rate gives the percentage of *D. suzukii* or *D. melanogaster* larvae that have a visible encapsulation of an *A. japonica* egg (Figure 2). After 24 h of parasitisation, we found no significant difference in the encapsulation rate between the two seasonal phenotypes of *D. melanogaster* larvae. After 48 h, we found that the winter phenotype larvae had a significantly higher encapsulation rate than the summer phenotype larvae. For *D. suzukii* larvae, we found no significant difference in the encapsulation rate at either time point.

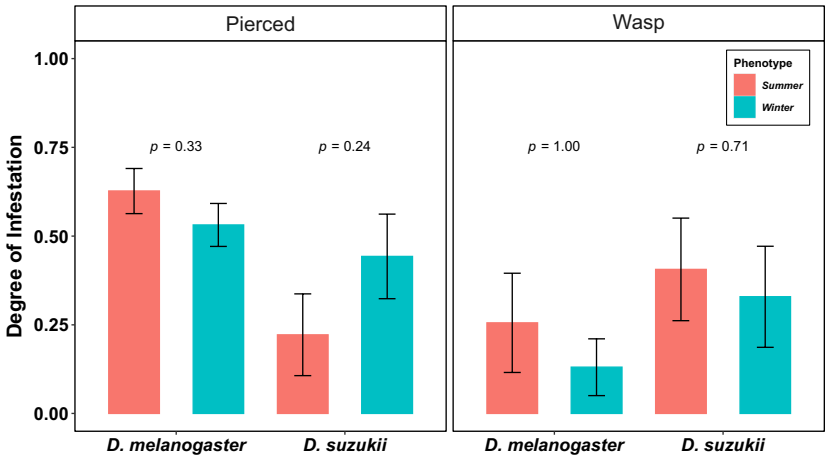

FIGURE 1 Degree of infestation (DI)—the proportion of larvae that could not develop into adult flies due to treatment (pierced or parasitisation treatment) compared with a control group—for larvae of summer (red) and the winter (blue) phenotype of *Drosophila melanogaster* and *Drosophila suzukii* that were either pierced with a needle (pierced) or parasitised by *Asobara japonica* (parasitised) (Wilcoxon rank-sum test).

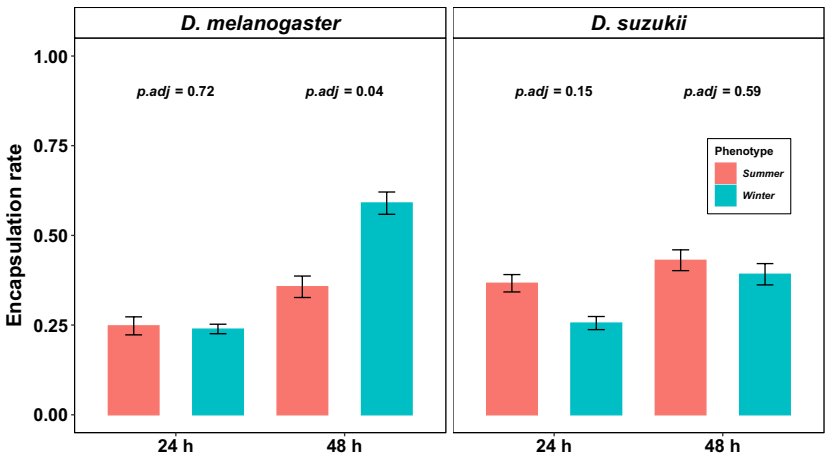

FIGURE 2 Encapsulation rate—the proportion of larvae with visible encapsulations—for larvae of the winter and the summer phenotype of *Drosophila melanogaster* and *Drosophila suzukii*. Encapsulations were counted 24 and 48 h after possible parasitisation. The seasonal phenotype of the larvae was either summer (red) or winter (blue) (Wilcoxon rank-sum test).

TABLE 1 Circulating haemocyte counts—generalised linear mixed effect model (family = negative binomial, link = logit, Petri dish ('repetition') as a random factor) output quantifying the effect of the phenotype (winter, summer) and the treatment (control, pierced, parasitisation by *Asobara japonica*) for *Drosophila suzukii* and *Drosophila melanogaster*.

Subset	Response	Terms	χ^2	*df*	*p*-Value
0 h					
D. suzukii	Haemocytes	Phenotype	5.00	1	**0.03**
D. melanogaster	Haemocytes	Phenotype	0.57	1	0.45
24 h					
D. suzukii	Haemocytes	Treatment	5.21	2	0.07
		Phenotype	1.26	1	0.26
D. melanogaster	Haemocytes	Treatment	0.01	2	0.99
		Phenotype	1.41	1	0.24
48 h					
D. suzukii	Haemocytes	Treatment	11.25	2	**0.004**
		Phenotype	5.49	1	**0.02**
D. melanogaster	Haemocytes	Treatment	20.95	2	**<0.001**
		Phenotype	1.05	1	0.31

Note: Significant values (*p*-value <0.05) are highlighted in bold. The response 'haemocytes' refers to the total number of haemocytes, with no differentiation of the haemocyte type.

3.1.3 | Haemocyte response of *Drosophila suzukii* and *Drosophila melanogaster* to parasitisation of *Asobara japonica*

In the second instar of *D. suzukii*, we found significant differences between the seasonal phenotypes in the number of haemocytes. Before treatment assignment (0 h), more haemocytes were counted in the summer phenotype than in the winter phenotype. After 24 h, neither the phenotype nor the treatment affected the number of haemocytes. After 48 h, the seasonal phenotype affected the number of haemocytes in *D. suzukii*, with a higher number of cells found in the summer phenotype. At that time, the treatment also influenced the number of haemocytes in *D. suzukii* larvae (Table 1 and Figure 3). Here the 'parasitised' treatment group had a significantly higher number of haemocytes than the 'pierced' treatment group. All other treatment groups were not significantly different from each other (Table S1 and Table 2).

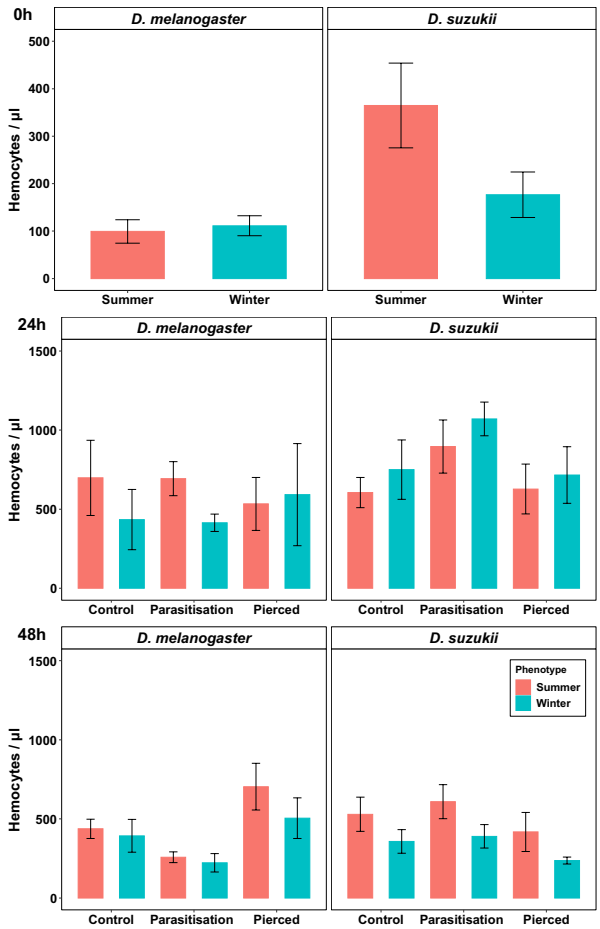

FIGURE 3 Haemocyte count—the number of cells in the haemolymph of *Drosophila melanogaster* or *Drosophila suzukii* larvae. Larvae were pierced or offered for parasitisation or untreated. The seasonal phenotype of the larvae was either summer (red) or winter (blue).

The haemocyte counts of *D. melanogaster* larvae were not affected by the seasonal phenotype at any time point (Table 1). But the treatment affected the number of haemocytes at 24 h in *D. melanogaster*. The parasitised treatment group had a significantly lower number of haemocytes than the control and the pierced treatment group (Table S2).

Looking at the different types of haemocytes separately, we found that, in *D. suzukii*, the number of podocytes and the crystal cells were significantly affected by the phenotype at 48 h. The summer phenotype had significantly more crystal cells than the winter phenotype (Table S2). In *D. melanogaster*, the phenotype affected the number of podocytes of the second instar larvae (0 h). The winter phenotype larvae had significantly more podocytes than the summer phenotype larvae.

The treatment affected the number of podocytes in *D. suzukii* at the time point 24 h. The parasitisation treatment had significantly more podocytes than the control. At 48 h, the number of plasmatocytes, crystal cells and lamellocytes counts were significantly affected by the treatment (Figures S2–S5). The control group had significantly more plasmatocyte counts than the pierced treatment group. The parasitisation treatment group had significantly more counted crystal cells than the pierced or the control. The same significant differences were observed for the lamellocytes.

For *D. melanogaster* larvae, the treatment significantly affected the number of podocytes and the crystal cell counts at 24 h. More podocytes were found in the parasitisation treatment group than in the control group. In the pierced treatment, significantly more

TABLE 2 Phenoloxidase activity—GLMM (family = binomial, link = logit, 'repetition' (Petri dish) as a random factor) output quantifying the effect of the Drosophila phenotype (winter, summer) of *Drosophila suzukii* and *Drosophila melanogaster* and the treatment (control, pierced, parasitisation by *Asobara japonica*) on the activity of the immune enzyme phenoloxidase.

Time	Terms	χ^2	*df*	*p*-Value
D. suzukii				
0 h	Phenotype	0.55	1	0.46
24 h	Treatment	0.36	2	0.84
	Phenotype	0.69	1	0.41
48 h	Treatment	17.44	2	**<0.001**
	Phenotype	0.78	1	0.38
D. melanogaster				
0 h	Phenotype	2.63	1	0.11
24 h	Treatment	0.41	2	0.82
	Phenotype	1.78	1	0.18
48 h	Treatment	2.28	2	0.32
	Phenotype	0.19	1	0.67

Note: Due to the non-independence of samples from the same Petri dish, we included the ID of the Petri dish as a random factor. Significant values are indicated in bold.

crystal cells were counted than in the parasitisation treatment. At 48 h, the treatment significantly affected the number of plasmatocytes, crystal cells, and lamellocytes. The pierced treatment group had significantly more plasmatocytes than the parasitisation treatment group. The pierced treatment had significantly more crystal cells than the control and parasitisation treatment groups. The control group had significantly more crystal cells than the parasitisation treatment group. For the number of lamellocytes, we found that the pierced treatment group had significantly more than the parasitisation or the control groups.

3.1.4 | Phenoloxidase activity

Not affected by the seasonal phenotype at any time point (Table 2, Figure 4, Table S5).

The treatment (control, parasitisation or pierced) also did not affect the phenoloxidase activity in either fly species. The only exception was the phenoloxidase activity of *D. suzukii* at 48 h. Here, the wasp treatment group had significantly lower phenoloxidase activity than the control or pierced treatment groups.

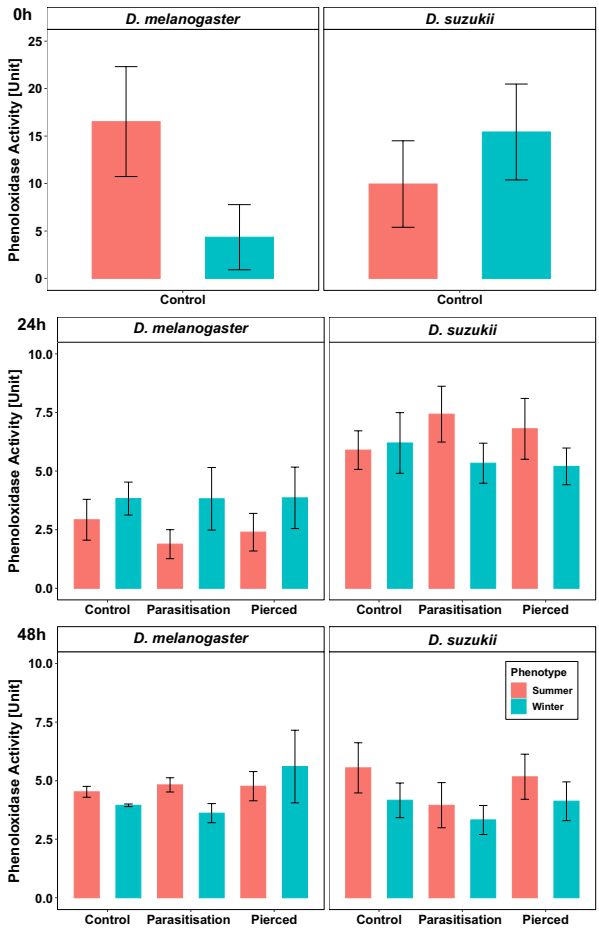

FIGURE 4 Phenoloxidase activity—the ratio of phenoloxidase activity to the total protein in the haemolymph of *Drosophila melanogaster* or *Drosophila suzukii* larvae. Larvae were pierced or offered for parasitisation or untreated. The seasonal phenotype of the larvae was either summer (red) or winter (blue).

3.2 | Pupae

3.2.1 | Parasitisation success of the pupal parasitoid *Trichopria drosophilae*

Parasitisation success is the number of emerged wasps from previously parasitised pupae. The number of parasitised pupae was for *D. suzukii* at 24°C in summer larvae 98 and 95 in winter; at 15°C in summer, 49 and 46 in winter. For *D. melanogaster*, it was at 24°C in summer, 286, 255 in winter; at 15°C in summer, 11, 16 in winter. The parasitisation success of the pupal parasitoid *Trichopria drosophilae* was significantly different between the two seasonal phenotypes of both fly species *D. melanogaster* and *D. suzukii* pupae at a parasitisation temperature of 24°C (Figure 5). The parasitoid had a higher parasitisation success in the winter phenotype of pupae of *D. suzukii* than in the summer phenotype. In *D. melanogaster*, the summer phenotype was parasitised more successfully than the winter phenotype.

At a parasitisation temperature of 15°C, the parasitisation success of the two seasonal phenotypes was not significantly different for either fly species.

The temperature had no significant effect on the parasitisation success of the *D. suzukii* summer ($p = 0.73$) and winter phenotype ($p = 0.25$). The *D. melanogaster* summer phenotype was significantly more successfully parasitised at 24°C than at 15°C ($p = 0.002$) (Tables S3 and S4). The parasitisation success in *D. melanogaster* winter phenotypes did not differ significantly between those two temperatures ($p = 0.82$).

3.3 | Female adults

3.3.1 | Haemocytes

Adults of the winter phenotype of both female *D. suzukii* and *D. melanogaster* had a significantly higher number of haemocyte counts than female adults of the summer phenotype (Figure 6). In both fly species, the number of haemocytes was much higher in the winter phenotype. The number of haemocytes did not differ between species (winter phenotype $p = 0.72$, summer phenotype $p = 0.59$).

3.3.2 | PO activity

On average, the summer phenotype of *D. melanogaster* had twice the phenoloxidase activity of the winter phenotype. However, the two phenotypes did not statistically differ from each other (Figure 7). In *D. suzukii*, the phenoloxidase activity was equal in both phenotypes. Although not statistically significant, the

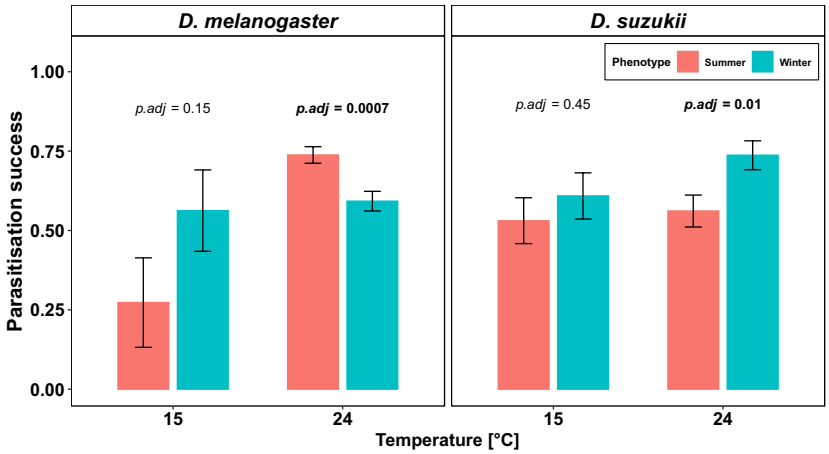

FIGURE 5 Parasitisation success (the proportion of hatched parasitoids) of *Trichopria drosophilae* from pupae of the summer (red) and winter phenotype winter (blue) of *Drosophila melanogaster* and *Drosophila suzukii* at 15 and 24°C (Wilcoxon rank-sum test).

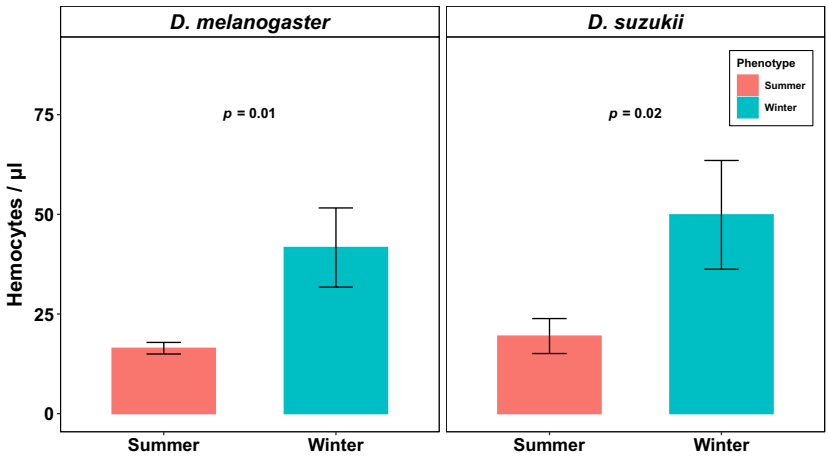

FIGURE 6 Haemocyte count (the number of cells in the haemolymph) of adult females of the summer phenotype (red) and the winter phenotype (blue) of *Drosophila melanogaster* and *Drosophila suzukii* (Wilcoxon rank-sum test).

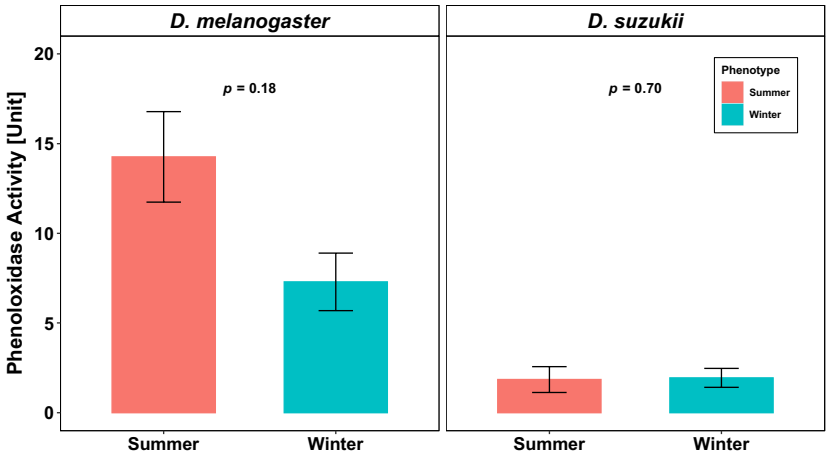

FIGURE 7 Phenoloxidase activity—the ratio of phenoloxidase activity and total protein of the haemolymph of female *Drosophila melanogaster* and female *Drosophila suzukii* adults. The seasonal phenotype of the adults was either summer (red) or winter (blue) (Wilcoxon rank-sum test).

phenoloxidase activity in the *D. melanogaster* winter phenotypes was almost four times higher than that in the *D. suzukii* winter phenotype. Similarly, for the *D. melanogaster* summer phenotype, the phenoloxidase activity was almost eight times higher than that in *D. suzukii* summer phenotype.

4 | DISCUSSION

In this study, for the first time, the immune system responses to parasitisation of the summer and winter phenotype of different life stages of *D. suzukii* were compared. We found differences

between phenotypes for all life stages, but these differences were inconsistent among the different life stages. Furthermore, we found differences in the immune response between *D. suzukii* and *D. melanogaster*.

First, we looked at the larvae stage. Our results show that at 0 h (second instar larvae), the summer phenotype of *D. suzukii* has a higher haemocyte count than the winter phenotype. This difference could also be found at 48 h after the parasitisation across all treatments (control, pierced, parasitisation). However, the haemocyte counts at 24 h (third instar larvae) after parasitisation do not differ. Thus, at 24 h, when the absolute haemocyte count is highest in *D. suzukii*, the differences between the phenotypes disappear. As we observed the onset of encapsulation at 24 h after parasitisation, the reduced haemocyte count at this time point could be due to a more pronounced encapsulation, because the capsules are an aggregation of haemocytes (Dubovskiy et al., 2016).

Another possible explanation for the reduction in haemocytes could be the temperature: during the parasitisation experiment, we kept both phenotypes at 24°C to keep the wasps active and the results comparable. However, this temperature also meant an increase for the winter phenotype larvae, which were previously kept at 15°C. The effect of temperature on the immune system has only been described for cold temperatures: Salehipour-Shirazi et al. (2017) found an increase in haemocytes in *D. melanogaster* exposed to acute cold. Whether such an increase in temperature, as in our experiment, leads to a similar increase in haemocyte counts should be the subject of further studies.

The immune resistance of *Drosophila* larvae to parasitoid eggs is associated with a high host haemocyte load (Kacsoh & Schlenke, 2012; Poyet et al., 2013). However, our study did not observe a higher immune resistance, as the encapsulation rates and the degree of infestation of the two seasonal phenotypes were similar, also we observed a higher cellular immunity of the summer phenotype. The effect that a high cell count results in higher immunity has also been observed in different strains of *D. suzukii* (Kacsoh & Schlenke, 2012; Poyet et al., 2013). Compared with *D. melanogaster* which has a lower immunity than *D. suzukii*, the haemocyte load in *D. suzukii* strains was five to eight times higher than that in *D. melanogaster* larvae (Poyet et al., 2013). However, in our study, the haemocyte count of the summer phenotype was only twice as high as the winter phenotype. The smaller difference between the phenotypes could explain why the higher haemocyte load did not affect the immunity of the *D. suzukii* summer phenotype in our study.

Furthermore, *Drosophila's* encapsulation process mainly depends on the haemocyte cell type lamellocytes (Binggeli et al., 2014; Dudzic et al., 2015; Vlisidou & Wood, 2015). At 0 h (second instar), lamellocytes were only found in the summer phenotype. At 24 and 48 h post-parasitisation, this cell type did not differ between the seasonal phenotypes, which further explains why we did not find a higher encapsulation rate in the summer phenotype. Another factor that influences the encapsulation is the fly strain, as the encapsulation rate against parasitoids can vary greatly between the *D. suzukii* and the *D. melanogaster* strains (Gerritsma et al., 2013; Kacsoh & Schlenke, 2012). Therefore, it could be that other strains show different encapsulation rates between the two phenotypes.

Drosophila melanogaster phenotypes exhibited no differences in total haemocyte counts or lamellocytes, crucial for encapsulation. Parasitised larvae showed consistent infestation and encapsulation rates across phenotypes. Previous studies noted increased haemocytes, particularly lamellocytes and crystal cells, in response to parasitisation in *D. melanogaster* larvae (Eslin & Prevost, 1998; Kacsoh & Schlenke, 2012). Our experiment confirmed a similar response. In *D. suzukii*, a trend towards higher haemocyte counts in the parasitisation treatment was observed, primarily due to increased lamellocytes and crystal cells after 48 h. This suggests limited impact of wasp venom on *D. suzukii's* immune system. In contrast, *D. melanogaster* exhibited reduced haemocyte counts, especially lamellocytes and crystal cells, indicating the potent effect of wasp venom, consistent with findings by Kacsoh and Schlenke (2012). Overall, haemocyte count differences were more associated with treatment (pierced, control, or parasitisation) than phenotype.

Interestingly, we found encapsulations in *D. melanogaster* and *D. suzukii* as early as 24 h (third instar larvae) after parasitisation. Other studies observed no encapsulation at all when *D. suzukii* larvae were parasitised by *A. japonica* (Iacovone et al., 2018; Poyet et al., 2013) and a delayed encapsulation (compared to *D. melanogaster* larvae) when parasitised by *Leptoplina heterotoma* or *Leptoplina boulardi* (Iacovone et al., 2018). One explanation could be the strains of flies and parasitoids, as we used a different strain of *D. suzukii* and probably also a different strain of *A. japonica* than those studies. As it has been observed that the parasitisation success can vary widely between different parasitoids and *Drosophila* strains from different geographical regions (Gerritsma et al., 2013; Iacovone et al., 2018; Poyet et al., 2013), it is very likely that this factor alone could explain the early encapsulation in our study.

Another important factor in the immunity of *Drosophila* larvae is phenoloxidase (PO), as it contributes to the melanisation process (Dudzic et al., 2015; González-Santoyo & Córdoba-Aguilar, 2012; Moreau et al., 2000; Tang, 2009). We found no difference in PO activity between the seasonal phenotypes in both species. Interestingly, we found a difference between the seasonal phenotypes of *D. suzukii* in the number of crystal cells containing the substrate and enzymes of the phenoloxidase cascade (Carton et al., 2008) at 48 h after parasitisation. However, this difference did not result in higher phenoloxidase activities. Furthermore, the parasitised treatment group of *D. suzukii* had a significantly lower phenoloxidase activity than the control or the pierced treatment groups. This low activity could be due to advanced melanisation of the parasitoid egg in the larvae, where the PO is a key enzyme in melanin biosynthesis (Tang, 2009). In general, there is often no clear correlation between phenoloxidase activity and insect immunity (González-Santoyo & Córdoba-Aguilar, 2012).

We can conclude, that for the larval stage, the difference in haemocyte load in the second instar or 48 h later did not result in any clear immunity benefits, such as a higher encapsulation rate or degree of the infestation when the larvae were parasitised.

In contrast to the larval parasitisation experiment, we found a difference in parasitisation success in the pupae of the *D. suzukii* phenotypes. Parasitisation at 24°C by the pupal parasitoid *T. drosophilae* resulted in a significantly higher parasitisation success in the winter phenotype compared with the summer phenotype. In conclusion, at 24°C, the winter phenotype pupae are more susceptible to parasitism than are the summer phenotype pupae. This finding raises the question of why the two phenotypes have similar degrees of infestation in the larval parasitisation experiment but such different levels of parasitisation success in the pupal stage. One explanation could be the complete morphological change during metamorphosis. Cellular immunity changes at the onset of metamorphosis, resulting in the release of haemocytes by the lymph glands. These cells are part of the metamorphosis when they ingest doomed larval tissues (Holz et al., 2003; Lanot et al., 2001). Therefore, different outcomes between larvae and pupae, as we observed, are possible and, thus, make it difficult to compare these two life stages.

In addition, the pupal parasitoids have evolved different strategies to circumvent the host immune system than larval parasitoids. This difference could cause the observed different levels of parasitisation success of pupa and larval stages. In some species, such as *Asobara tabida*, the egg can stick to host tissue by proteins or special coatings that make the eggs sticky (Eslin & Prevost, 2000; Huang et al., 2021). When attached to host tissue, the egg is less likely to be attacked by haemocytes, which is one passive form of immune evasion. An active form of immune invasion is the venom that is injected during oviposition, which can suppress the host immune response, as shown in *Leptopilina heterotoma* (Huang et al., 2021; Moreau & Asgari, 2015). The virulence of the parasitoid can be species-specific and often also strain-specific (Cavigliasso et al., 2019). To our knowledge, it is unclear whether the parasitoid *T. drosophilae* also injects venom or whether its eggs have a sticky coating. The pupal ectoparasitoid *Pachycrepoideus vindemiae* is known to inject venom into the pupae (Yang et al., 2020). As an endoparasitoid, *T. drosophilae* lays its eggs in the host's hemocoel (Carton et al., 1986). This process means the egg is in contact with the host haemolymph in a similar way to the eggs of larval parasitoids. These behaviours make it likely that the *T. drosophilae* also injects venom, similar to larval parasitoids (Wertheim, 2022).

Temperature affects the immunity of *Drosophila* flies. Cavigliasso et al. (2021) observed a decrease in the encapsulation rate of parasitoid eggs with increasing temperature in *D. melanogaster* larvae. We observed a similar effect in the parasitisation success of the *Drosophila* pupae: With an increasing temperature, a higher parasitisation success of the parasitoid in the summer phenotype of *D. melanogaster* and in the winter phenotype of *D. suzukii* occurred. This effect means these pupae are probably less immune to parasitoids at higher temperatures. In the respective corresponding phenotype, the parasitisation success did not change with increased temperature.

The success of parasitisation relies on both the host's immune response and the parasitoid's ability to parasitise. Temperature, which affects this ability, has been noted in previous studies (Cavigliasso et al., 2021). Further studies evaluating the temperature impact on *T. drosophilae* parasitisation are warranted. Adult females of the winter phenotype in *D. suzukii* and *D. melanogaster* unexpectedly exhibited significantly more haemocytes than their summer counterparts. This contradicts the larval development trend, where *D. suzukii's* winter phenotype had similar or lower haemocyte loads. In *D. melanogaster*, adult haemocytes are primarily embryonic and lymph gland-derived, with little haematopoiesis in adulthood (Boulet et al., 2021; Holz et al., 2003). The observed disparity in haemocyte proportions between adults and larvae suggests potential differences in the phenotypes' ability to maintain haemocytes during metamorphosis, influenced by developmental temperatures. The winter phenotype, developing at 15°C, demonstrated an immunity advantage, as seen in our pupal parasitisation experiment for *D. suzukii*.

Alternatively, the differing decline of haemocytes with age in the two phenotypes may provide an explanation. In *Drosophila*, haemocyte numbers generally decrease with adult age, a phenomenon observed more prominently in females than males (Boulet et al., 2021; Mackenzie et al., 2011; Sanchez Bosch et al., 2019). Surprisingly, adult female flies exhibited lower haemocyte counts than their larval-stage counterparts. This discrepancy suggests a potentially higher decline in haemocytes with age in the summer phenotype compared with the winter phenotype, warranting further analysis.

We found a difference when we examined the phenoloxidase activity. In *D. suzukii*, the phenotypes were not different in PO activity. Additionally, we found a much lower PO activity than in *D. melanogaster*. These findings are unexpected. As *D. suzukii* larvae have a much higher haemocyte load than *D. melanogaster* larvae and a much higher immunity against parasitoids (Kacsoh & Schlenke, 2012; Poyet et al., 2013), we anticipated they would also have a higher PO activity. However, also González-Santoyo and Córdoba-Aguilar (2012) argued in their review that PO activity in insects does not seem to be an indicator of host resistance but rather of host condition, as it is a costly trait. Adult *D. melanogaster* flies live more frequently in food patches with higher population densities than *D. suzukii* (personal observation), and *D. suzukii* avoids competition by shifting the oviposition preference to ripe fruits (Kidera & Takahashi, 2020). In addition, the contact of the fly with microbes should be higher in rotten food patches than in ripe fruits. As a result, the adult *D. suzukii* may have a lower phenoloxidase activity because it is very costly, and the immune challenge is lower in *D. suzukii* adults than in *D. melanogaster* adults.

5 | CONCLUSION

Our study found that differences in immunity between the seasonal phenotypes of *D. suzukii* larvae have no measurable effect on the success of a parasitoid at the larval stage. Still, we suggest further research on the winter phenotype, as other factors than the immunity can affect the parasitisation of *D. suzukii* larvae.

Unexpectedly, we discovered a high haemocyte load in adult females of *D. suzukii* and *D. melanogaster* of the winter seasonal phenotype. This high haemocyte load gives the fly a stronger immunity during overwintering. As far as we know, it is unclear why such a high level of immunity is required for overwintering female flies. At the pupal stage, our study shows that a pupal parasitoid will be more successful on the winter seasonal phenotype of *D. suzukii* when it develops at higher temperatures.

Our results have implications for integrated pest management implementation, where an early release of larval parasitoids during the growing season is crucial to reduce early pest populations. The release of pupal parasitoids may be particularly effective in areas and years where spring temperatures rise rapidly, as the winter phenotype of *D. suzukii* pupae is more susceptible to parasitisation under such conditions. With global warming, these abiotic conditions are expected to become more common in the future.

AUTHOR CONTRIBUTIONS

Benedikt J. M. Häussling: Conceptualization; methodology; software; data curation; investigation; validation; formal analysis; supervision; visualization; project administration; writing – original draft; writing – review and editing. **Nathalie Rausch:** Methodology; investigation. **Emely K. Klüsener:** Investigation; methodology. **Johannes Stökl:** Conceptualization; project administration; supervision; funding acquisition; writing – original draft; writing – review and editing; formal analysis; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at https://datadryad.org/stash/share/jfWkAQvhoG0MlV0Dowq81SNtHIex9D7sVgHchcTlaCo, reference number doi: 10.5061/dryad.0cfxpnw91.

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Statutory declarations

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