Biotechnological Control Strategies for Managing *Drosophila suzukii*

By

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Dissertation

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" in carrying out the investigations described in the dissertation."

Gießen, 2025

Signature of the candidate

Acknowledgment

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(وَإِذْ تَأَذَّنَ رَبُّكُمْ لَئِن شَكَرْتُمْ لَأَزِيدَنَّكُمْ) صدق الله العظيم

فالحمد لله رب العالمين حمداً كثيراً

As a child, my mother asked me what I want to be when I grew up. My answer was a scientist so I can create Pokémon… Unfortunately, although I became a scientist creating Pokémon is still out of reach. I do know how to manage a fruit fly infestation, so…. close enough? I was always described as a curious child, and this dissertation is the magnum opus of my scientific journey shaped by that curiosity.

This journey was filled with ups and downs, satisfactions and frustrations, joys and disappointments. But I would like to acknowledge and thank the people who shared and helped shape it. First, to my father and mother, who always showed love and support for what I'm doing. And to my siblings and friends, who are now only allowed to address me as Dr. They are who shaped me (for better or worse) and the main reason I call Jordan home, their love and support are a comfort I could always fall back on. Second, the new friends made here in Germany, who made life as an Ausländer much more tolerable and helped me grow in ways I couldn't imagine. Their friendship was accompanied with many new experiences. Finally, to my first scientific family at Philadelphia University, who took a chance on me, and made me believe I can achieve more than I think I can. I would like to especially thank Haneen whose constant support and friendship was a lifeline, Frisca and Sandra for the fun tea time, and the gamers of IME Ale, Lui, and Maurice.

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In the final words of this acknowledgment, I would like to dedicate this thesis to the memory Mohammad Jamal Al-Din Abdelhafiz and Tariq Akilah, two great men who I miss dearly and helped shape me into the person I am today.

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Summary

Drosophila suzukii (*D. suzukii*) is an invasive pest native to East Asia that has established itself across much of the world. Unlike most *Drosophila* species, which prefer decaying organic material for reproduction, *D. suzukii* targets fresh and ripening fruits, causing significant agricultural damage. Larval feeding and subsequent secondary infections can result in complete crop loss. Current control strategies rely heavily on chemical insecticides, which pose environmental risks and affect non-target organisms. This underscores the urgent need for safer and more sustainable alternatives.

This dissertation investigates three biotechnological control strategies against *D. suzukii*. The first approach enhances the Sterile Insect Technique (SIT), a method that reduces pest populations through the release of sterilized males. Three key advancements are presented: (1) development of a non-destructive sexing method based on pupal weight differences, (2) identification of optimal X-ray sterilization conditions (90 kV/40 Gy), and (3) implementation of a temperature-based sterilization technique utilizing the natural thermosensitivity of *D. suzukii* males. The second strategy focuses on characterizing La Jolla virus (LJV), a candidate for virus-based biocontrol. The study examines natural transmission routes, including airborne, venereal, oral, and fecal, and investigates the virus's pathology. LJV infection was shown to affect feeding behavior, nutrient absorption, fecundity, and egg-to-adult viability, offering insight into its potential as a biological control agent. The third approach explores RNA interference (RNAi) as a control tool in two contexts: enhancing SIT by generating sterile male-only populations through gene silencing, and deploying RNAi as a biopesticide by targeting essential genes. However, under the tested conditions, RNAi did not yield significant effects in either application.

Collectively, the findings provide valuable contributions to the development of targeted, environmentally friendly control methods for *D. suzukii*, highlighting both promising advances and existing limitations in the field of biological pest management.

Zusammenfassung

Drosophila suzukii (*D. suzukii*) ist ein invasiver Schädling, der endemisch in Ostasien ist und sich in weiten Teilen der Welt etabliert hat. Im Gegensatz zu den meisten *Drosophila*-Arten, die sich bevorzugt in verrottendem organischem Material vermehren, befällt *D. suzukii* frische und reifende Früchte und verursacht dadurch erhebliche landwirtschaftliche Schäden. Larvenfraß und nachfolgende Sekundärinfektionen können zu vollständigen Ernteverlusten führen. Aktuelle Bekämpfungsstrategien basieren stark auf chemischen Insektiziden, die Umweltrisiken bergen und Nichtzielorganismen beeinträchtigen. Dies unterstreicht den dringenden Bedarf an sichereren und nachhaltigeren Alternativen.

Diese Dissertation untersucht drei biologische Bekämpfungsstrategien gegen *D. suzukii*. Der erste Ansatz verbessert die Sterile Insekten Technik (SIT), eine Methode zur Reduzierung von Schädlingspopulationen durch den Einsatz und Freisetzung sterilisierter Männchen. Drei wichtige Fortschritte werden vorgestellt: (1) Entwicklung einer zerstörungsfreien Methode zur Geschlechtsbestimmung basierend auf Gewichtsunterschieden der Puppen, (2) Identifizierung optimaler Bedingungen für die Röntgensterilisation (90 kV/40 Gy) und (3) Implementierung einer temperaturbasierten Sterilisationstechnik unter Ausnutzung der natürlichen Thermosensitivität von *D. suzukii*-Männchen. Die zweite Strategie konzentriert sich auf die Charakterisierung des La-Jolla-Virus (LJV), einem Kandidaten für die virusbasierte biologische Schädlingsbekämpfung. Die Studie untersucht natürliche Übertragungswege, darunter aerogen, venerisch, oral und fäkal, und erforscht die Pathologie des Virus. Es zeigte sich, dass eine LJV-Infektion das Fressverhalten, die Nährstoffaufnahme, die Fruchtbarkeit und die Lebensfähigkeit vom Ei bis zum adulten Tier beeinflusst, was Einblicke in das Potenzial des Virus als biologisches Schädlingsbekämpfungsmittel bietet. Der dritte Ansatz untersucht RNA-Interferenz (RNAi) als Bekämpfungsinstrument in zwei Kontexten: Verbesserung der SIT durch Gen-Silencing und Einsatz von RNAi als Biopestizid durch gezielte Behandlung essentieller Gene. Unter den getesteten Bedingungen zeigte RNAi jedoch in keiner der beiden Anwendungen signifikante Effekte.

Zusammenfassend liefern die Ergebnisse wertvolle Beiträge zur Entwicklung gezielter, umweltfreundlicher Bekämpfungsmethoden für *D. suzukii* und verdeutlichen sowohl vielversprechende Fortschritte als auch bestehende Einschränkungen im Bereich des biologischen Schädlingsmanagements.

I Introduction

1.1 *Drosophila suzukii* biology and morphology

The genus *Drosophila* (Order: Diptera, Family: Drosophilidae) encompasses a diverse array of small fruit fly species, characterized by several shared traits. They typically exhibit a short life cycle of approximately 10 days, comprising the egg, larva, pupa, and adult stages (Figure 1). Their primary food source is usually fermented and spoiled fruits (Keesey, 2022; Jennings, 2011; Hales et al., 2015).

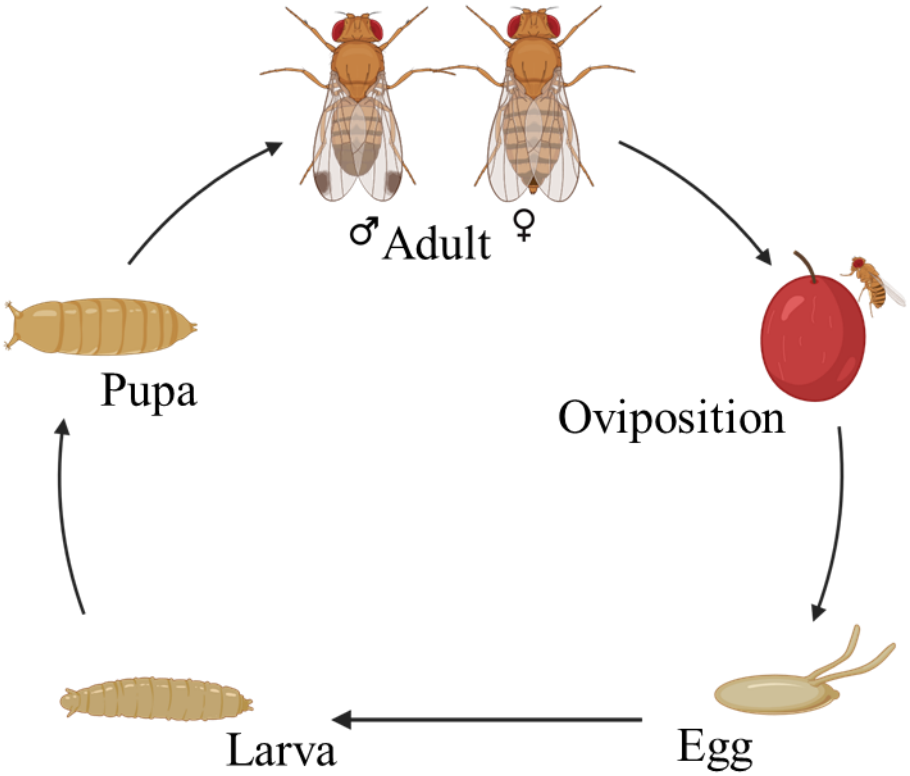


Figure 1. Typical life cycle of Drosophila suzukii (Figure generated by BioRender.)

A particularly noteworthy species is *Drosophila suzukii*, first described in Japan in 1931 by Matsumura (Kanzawa, 1939). *D. suzukii* can be distinguished from other *Drosophila* species by distinct phenotypic and behavioral traits. A notable feature of male flies is the prominent dark spot on the wing tips, hence the name "spotted wing *Drosophila*" (Figure 2D-E) (Walsh et al., 2011; Hauser, 2011). Additionally, female flies prefer to oviposit on fresh ripening fruit (Atallah et al., 2017), utilizing their serrated ovipositor to puncture the fruit's skin and oviposit

their eggs within (Figure 2A-C). The puncture site often leads to secondary bacterial and fungal infections and attracts other insects (Asplen et al., 2015). The larvae then reside inside the fruit, which offers protection from external threats and most control measures.

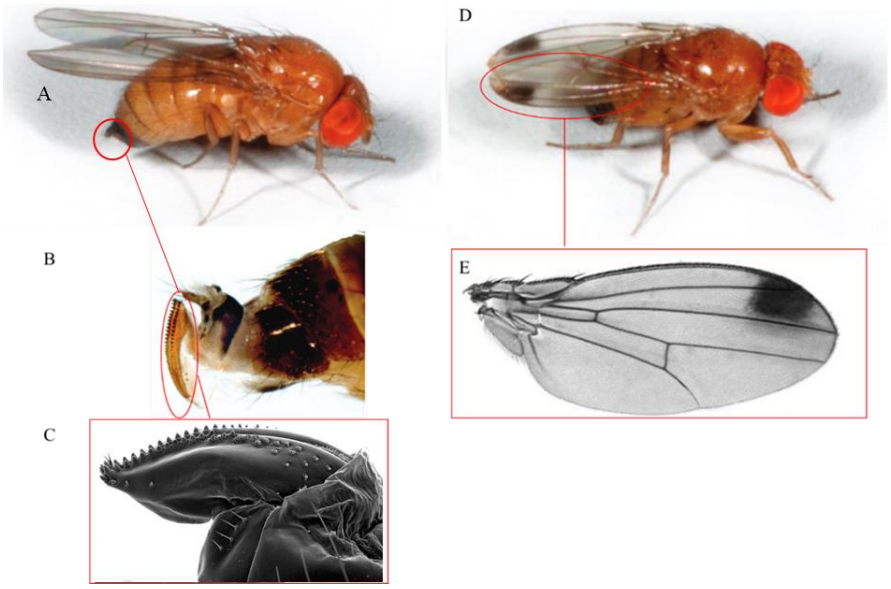


Figure 2. (A) Female *Drosophila suzukii* (Walsh et al., 2011). (B) The ovipositor of the female (Walsh et al., 2011). (C) Microscopic close-up of the ovipositor (Hauser, 2011). (D) Male *Drosophila suzukii* (Walsh et al., 2011). (E) Microscopic close-up of a male wing showing the distinguishing black spot (Hauser, 2011).

These factors, combined with the short life cycle, broad climatic tolerance, and the extensive range, constitute *D. suzukii* as a highly destructive pest. Consequently, infestations can rapidly become unmanageable, rendering entire harvests unmarketable (Figure 3) (Little et al., 2020; Kenis et al., 2016).

Figure 3. (A) The puncture in a cherry caused by a female *Drosophila suzukii* oviposition (Walsh et al., 2011). (B) A healthy blackberry compared to a spoiled blackberry following oviposition (Asplen et al., 2015).

1.2 The invasion of *Drosophila suzukii*

Although native to East Asia, *D. suzukii* has rapidly disseminated across much of the globe (Andreazza et al., 2017). The first report outside its endemic region was in the 1980s, when it

was identified in Hawaii, though it did not cause significant damage (Kaneshiro, 1983). A few decades later, it was discovered on ripening crops of strawberries, cherries, and blueberries in California, USA, marking the onset of its rapid spread across North America (Hauser, 2011).

Reports of *D. suzukii* in Europe began in 2009, initially found in Spain, Italy, France, and Switzerland (Calabria et al., 2012; Grassi et al., 2011). Subsequently, in 2011, it was reported in Germany (Vogt et al., 2012). From 2012 to 2013, *D. suzukii* had been detected in most of North America, Central Europe, and the Mediterranean regions. Detections in South America were reported from 2011 to 2017, and parts of Africa in 2013. Presently, *D. suzukii* is found on every continent besides Oceania and Antarctica (Figure 4) (Cini et al., 2012; Rota-Stabelli et al., 2020; Garcia et al., 2022; Langille et al., 2016; Kwadha et al., 2021; Ouantar et al., 2020).

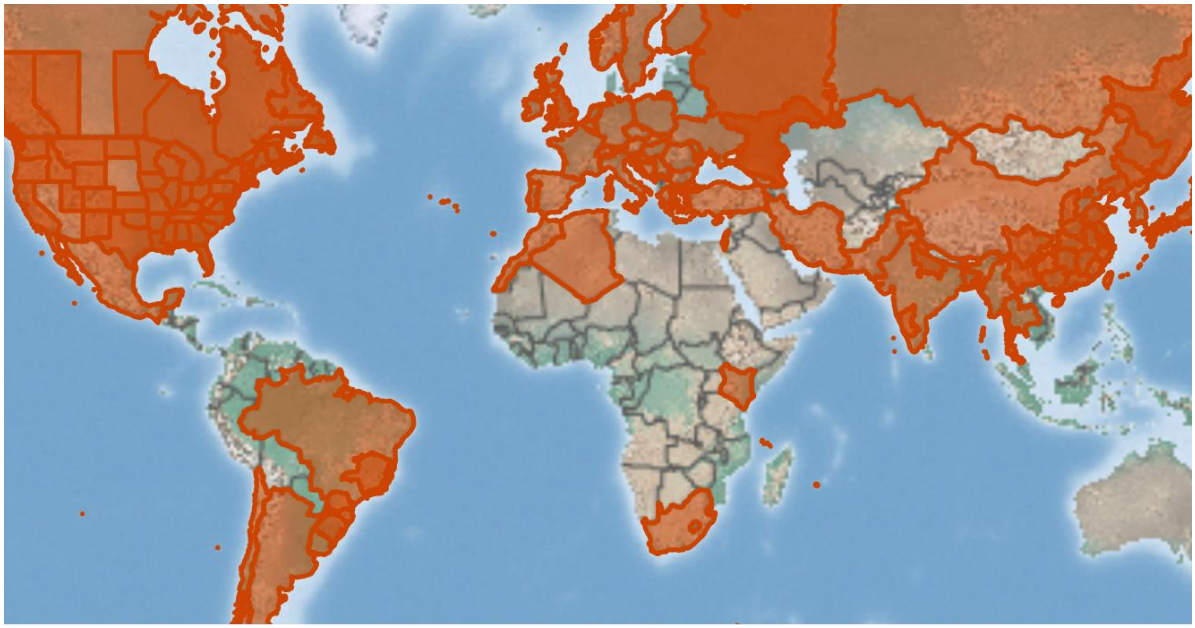


Figure 4. World map of the confirmed distribution (highlighted countries) of *Drosophila suzukii* (https://www.cabidigitallibrary.org/doi/10.1079/cabicompendium.109283#sec-8)

1.3 Economic impact of *Drosophila suzukii*

The widespread presence of *D. suzukii* has resulted in substantial economic losses, calculated by the damage to crops and the additional costs of pest control (De Ros, 2024). The extent of crop damage caused by *D. suzukii* can vary significantly, ranging from negligible to catastrophic. Several studies have estimated the economic impact of *D. suzukii* to be in the millions (Bolda et al., 2010; Knapp et al., 2020). However, accurately estimating these costs is challenging, as crop damage can occur both pre- and post-harvest (Knapp et al., 2020). In 2011, a study reported losses of about 3 million euros due to *D. suzukii* damage in fruit sales in Trento,

Italy (Simoni et al., 2011). In Oregon, the United States, the costs of pest control increased from 1 million USD to 15 million USD following the invasion of *D. suzukii* in 2013. In certain instances, control measures have incurred additional costs ranging from $250 to $350 per acre (Werts & Green, 2014). Similarly, in 2016, the estimated costs of damage to peaches and figs were 21.4 and 7.8 million USD, respectively, due to infestations (Benito et al., 2016). Table 1 contains a summary of estimated damages in various regions in the world.

Table 1. Summary of the financial damage caused by *D. suzukii* in the United States, Italy, and Switzerland.

Region / Country / Crop	Year(s)	Estimated Yield Loss / Context	Estimated Damage / Cost	Reference
Trento, Italy (fruit sales)	2011	Losses from crop damage due to *D. suzukii* infestation	€3 million (~$3.2 million)	Simoni et al., 2011
Oregon, USA (various crops)	2013	Increase in pest control costs post-invasion	$1 million → $15 million	Werts & Green, 2014
USA (general)	2013	Additional control costs per acre	$250–$350 per acre	Werts & Green, 2014
USA (peaches)	2016	Estimated damage from *D. suzukii* infestation	$21.4 million	Benito et al., 2016
USA (figs)	2016	Estimated damage from *D. suzukii* infestation	$7.8 million	Benito et al., 2016
Switzerland (sweet cherry)	~2016–18	Infestation and inspection costs per hectare	Up to 70000$ per hectare	Mazzi et al., 2017

1.4 Control of *Drosophila suzukii*

Control of *D. suzukii* has relied on conventional broad-spectrum chemical synthetic insecticides, such as pyrethroids, carbamates, and organophosphates. However, these insecticides are known to have adverse effects on the environment and ecosystems (Shawer, 2020), often impacting non-target organisms, including beneficial arthropods, mammals, birds, and reptiles (Wan et al., 2025). They also significantly impact human health, through both direct and indirect exposure (Ahmad et al., 2024). Additionally, *D. suzukii* has been reported to develop resistance to these insecticides (Shawer, 2020). An effective alternative to synthetic chemical insecticides is the use of natural bacterial-based spinosyns (Spinosad and Spinetoram), which have demonstrated efficacy in controlling *D. suzukii*. Nevertheless, their application must be limited throughout the year due to insecticide resistance (Deans & Hutchison, 2022; Tabuloc et al., 2024). Most insecticides are applied as spray coating over fruits, providing protection against adult flies but not larvae, as they do not come into contact with the fruit surface. This requires more frequent applications, particularly closer to harvest as fruits ripen, resulting in high insecticide residues on the fruit. (Shawer, 2020; Deans & Hutchison, 2022; Tabuloc et al., 2024).

The negative consequences of chemical insecticides underscore the urgent need for alternative, more sustainable, and holistic control strategies. Integrated Pest Management (IPM) is an ecologically-based strategy for pest control that combines multiple complementary methods, biological, cultural, mechanical, behavioral, and, chemical methods when necessary, in a coordinated and sustainable manner. The goal of IPM is to manage pest populations below

economically damaging levels while minimizing risks to human health, beneficial organisms, and the environment (Barzman et al., 2015; Kogan 1998). Cultural practices aim to mitigate the impact of a *D. suzukii* infestation by implementing unfavorable conditions for the pest, limiting access to host fruit. These practices include timely harvesting, pruning to reduce canopy humidity, mulching, exclusion netting, sanitation measures, and removal of fallen or infested fruit which help to disrupt the pest's life cycle (Schöneberg et al., 2021, Tait et al., 2021).

Biological pest control methods are valued for their specificity and diversity. If implemented well, they are an effective addition to any pest control program. Traditionally, it involves the use of antagonists to manage specific pests, including natural predators, parasitoids, and various pathogens (bacteria, fungi, and viruses) (Stenberg et al., 2021). In addition, the definition of autocidal biological control has been expanded to encompass genetic manipulation and the modification of pest populations in such a manner that the pests themselves become the primary agents of their population collapse. Techniques that align with this definition include the Sterile Insect Technique (SIT) and the release of insects carrying dominant lethal genes (RIDL) (Fryxell et al., 1995).

The application of biological control against *D. suzukii* remains a subject of ongoing research interest. For example, several parasitoid wasps, such as *Ganaspis brasiliensis*, have been identified to exploit the egg, larval, and pupal life stages of *D. suzukii* as hosts (Fellin et al., 2023). The findings demonstrate the successful parasitization of *D. suzukii* by wasps at varying rates, highlighting the adaptability of local wasps to this invasive pest (Häussling et al., 2022; Jarrett et al., 2022; Bezerra Da Silva et al., 2019; Mazzetto et al., 2015).

Additionally, *D. suzukii* has been shown to be susceptible to various entomopathogens, including bacteria (Bing et al., 2024; Bing et al., 2020; Hiebert et al., 2020), fungi (Cossentine et al., 2016; Cuthbertson & Audsley, 2016; Naranjo-Lázaro et al., 2014; Urbaneja-Bernat et al., 2020; Becher et al., 2017), nematodes (Cuthbertson & Audsley, 2016; Ibouh et al., 2019; Garriga et al., 2017; Hübner et al., 2017), and viruses (Lee & Vilcinskas, 2017; Linscheid et al., 2022). All pathogens were isolated from the environment and have demonstrated the capacity to induce mortality in *D. suzukii*. The remaining challenge for these pathogens lies in their large-scale cultivation and application (Bravo & Soberon, 2023). Furthermore, it is essential to formulate them to withstand environmental factors such as temperature, UV radiation, and weather conditions (Negi et al., 2023).

While there are many biological control methods described for *D. suzukii*, this dissertation focuses specifically on three approaches: SIT, virus-based control, and RNA

interference (RNAi). These strategies were selected due to their promising potential for species-specificity, scalability, and compatibility with integrated pest management programs. Unlike many traditional biological control agents, these methods offer the possibility of precise population targeting with minimal impact on non-target organisms and the surrounding environment. Furthermore, SIT, viruses, and RNAi align with the concept of autocidal control, where the pest population contributes to its own suppression. Given the increasing demand for sustainable and innovative pest control tools, these methods represent a critical area of ongoing research and development. Their evaluation in this dissertation aims to explore their feasibility, effectiveness, and role in future *D. suzukii* management strategies.

1.5 The Sterile Insect Technique: History and application against *Drosophila suzukii*

SIT is a pest management strategy involving the repeated release of large numbers of sterile insects into the environment. These releases aim to inundate the wild pest population with sterile mates, ultimately leading to population suppression or eradication. Insect sterilization can be achieved by ionizing radiation (Yamada et al., 2023), by chemical methods, or by genetic methods (Baxter, 2016). In the initial years of application of this technique, insects were sterilized using ionizing radiation from X-rays or gamma rays (Kaboré et al., 2023), which induced a dominant lethal mutation in the DNA of their reproductive cells (Bushland & Hopkins, 1951). While gamma radiation is highly effective, it generates nuclear waste and is subject to strict regulations (Kaboré et al., 2023). In addition, the improvements in X-ray technology make it a viable alternative. Chemical sterilization is also avoided due to the toxic waste it produces. Genetic sterilization, while efficient and effective, can be complex for similar reasons, and it also faces public hesitancy regarding the use of genetically modified organisms (GMOs).

The concept of SIT was independently conceived by several scientists in the 1930s and 1940s, but it was further developed in the 1940s by Edward F. Knipling and Raymond C. Bushland. By the 1950s, SIT was implemented in the United States to control *Cochliomyia hominivorax*, or the New World screwworm, successfully leading to its eradication (Klassen & Curtis, 2005). Since then, SIT has been applied to various pests, including Mediterranean fruit flies *Ceratitis capitata* (Plá et al., 2021), *Glossina* flies (Vreysen et al., 2000), Mexican fruit flies *Anastrepha ludens* (Cancino et al., 2023), codling moths *Cydia pomonella* (Thistlewood & Judd, 2019), and various mosquito species (Bouyer et al., 2024). While complete eradication

is not always feasible, SIT is highly effective for population suppression, particularly when integrated with other pest management strategies.

The advantages of SIT include: high specificity, environmental safety, and ecological soundness. Moreover, with a well-established system, it can be cost-effective (Marec & Vreysen, 2019; Kandul et al., 2019), making it one of the more effective methods for pest control. There are three key components required for an effective SIT system (Figure 5): an efficient mass-rearing system, a reliable and cost-effective sexing method, and a safe sterilization process (Kapranas et al., 2022). The mass-rearing system should be cost-effective and not labor-intensive due to the large number of sterile insects that will be periodically released (Parker et al., 2020).

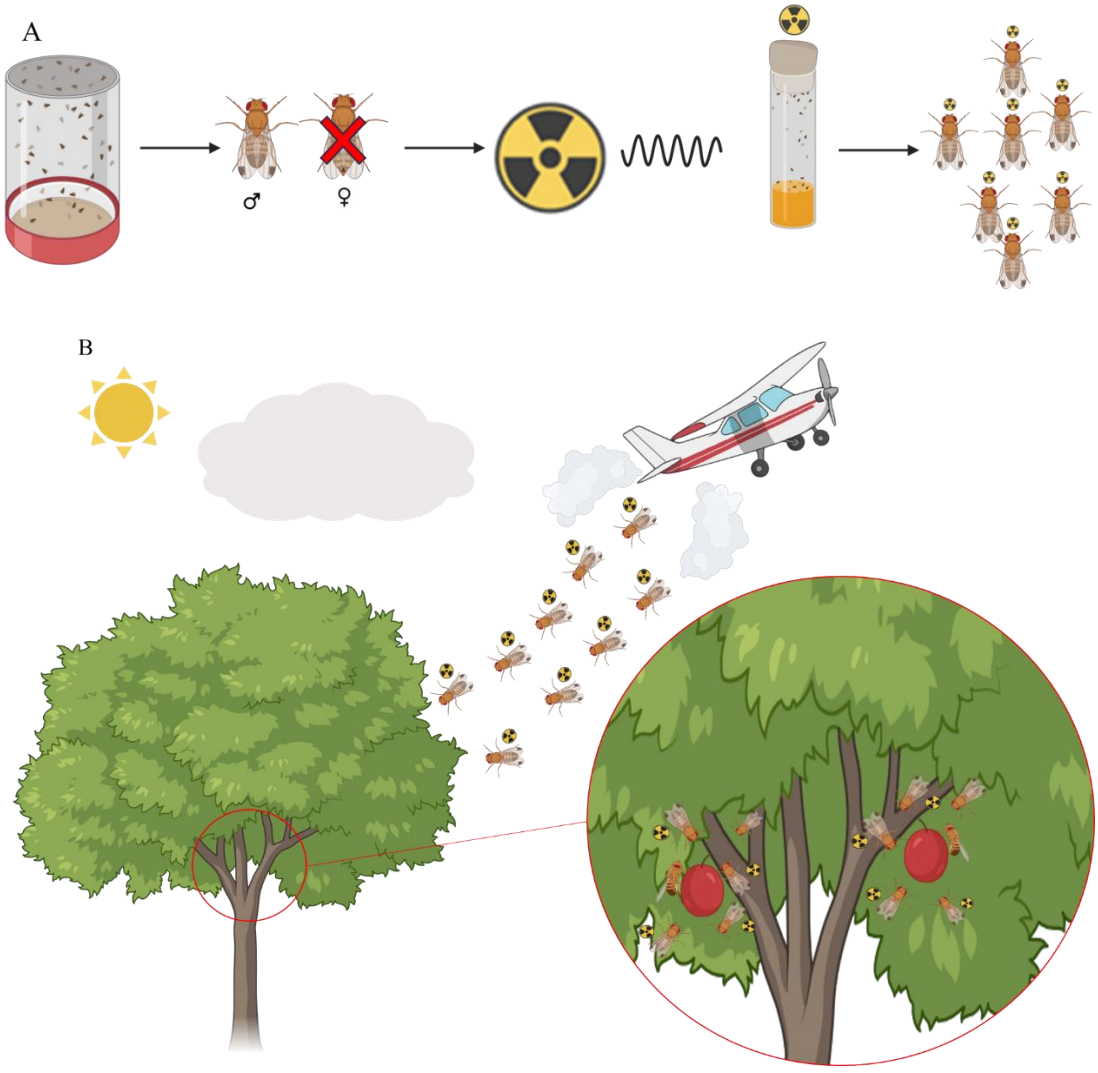


Figure 5. The typical process of SIT. This illustration depicts: (A) mass-rearing, sexing, and sterilization. (B) The aerial release of and the mode of action of the sterile male insects. Insects are reared in laboratory conditions, males are separated from females, sterilized with radiation, and subsequently released by aircraft over infested agricultural areas to suppress wild populations (Figure generated by BioRender).

Sex separation is important for efficiency, as it eliminates the potential for intermating of the sterile insects. Additionally, it prevents any nuisance caused by females, such as physical damage caused to the fruit by oviposition of *D. suzukii* and *Ceratitis capitata*, or the bites of blood-feeding mosquitoes (Mashatola et al., 2018; Nguyen et al., 2021; Cayol et al., 1994). Sexing can be based on size, since many species exhibit sexual dimorphism, with one sex consistently larger (Papathanos et al., 2009). Sexing can also be achieved through genetic modification or engineering. Insects can be engineered to express fluorescent proteins (Davydova et al., 2023), and to carry conditional female-lethal systems (Heinrich & Scott, 2000; Schetelig et al., 2021). However, the use of GMOs is difficult due to varying laws and regulations across regions (Ichim, 2021). Furthermore, public opinion on the unrestricted use of GMOs remains widely disputed (Ichim, 2021). Finally, sterilization should be environmentally safe while preserving the health and competitiveness of the insect, and it should comply with the laws and regulations of the region (Kapranas et al., 2022).

The SIT is a suitable control method for *D. suzukii*. Its rapid life cycle facilitates mass-rearing (Sassù et al., 2019; Aceituno-Medina et al., 2020), and the polyandrous mating behavior of the females increases the success rate of suppression (Chen et al., 2022; Krüger et al., 2019). Additionally, it has been sterilized using several different approaches (gamma radiation, genetic modification, and X-ray) without significant loss of fitness (Lanouette et al., 2017; Witherbee et al., 2017; Follett et al., 2014). X-ray sterilization, however, is a very attractive method due to the ease of use and implementation. Data obtained from field trials further support the use of SIT against *D. suzukii*, where populations were successfully suppressed in both conducted studies (Homem et al., 2022; Gard et al., 2023). However, optimizing the sterilization process by determining the minimum effective X-ray dose and energy level would further improve the feasibility of this technique by reducing costs and preserving insect quality. Additionally, developing alternative sterilization methods that eliminate the need for radiation, such as temperature-based approaches, could enhance the overall efficiency and scalability of SIT programs. Another remaining challenge in the application of SIT for the control of *D. suzukii* is the development of a reliable sexing system that does not rely on genetic modification. Although genetic sexing methods, such as sperm marking, CRISPR-mediated mutagenesis, and conditional female lethality, are highly accurate and well-established (Ahmed et al., 2019; Schetelig et al., 2021; Li et al., 2017), their widespread adoption is constrained by regulatory variability and public concerns, making standardization across regions difficult.

1.6 Viral Biocontrol: History and application in *Drosophila suzukii* management

Viruses are typically submicroscopic, infectious agents that exist in almost all ecosystems on earth. They require living cells for replication and are capable of infecting a wide range of organisms, from the simplest prokaryotes to the most complex eukaryotes (Grinin & Grinin, 2025). Entomopathogenic viruses, with host specificity often reaching down to the species level, play a crucial role in the natural regulation of insect populations. This phenomenon has been the subject of research since the early 20th century, with numerous viruses being evaluated as potential pest control agents. A significant milestone was achieved in 1970 when the first virus-based pesticide was registered in the United States to control the cotton bollworm *Helicoverpa zea* (López-Ferber, 2020). Since that time, additional virus-based pesticides have been approved, and research in this area continues to the present. The typical method for discovering entomopathogenic viruses involves collecting insects from natural environments, identifying symptoms, and isolating viruses from these samples (Liu & Bonning, 2011).

Entomopathogenic viruses are of considerable interest due to their specificity and environmentally friendly nature, as they can persist for extended periods, making them valuable components of IPM programs (Nikhil Raj et al., 2022). These viruses are classified based on their genetic material, either DNA or RNA (Eberle et al., 2012). Several challenges impede the successful commercialization of entomopathogenic viruses as biopesticides. The specificity of insect viruses often limits their appeal for investment, as they typically target only a single pest species or genus. Additionally, the time required for viruses to lethally affect their hosts, usually ranging from 4 to 14 days, poses a challenge. The persistence of viruses is limited by environmental factors such as temperature, weather, and ultraviolet radiation, which can inactivate them (Tadesse Mawcha et al., 2024). Furthermore, for a viral control method to be effective it needs to significantly affect the population of the target insect, and the mass production of insect viruses is cost-intensive due to the necessity of maintaining large insect numbers for production (Nikhil Raj et al., 2022). Moreover, insects can develop resistance to viruses (Fuxa, 1993). However, given their high host specificity, minimal non-target effects, and potential for environmentally stable persistence, insect viruses represent a robust and ecologically sound strategy for pest management.

These characteristics underscore the scientific and practical value of continued investment in virus-based biocontrol research within IPM frameworks. The search for viral antagonists of *D. suzukii* has been of significant interest since its invasion, with discovery efforts typically beginning by screening moribund wild flies. For instance, one study utilized metagenomic analysis to identify viral RNA associated with *D. suzukii* (Medd et al., 2018). Another study identified candidates through a continental screening aimed at investigating the interaction of Wolbachia with *D. suzukii* viruses (Dudzic et al., 2025). Additionally, model viruses such as *Drosophila* C virus (DCV), Cricket paralysis virus (CrPV), and Flock house virus (FHV) have been found to be lethal upon injection (Lee & Vilcinskas, 2017). *Drosophila* A virus and La Jolla virus were both identified in field screens conducted in Germany (Carrau et al., 2018). La Jolla virus (LJV), a positive-sense single-stranded RNA virus that was initially discovered in a metagenomic approach in *Drosophila melanogaster* (Webster, 2015), has garnered particular interest due to its ability to be administered orally and demonstrated rapid lethality when ingested (Linscheid et al., 2022). To test if LJV is a viable biocontrol option, several key questions have to be addressed, such as its persistence within fly populations, its impact on population dynamics, modes of transmission, and the effects of infection on feeding behavior.

1.7 RNA Interference: Mechanisms and applications in pest and *Drosophila suzukii* control

RNAi represents a conserved biological response to double-stranded RNA (dsRNA) functioning as a defense mechanism against pathogenic or foreign nucleic acids (Matzke et al., 2005). The induction of RNAi can occur either endogenously or exogenously, contingent upon the source of the dsRNA (Dave et al., 2003). During the 1990s, several researchers reported the phenomenon of RNAi. In 1990, Napoli and Jorgensen made contributions in this field while attempting to produce violet petunias through the co-suppression of the transgene and the endogenous gene coding for chalcone synthase. In 1992, Romano and Macino observed a similar occurrence in *Neurospora crassa*, where the introduction of homologous RNA sequences resulted in the quelling of the endogenous gene (Sen & Blau, 2006). Guo and Kemphues noted the degradation of par-1 mRNA by introducing either sense or antisense RNA to the gene in *Caenorhabditis elegans* (*C. elegans)*. Finally, in 1998, Fire and Mello published a seminal paper elucidating this silencing trigger in *C. elegans,* concluding that the trigger for RNAi is dsRNA rather than ssRNA. RNAi is activated by viral dsRNA, aberrant transcripts from repetitive sequences such as transposons, or host-derived pre-microRNA (pre-miRNA),

all of which are processed by the cell's intrinsic RNAi machinery to regulate gene expression and maintain genome stability. In contrast, exogenous RNAi is induced by the introduction of externally synthesized dsRNA, short interfering RNAs (siRNAs), or hairpin RNAs (hpRNAs), which are extensively used in research and biotechnology (Sen & Blau, 2006).

Transgenic plants can be engineered to express dsRNA endogenously to silence specific genes controlling target traits; however, maintaining such plants is labor-intensive, and their classification as GMOs limits their widespread use (Dalakouras et al., 2020; Zhai et al., 2009). Consequently, exogenous RNAi is considered a more practical and socially acceptable alternative, offering a non-GMO approach to gene silencing while achieving similar effects.

RNAi (Figure 6) is initiated by processing long dsRNA with the enzyme Dicer into short RNA fragments. In animals, Dicer processes these dsRNAs in the cytoplasm into siRNAs or pre-miRNAs into mature microRNAs (miRNAs), typically 21–25 nucleotides in length. These small RNAs are then loaded into the RNA-induced silencing complex (RISC), where one strand (the guide strand) is retained. The Argonaute family proteins, particularly Argonaute 2 (Ago2), form the catalytic core of RISC. Ago2 binds the guide strand and facilitates recognition of complementary messenger RNA (mRNA) targets. Depending on the degree of complementarity, Ago2 can either directly cleave the target mRNA (slicer activity) or recruit additional factors to mediate translational repression, ultimately silencing gene expression (Sontheimer, 2005; Wilson & Doudna, 2013). In animals, there are three main RNAi pathways: the microRNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA) pathways (Formaggioni et al., 2024).

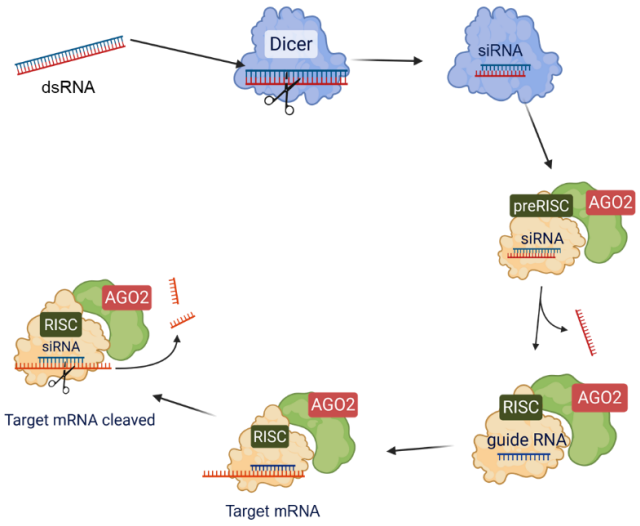


Figure 6. A visual and simplified representation of the RNAi pathway. Dicer and the RISC complex process dsRNA into siRNAs, which guide cleavage of target mRNA, ultimately leading to silencing. (Figure generated by BioRender. Creator: Krish Mistry).

Insects employ the siRNA pathway to defend against viral infections by silencing and neutralizing foreign genetic material. This natural defense mechanism can be harnessed by introducing exogenous dsRNA to target specific endogenous insect genes (Vogel, et al., 2019; Kingsolver et al., 2013). This strategy has revolutionized invertebrate molecular biology research by enabling loss-of-gene-function studies in non-model species, ultimately leading to the discovery that silencing essential genes in insects can result in mortality. This insight has become a powerful tool for pest control (Christiaens et al., 2020).

Gene-based pest control represents a safer and environmentally sustainable alternative to conventional insecticides (Grilli et al., 2021). By targeting the sequences of species-specific genes, the risk of affecting non-target organisms is significantly reduced (Arora et al., 2021). Furthermore, its organic nature ensures minimal environmental and ecological impact (Christiaens et al., 2020). However, several considerations must be addressed prior to the deployment of dsRNA as a biopesticide. These include delivery methods, such as soaking or oral ingestion, which render it suitable for application as a sprayable pesticide (Cedden & Bucher, 2024). It is imperative that this spray is formulated to remain sufficiently stable in the environment to fulfill its insecticidal role, and sufficiently degradable to avoid long-term residues in soil and water, and resistant to degradation to RNases present in many insects (Cedden & Bucher, 2024). Additionally, delivery via transgenic plants, although not yet widely adopted, is another viable option. A critical factor to consider is the dependency of RNAi success on the selection of an appropriate target gene (Cedden & Bucher, 2024). Moreover, the production costs associated with RNAi can be substantial (Zhu & Palli, 2020).

The first commercially available RNAi pest control was introduced in 2017, following the U.S. Environmental Protection Agency's approval of Monsanto's transgenic maize. This maize synthesizes dsRNA targeting the SNF7 gene in the Western Corn Rootworm (*Diabrotica virgifera virgifera*), resulting in high mortality rates even in low concentrations (Christiaens et al., 2020). Since then, two additional RNAi-based control products have received commercial approval (Liu et al., 2025). The most frequently targeted pest species for RNAi applications belong to the orders Hemiptera, Coleoptera, Lepidoptera, and Diptera (Vogel et al., 2019).

The application of RNAi for *D. suzukii* control is not yet in practice. Despite the identification and silencing of numerous potential target genes (Yoon et al., 2023), the

efficiency remains suboptimal, as reflected by low lethality, particularly in adults (Taning et al., 2016). This inefficacy may be attributed to the non-systemic nature of RNAi in *D. suzukii* (Abrieux & Chiu, 2016; Jarausch et al., 2018). Furthermore, the primary mode of administration is oral, which may lead to degradation and other complications. Nonetheless, extensive research is underway to improve delivery efficiency (e.g., lipid-based formulation, transgenic microorganisms expressing RNAi, and packaging RNAi with virus-like particles), and production (e.g., microbes expressing the dsRNA) (Murphy et al., 2016; Xue et al., 2024).

1.8 The aim and objectives of this study

This dissertation aims to contribute to the development of sustainable and species-specific biological and biotechnological control strategies for *D. suzukii* by investigating three distinct, complementary approaches: SIT, viral biocontrol using LJV, and RNAi.

The first section focuses on enhancing SIT as a viable management strategy for *D. suzukii*. It examines the optimization of X-ray-based sterilization, including the effects of varying radiation power outputs on sterility and fly fitness. In addition, it explores a novel thermal sterilization method using elevated incubation temperatures. The development of an efficient sexing system is also addressed, using pupal size as a potential morphological marker to enable sex separation during rearing.

The second section characterizes the biological and ecological potential of LJV as a viral biocontrol agent. This includes an in-depth investigation of its transmission pathways (airborne, oral, fecal, and venereal), its effects across generations, and the influence of infection on feeding behavior and overall host fitness. These findings further reinforce the feasibility of using LJV to suppress *D. suzukii* populations through natural or induced viral epidemics.

The final section evaluates RNAi as a gene-targeting tool for *D. suzukii* control. Two potential applications are explored, the first as a genetic support tool for SIT, targeting genes involved in sex determination and reproduction to enable the breeding of sterile, male-only populations; and the second as a species-specific biopesticide by silencing essential genes to induce mortality. Although this work remains unpublished, it offers foundational data for future RNAi-based pest control strategies. The findings will be elaborated upon in sections II and III.

II Summary of Publications and Unpublished Work

The chapters of this section summarize the results of research articles published as a first author in peer-reviewed journals during the tenure as a PhD student. Additionally, it includes a summary of work that is currently pending publication. The research conducted over three years as a PhD student focuses on the biological and biotechnological control of *D. suzukii* using SIT (Chapter I), entomopathogenic viruses (Chapter II), and RNAi (Chapter III).

Chapter I

Radioactivity and GMO-Free Sterile Insect Technology for the Sustainable Control of the Invasive Pest *Drosophila suzukii*

Ibrahim Abdelhafiz, Stefan Gerth, Joelle Claussen, Mareike Weule, Eva Hufnagel, Andreas Vilcinskas, and Kwang-Zin Lee

Adv Biol (Weinh). 2024 Jul;8 (7): e2400100. doi: 10.1002/adbi.20240010 PMID: 38797923

This study investigates the efficiency of sterilizing *D. suzukii* with X-ray and examines the influence of various parameters on this process. Four different energy outputs (255 kV, 160 kV, 130 kV, 90 kV) were tested, along with different radiation dosages (40 and 60 Gy). The sterilization efficiency was assessed by conducting several single mating experiments and evaluating the impact of the sterilization process on the fitness of the irradiated flies. It was observed that most energy outputs and dosages resulted in similar sterilization effects, with the exception of the 90 kV/40 Gy combination, which achieved near-perfect sterility with a slight reduction in lifespan (treatment average of offspring = 3.3107). This difference was attributed to the proximity of the flies to the radiation source. Subsequently, a novel temperature-dependent sterilization process was explored. Results indicate a high temperature of 30 °C induces sterility in the flies, albeit with a significant cost to fitness. Furthermore, the duration of exposure to the high-temperature environment directly influences the fertility and lifespan of the flies, with both increasing as incubation time at 30 °C decreased. Finally, an attempt at sexing was done by exploiting the potential size difference in the pupal stage between female (average weight = 1.057 mg) and male flies (average weight = 0.873 mg). Results indicate a significant size difference between females and males in the pupal stage, which can be utilized for sorting. However, further optimization is required for complete viability.

The results obtained advance the application of SIT against *D. suzukii*, contributing to mitigating the pest's impact.

Chapter II

La Jolla Virus: The Pathology and Transmission in Its Host *Drosophila suzukii*

Ibrahim Abdelhafiz, Tobias Kessel, Andreas Vilcinskas, and Kwang-Zin Lee

Viruses. 2025 Mar 13;17(3):408. doi: 10.3390/v17030408 PMID: 40143335 PMCID: PMC11945923

This study aims to further investigate and characterize LJV, reaffirming its suitability as a biocontrol agent. It investigates a chronically infected population of flies and the viral impact on it by testing several horizontal transmission routes, including airborne, venereal, oral, and fecal. The results indicate a high transmission rate through the oral/fecal route. An investigation into the impact of the virus on the fecundity of the flies and the success rate of infected offspring reaching adulthood was conducted, revealing a significant (~33%) reduction in the egg-to-adult success when infected with LJV. Additionally, an investigation into whether LJV alters the feeding behavior of the flies indicated that infection with LJV significantly affects female flies, rendering them less likely to search for food. There also appears to be an effect on feeding duration and interaction with food, although not statistically significant.

Overall, the results enhance the understanding of LJV and its population dynamics, by demonstrating the virus's persistence can adversely affect the population. These findings support the use of LJV as a tool within a broader integrated pest management plan against *D. suzukii*.

Chapter III

Unpublished work: RNAi for *Drosophila suzukii* Sex Manipulation, Sterility, and Control

This chapter summarizes the work conducted with RNAi and *D. suzukii*. RNAi was tested for two different applications. The first application sought to determine whether RNAi could enhance the SIT by sex manipulation and breeding gender-specific populations. Studies show how the genes *sex-lethal* (*sxl)* and *transformer* (*tra)* play an important role in differentiation into females, and *male-specific lethal-2 (msl-2)* for male differentiation. Also, genes believed to be responsible for fertility were targeted to see if sterile males can be generated. The second application explored the potential of RNAi as a biopesticide against *D. suzukii* by targeting and silencing genes essential for various functions and pathways. This chapter will briefly outline the process and the negative results that led to the project's termination.

Materials and methods:

dsRNA synthesis

Target genes were selected and primers were designed and ordered (Merck) using Geneious and NCBI primer design (all genes and rationale for targeting them are available in Table 2). All primers had the T7 sequence attached to them (TAATACGACTCACTATAGGGAG). *D. suzukii* RNA was extracted using TRIzol reagent (TRIzol™ - Invitrogen) following the protocol. cDNA was then synthesized (RevertAid First Strand – cDNA – Synthesis Kit – Thermo Fisher). PCR (GoTaq Green PCR Master Mix - Promega) was then performed to obtain all selected products, which were confirmed by sequencing. The products were purified (Monarch Spin PCR & DNA Cleanup Kit – New England Biolabs) and then cloned into E. coli (NEB 5-alpha High Efficiency Competent E. coli and pGEM®-T Easy Vector System – New England Biolabs). Successful cloning was confirmed by selecting white colonies from MacConkey agar containing 1% ampicillin (10 mg/ml). A single colony was then picked and incubated overnight in 5 ml LB broth containing 1% ampicillin in a shaking incubator at 250 rpm at 37 °C. Plasmids were extracted from the overnight culture the following day using (Monarch Spin Plasmid Miniprep Kit – New England Biolabs). PCR was performed again and products were extracted and purified from 1% agarose gel (Monarch Spin DNA Gel Extraction Kit – New England Biolabs). PCR was performed and purified for a final time; the products were then used for dsRNA synthesis. dsRNA synthesis was done using the MEGAscript™ T7 Transcription Kit (Thermo Fisher) and purified via phenol–chloroform extraction.

Using RNAi for a sex manipulation of *D. suzukii*

Two different methods were conducted to confirm whether RNAi can be used to manipulate the sex of the flies. The first method involved injecting the abdomen of virgin female flies aged 3-7 days with dsRNA (4 µg/µl). The females were then allowed to rest for 3 days before being paired with 3 males of the same age for mating for 18 days (the medium was exchanged every 6 days and dead males were replaced). Following the mating, the offspring resulted from the pairing were sorted and counted. Additionally, virgin females and males were isolated for a subsequent mating to observe any potential effects in the F2 generation. The F2 pairings were conducted by pairing one F1 female with three wild-type (WT) males, and three F1 males with one WT female.

The second method involved feeding the larvae, aged 3-4 days, of *D. suzukii* with dsRNA. Twenty larvae were added to 50 µl of water and starved for three hours. Post-starvation, the larvae were placed on 50 µg of standard fly food mixed with 16 µl of dsRNA solution. The dsRNA was in a solution to prevent degradation by gut RNases. This solution was composed of 8 µl of 2 µg/µl dsRNA + 7 µl of sucrose solution (20% sucrose + 10 mM Tris, pH = 7.5 + 0.05 mM spermidine) + 1 µl of Lipofectamine (Adopted from Taning et al., 2016). An alternative solution replacing Lipofectamine with DMSO was also tested. The larvae were allowed to feed for five hours, then transferred to standard food tubes to reach adulthood. The sex of the adults was recorded, and they were isolated for pairings. Ten treated males were singly paired with ten WT virgin females, and ten WT males were singly paired with ten treated virgin females.

Using RNAi for the generation of sterile males

For sterility assessment, adult males were injected with 4 µg/µl of dsRNA and rested for 3 days, then each injected male was paired with a single virgin WT female. Mating proceeded as before, with dead females being replaced, and offspring were counted post-mating. The offspring of this mating were then isolated and paired to assess the F2. Ten treated males were singly paired with ten WT virgin females, and ten WT males were singly paired with ten treated virgin females. An experiment with larvae was also conducted as previously described, and then paired with 10 WT counterparts to assess fertility.

Using RNAi as a biopesticide

For the lethality test, twenty adult female flies were injected with 200 nl of dsRNA (80 ng/µl concentration). They were then placed on food and had their lifespan measured.

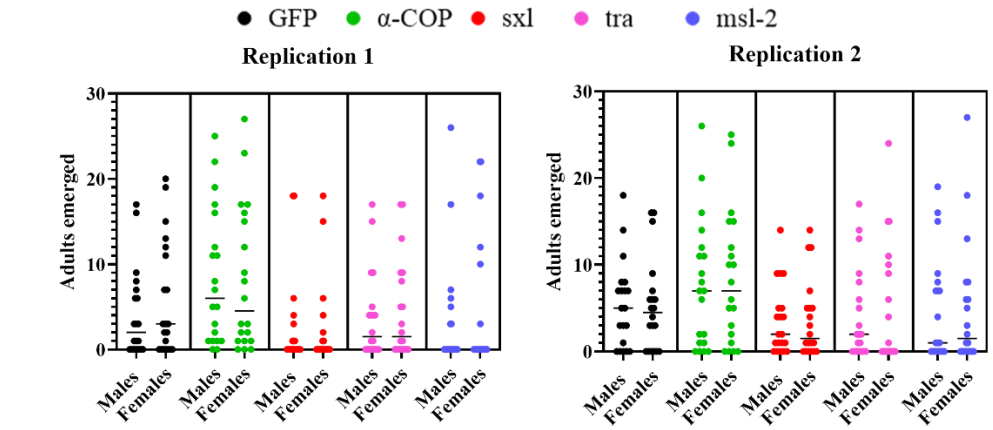
Table 2. Table of all selected genes for different tests. Table highlights the reason behind picking the genes and their functions function

Target gene	Reason of targetting	Function	Sequence of primers	Length
sxl	Generate male only in fly stock	Gene essential for female development (Meise et al., 1998)	3'- TCATCGCAGCATCGATAGGTC -5' 5'- TTATCGCCATCGTTGTTCCCA -3'	379
tra	Generate male only in fly stock	Sex determination (Baker & Wolfner, 1988)	3'- TCTGTGCAACGAGATTCCCAT -5' 5'- CGATGGTCGCTGGTATCCAAA -3'	444
msl-2	Generate female only in fly stock	Responsible for dosage compensation in male flies (Kelley et al., 1995)	3'- CGGTGTATTTGAAGGTGACGC -5' 5'- GAGATGTTGGAACCCTCACGT -3'	445
α-COP	Positive control to confirm RNAi is functional	Codes essential protein (Taning et al., 2016)	3'- GAATTACAAGACGGCCGCC -5' 5'- AACTAAACTAAGGGGTCTCGC -3'	397
soti	Sterility	Late stages of spermatid development (Barreau et al., 2008)	3'- CAGGGATCCCAATGATGAGCT -5' 5'- ATCGATCTTCTGAGGTTCCGC -3'	484
can	Sterility	Encodes a testis-specific TBP-associated factor essential for spermatogenesis. (White-Copper, 2010)	3'- CCGTTCGTTTGTGGTGTCTTC -5' 5'- GTCCACCCACCACCAATATGT -3'	495
sa	Sterility	Encodes a nuclear protein involved in male meiosis (White-Copper, 2010)	3'- ACAGATTCCGTGAGATTGCCA -5' 5'- TGTCCTTCTCAAAGACCTCGC -3'	549
CG15189	Sterility	Not entirely known, but testis spicific (White-Cooper, 2010)	3'- TGTCCGTGTGAATAGCTTCGT -5' 5'- GTTCATTCACAGCCGGGACTT -3'	477
CG5062	Sterility	Not entirely known, but testis spicific (White-Cooper, 2010)	3'- GAGGCCGATGAGGATGTGAAT -5' 5'- CGGCCCGTTTATACTCATCCA -3'	418
Vha26	Positive control to confirm RNAi is functional and induces lethality	Encodes the 26 kDa E subunit of the vacuolar-type H^+-ATPase (Murphy et al., 2016)	3'- TGGAGCAGTACAAGGCC -5' 5'- AACAGGGCGTTACGAATC -3'	193
yTub23C	Positive control to confirm RNAi is functional and induces lethality	Encodes a γ-tubulin isoform crucial for microtubule organization and function (Murphy et al., 2016)	3'- ACGCTAAGTCGGAGGAC -5' 5'- ACAGTATTAACATACATGCG -3'	183
Ssk	Test if induction is lethal	Critical for the proper formation of invertebrate smooth septate junctions (Yoon et al., 2022)	3'- ACGTGGTTGGCTGCATTA -5' 5'- CTCCGAGTTCACATAGAGGAATC -3'	101
Dh31-R1	Test if induction is lethal	Codes protein involved in G protein-coupled receptor signaling pathwayS (Yoon et al., 2022)	3'- GGATTCGACCCAGCAAGATT -5' 5'- TTACGCAGGTGGTGTAGTTG -3'	106
mth	Test if induction is lethal	Codes essential G-protein-coupled receptors involved in lifespan (Yoon et al., 2022)	3'- GTTCCCGAATGGTTCGTATCT -5' 5'- CTTATGTGGCTCTCCACCTTT -3'	117
NPFR1	Test if induction is lethal	Integral part of the sensory system that mediates food signaling, providing the neural basis for the regulation of food response (Yoon et al., 2022)	3'- GTGAGTCAGGCCACTCTTT -5' 5'- GACATGCTCTGGGAGTTCTT -3'	110
mayo	Test if induction is lethal	Enable G protein-coupled receptor activity and mechanoreceptor activity (Contreras et al., 2023)	3'- CGGAGGAATCCACTGAAACTAA -5' 5'- CTGATCGTGGTTGCTGACTAT -3'	98
ktch	Test if induction is lethal	Enable G protein-coupled receptor activity and mechanoreceptor activity (flybase.com)	3'- GCCGCCGATGTCTTTAGTAT -5' 5'- TCCGTGGTCATGACTTTCTG -3'	116

Results

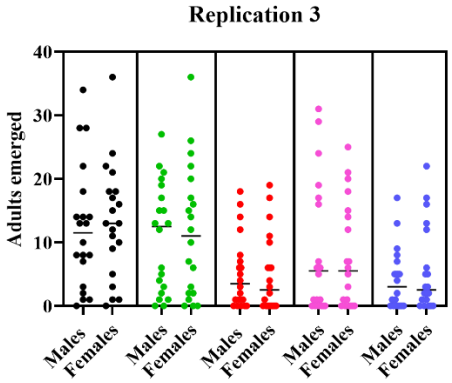
RNAi cannot be used to generate male-only populations

Our results indicate that RNAi is not suitable to manipulate the sex of the populations whether through adult injection, larval feeding, or F2 generation. The results of all the pairings can be seen in Figures 7-10, where the number of males and females were counted with no significant difference in the sex emergence apparent.



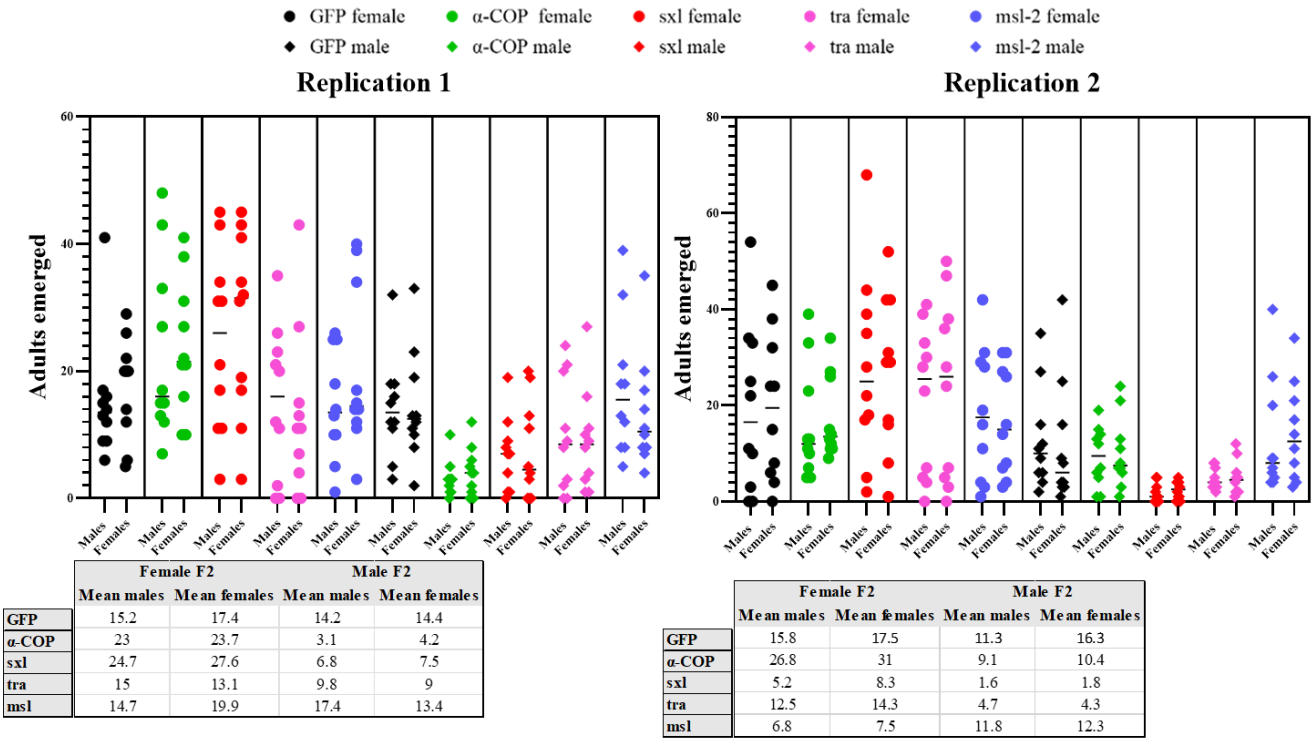
	Mean males	Mean females
GFP	4.05	5.9
α-COP	8.35	8.15
sxl	2.6	2.35
tra	3.75	4.5
msl	3.35	4.35

	Mean males	Mean females
GFP	5.35	5.2
α-COP	7.65	8.4
sxl	3.55	3.65
tra	4.15	4.75
msl	4.75	4.9



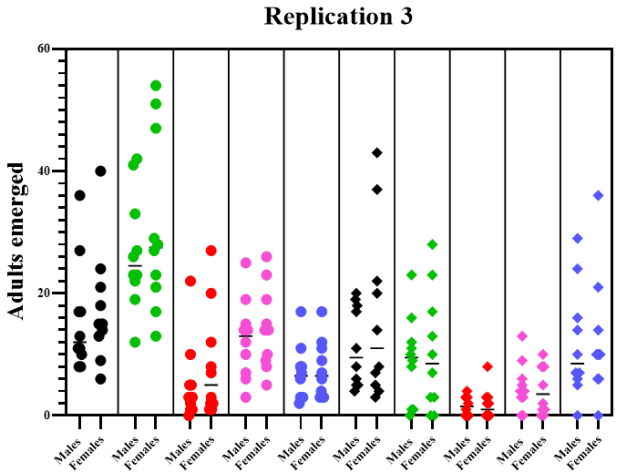
	Mean males	Mean females
GFP	12.3	13.25
α-COP	10.8	11.65
sxl	5.2	4.95
tra	8.45	7.75
msl	4.1	5.5

Figure 7. Results of single mating after injection of adult females with dsRNA, which show no significant difference between the emerged males and females. The dots in the figures represent the number of emerged adults, the line represents the median of the emerged adults. Tables represent the mean of emerged males and females from each crossing.



	Female F2		Male F2	
	Mean males	Mean females	Mean males	Mean females
GFP	15.2	17.4	14.2	14.4
α-COP	23	23.7	3.1	4.2
sxl	24.7	27.6	6.8	7.5
tra	15	13.1	9.8	9
msl	14.7	19.9	17.4	13.4

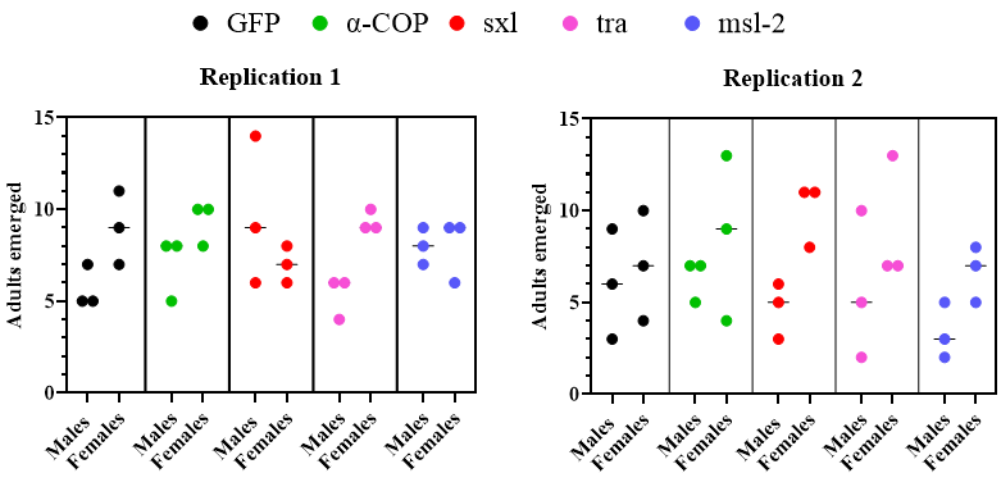
	Female F2		Male F2	
	Mean males	Mean females	Mean males	Mean females
GFP	15.8	17.5	11.3	16.3
α-COP	26.8	31	9.1	10.4
sxl	5.2	8.3	1.6	1.8
tra	12.5	14.3	4.7	4.3
msl	6.8	7.5	11.8	12.3



	Female F2		Male F2	
	Mean males	Mean females	Mean males	Mean females
GFP	19.2	19.6	12.8	11.5
α-COP	15.9	17.2	9.3	10.1
sxl	27.8	26.7	1.7	2.4
tra	21	23.8	4.7	4.9
msl	18.4	16.7	13	14.2

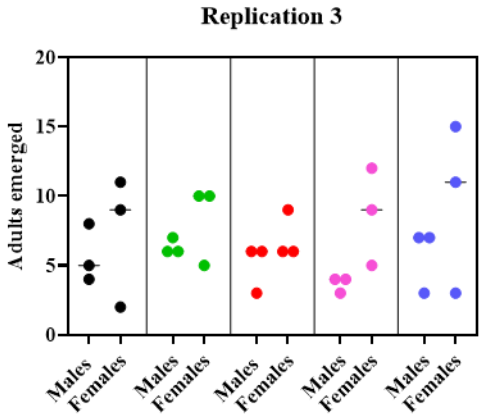
Figure 8. The F2 of the offspring from the adult injections. No significant difference observed between the emerged males and females. The dots in the figures represent the number of emerged adults, the line represents the median of the emerged adults. Tables represent the mean of emerged males and females from each crossing.

A



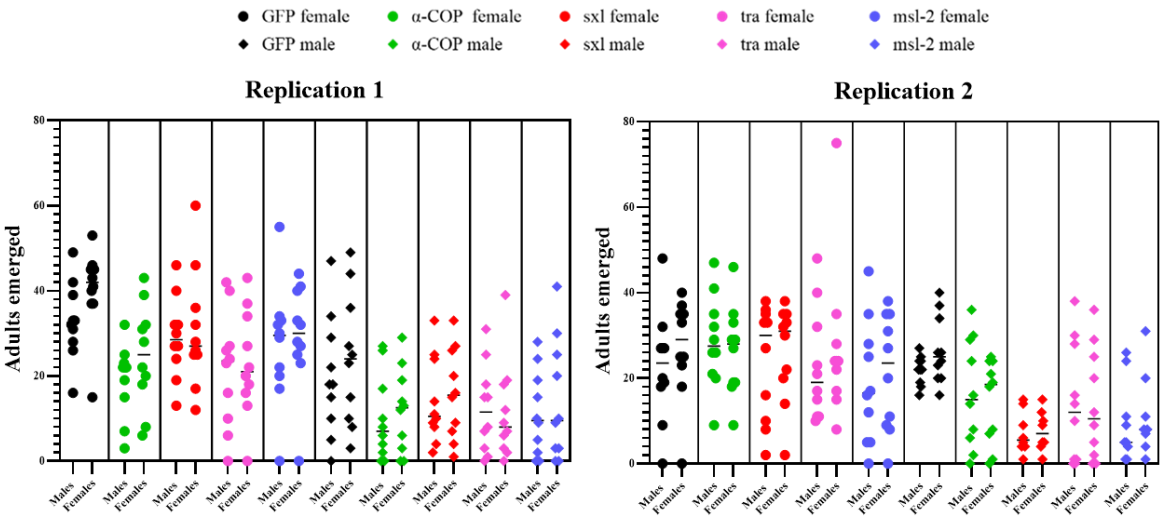
	Mean males	Mean females
GFP	5.667	9
α-COP	7	9.333
sxl	9.667	7
tra	5.333	9.333
msl	8	8

	Mean males	Mean females
GFP	6	7
α-COP	6.333	8.667
sxl	4.667	10
tra	5.667	9
msl	3.333	6.6667



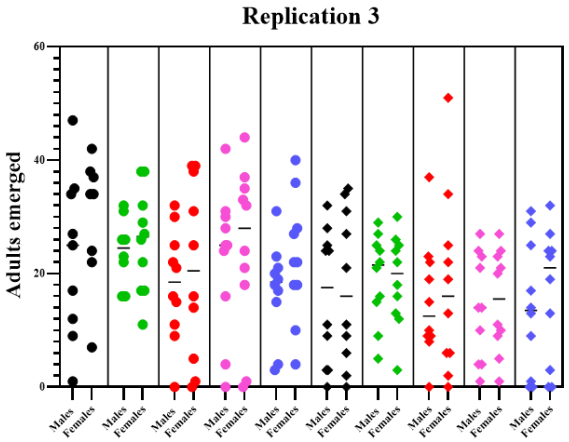
	Mean males	Mean females
GFP	5.667	7.333
α-COP	6.333	8.333
sxl	5	7
tra	3.667	8.667
msl	5.667	9.667

B



	Female Offspring		Male Offspring	
	Mean males	Mean females	Mean males	Mean females
GFP	32.9	40.2	19.8	24
α-COP	19	24.7	10	11.9
sxl	29	30.7	14	15.8
tra	21.4	23	21.3	11.5
msl	27.2	29.3	11.2	14.1

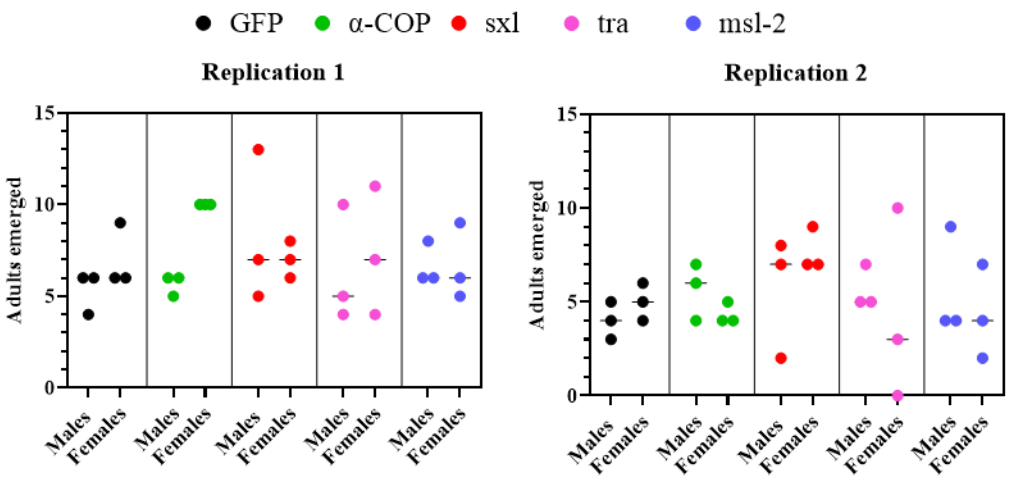
	Female Offspring		Male Offspring	
	Mean males	Mean females	Mean males	Mean females
GFP	22.7	27.1	21.9	26.6
α-COP	28.6	26.4	16.5	14.7
sxl	23.8	26.1	7.25	7.625
tra	22.8	27.2	13.8	13.8
msl	18.8	21.4	9.556	11.25



	Female Offspring		Male Offspring	
	Mean males	Mean females	Mean males	Mean females
GFP	23	29.75	15.9	17.6
α-COP	23.4	25.7	19.3	18.9
sxl	18.1	20.8	15.2	17.8
tra	22.5	24.5	14.2	15.1
msl	17.1	22.5	13.9	16.5

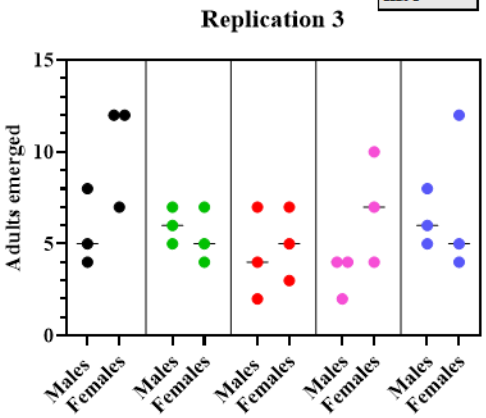
Figure 9. (A) Results of feeding larvae with dsRNA in Lipofectamine solution. No skewing towards males observed. Tables represent the mean of emerged males and females from the treated larvae. (B) The results of pairing up to 10 of the emerged males and females with their WT counterparts and the offspring from this mating. The results of this pairing also show no significant differences. The dots in the figures represent the number of emerged adults, the line represents the median of the emerged adults. Tables represent the mean of emerged males and females from each crossing.

A

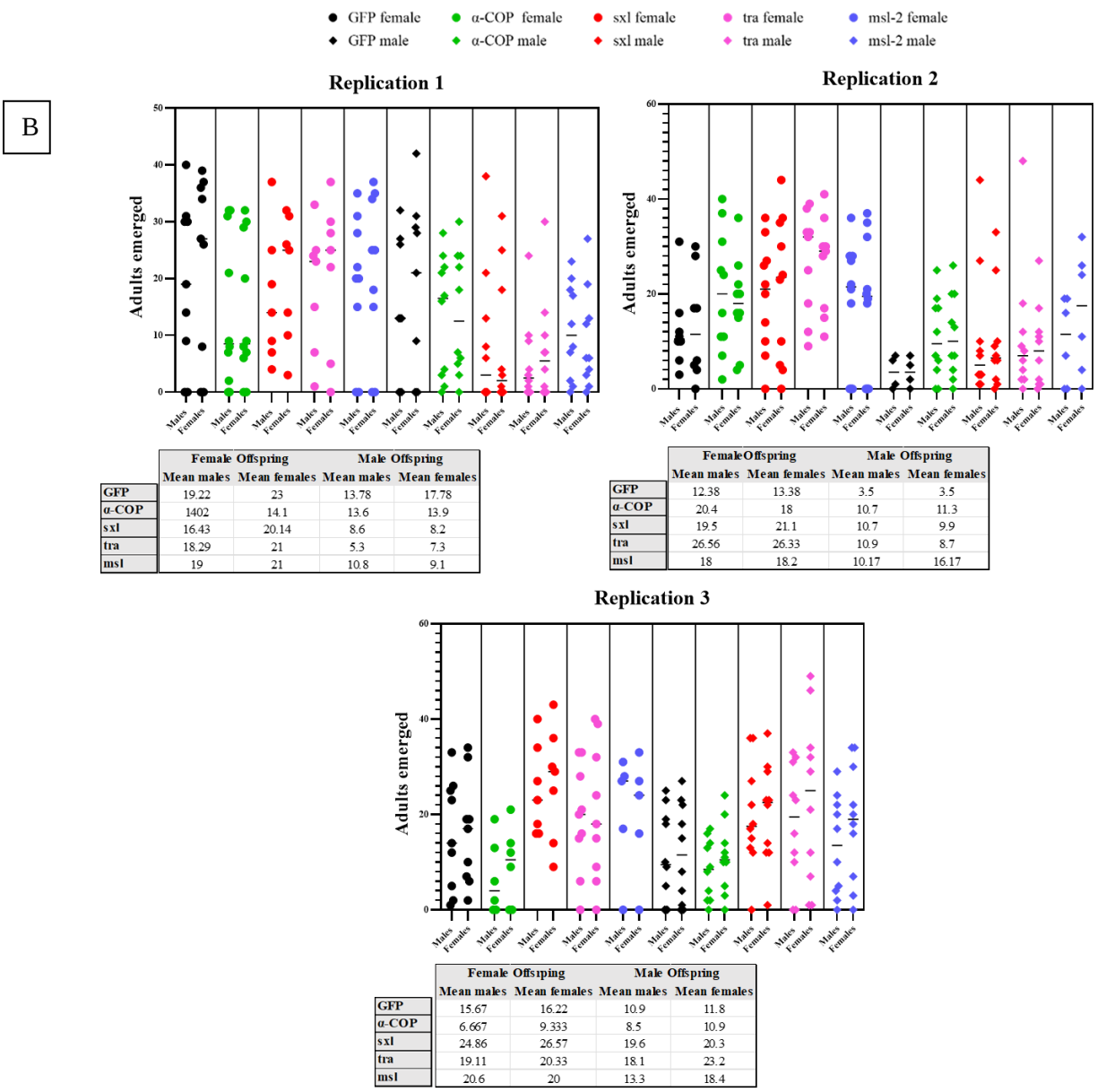


	Mean males	Mean females
GFP	5.333	7
α-COP	5.667	10
sxl	8.333	7
tra	6.333	7.333
msl	6.667	6.667

	Mean males	Mean females
GFP	4	5
α-COP	5.667	4.333
sxl	5.667	7.667
tra	5.667	4.333
msl	5.667	4.333



	Mean males	Mean females
GFP	5.667	10.33
α-COP	6	5.333
sxl	4.333	5
tra	3.333	7
msl	6.333	7



	Female Offspring		Male Offspring	
	Mean males	Mean females	Mean males	Mean females
GFP	19.22	23	13.78	17.78
α-COP	1402	14.1	13.6	13.9
sxl	16.43	20.14	8.6	8.2
tra	18.29	21	5.3	7.3
msl	19	21	10.8	9.1

	Female Offspring		Male Offspring	
	Mean males	Mean females	Mean males	Mean females
GFP	12.38	13.38	3.5	3.5
α-COP	20.4	18	10.7	11.3
sxl	19.5	21.1	10.7	9.9
tra	26.56	26.33	10.9	8.7
msl	18	18.2	10.17	16.17

	Female Offspring		Male Offspring	
	Mean males	Mean females	Mean males	Mean females
GFP	15.67	16.22	10.9	11.8
α-COP	6.667	9.333	8.5	10.9
sxl	24.86	26.57	19.6	20.3
tra	19.11	20.33	18.1	23.2
msl	20.6	20	13.3	18.4

Figure 10. (A) Results of feeding larvae with dsRNA in DMSO solution. No skewing towards males observed. Tables represent the mean of emerged males and females from the treated larvae (B) The results of pairing up to 10 of the emerged males and females with their WT counterparts and the offspring from this mating. The results of this pairing also show no significant differences. The dots in the figures represent the number of emerged adults, the line represents the median of the emerged adults. Tables represent the mean of emerged males and females from each crossing.

RNAi cannot induce sterility in *D. suzuki*i males

The results also confirm that dsRNA was unable to induce sterility whether administered to the adults by injection or to larvae by feeding. The results are shown in Figures 11-13, where there was no apparent reduction in the offspring.

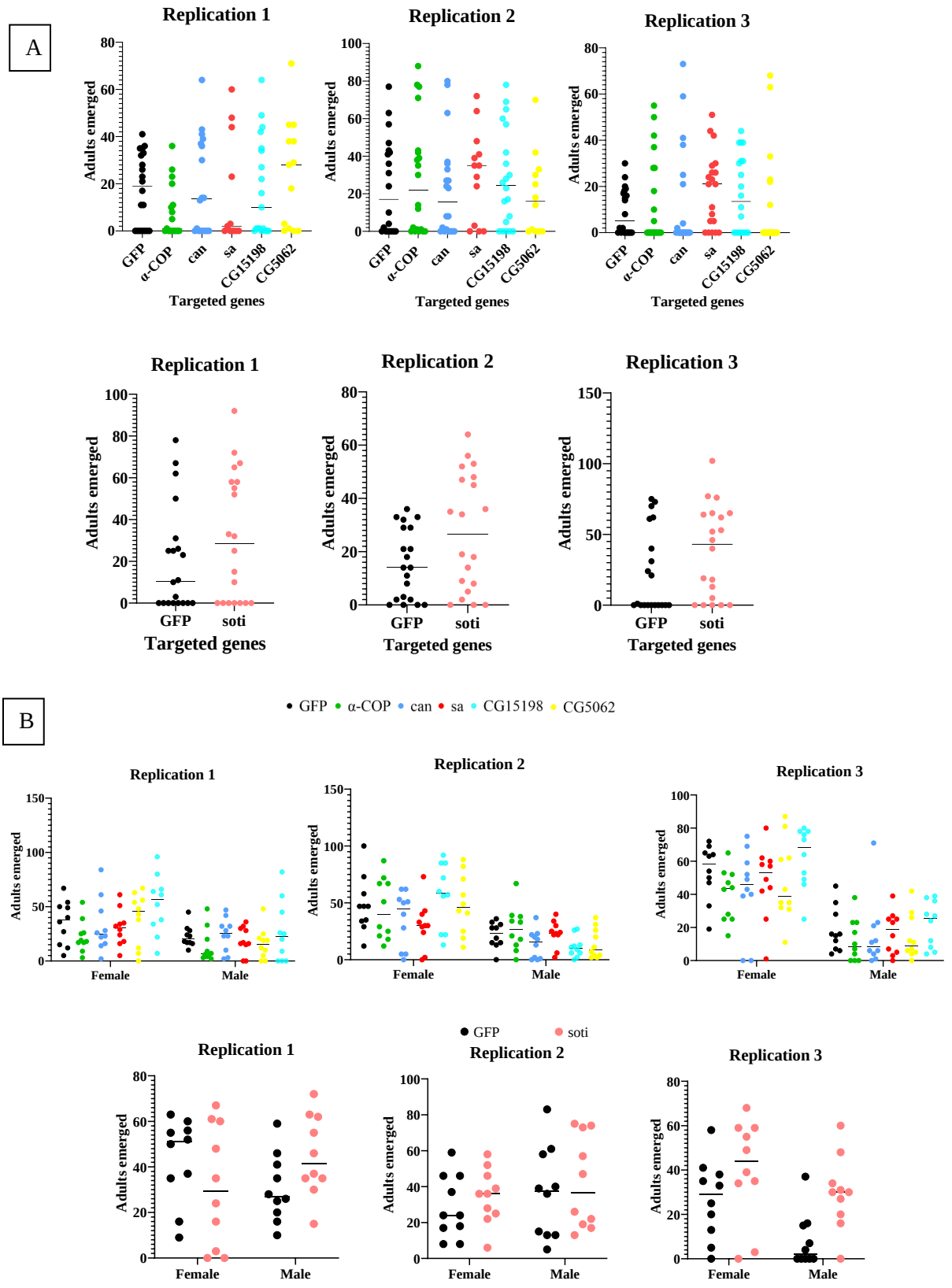


Figure 11. (A) Results of adult injection with dsRNA in an attempt to sterilize the males. Post-injection, males exhibited no sterility. GFP was used as a negative control. *soti* is presented in a separate graph as it was conducted at a later date. (B) The figure also illustrates the results of the single pairing of ten females and ten males from the

offspring of the initial pairing to assess potential sterility in the progeny. No sterility was observed. The dots in the figures represent the number of emerged adults, the line represents the median of the emerged adults.

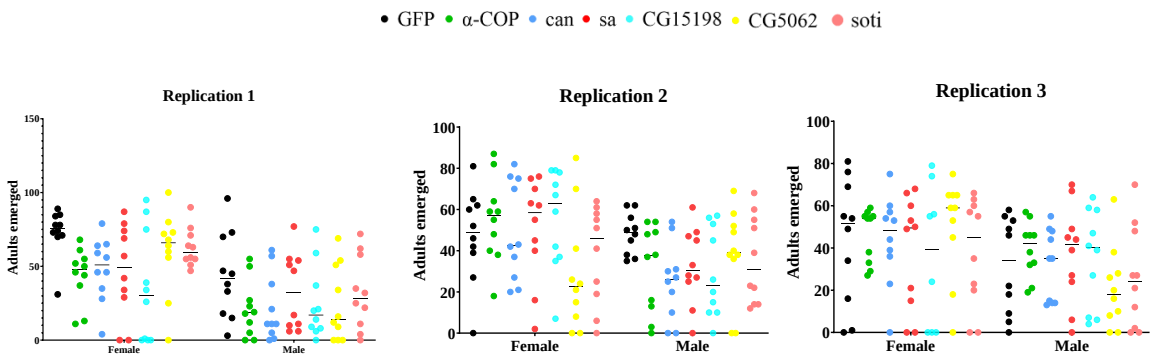


Figure 12. Results of feeding larvae with dsRNA in Lipofectamine solution. After development to adulthood, 10 solution-fed virgin females were crossed with 10 WT males, and 10 solution-fed males were crossed with 10 WT virgin females. No sterility was observed. Each dot represents the number of emerged adults in a replicate, and the horizontal line indicates the median number of emerged adults**.**

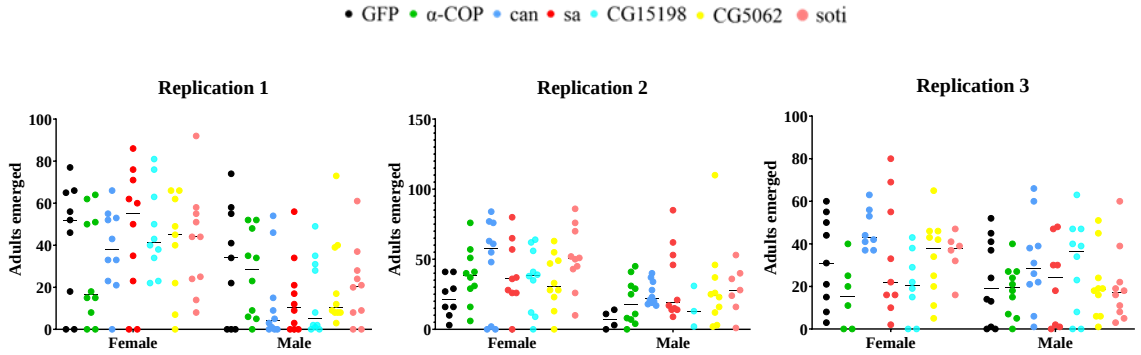


Figure 13. Results of feeding larvae with dsRNA in DMSO solution. After development to adulthood, 10 solution-fed virgin females were crossed with 10 WT males, and 10 solution-fed males were crossed with 10 WT virgin females. No sterility was observed. Each dot represents the number of emerged adults in a replicate, and the horizontal line indicates the median number of emerged adults.

Silencing the selected genes does not result in lethality

Several genes were selected as candidates for potential use as a biopesticide, all genes selected were responsible for essential functions in the flies (see Table 2). However, silencing these genes did not result in lethality. Figure 14 demonstrates the lifespan of the injected flies, where an LT50 was not achieved and the experiment was stopped at day 30.

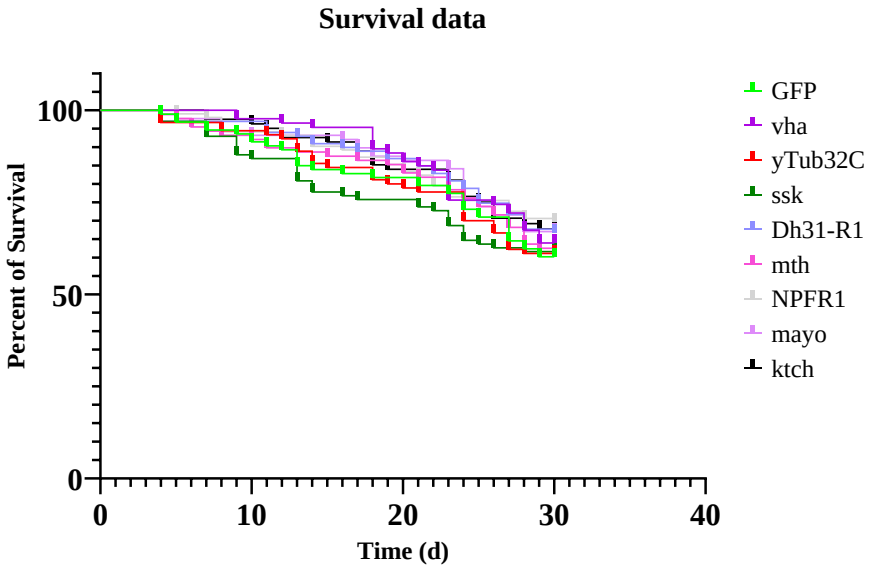


Figure 14. The survival test confirmed after injecting female adult flies with dsRNA. Results do not show a drop below 50% after 30 days, which was the point the test was stopped.

III Discussion

This dissertation investigates three distinct aspects of the biological and biotechnological control of *D. suzukii*, with the aim to enhance both understanding and practical application. Although each aspect yielded different conclusions, all were equally insightful. The first control method examined was SIT, a potentially powerful and effective strategy when applicable. SIT relies on three key parameters: mass-rearing, sterilization, and sorting. This dissertation focused on sterilization and sorting, as mass-rearing techniques are already well-established.

3.1.1 The sterile insect technique is a fitting method of control against *Drosophila suzukii*

Experimentation commenced with the evaluation of two sterilization approaches: X-ray and temperature-induced sterilization. Ionizing radiation, such as X-rays, can effectively sterilize insects, but minimizing its impact on insect fitness is crucial (Lux et al., 2003). Determining the optimal radiation dose is therefore essential, as lower doses tend to preserve fitness (Peccerillo et al., 2023). The required dose can vary based on factors such as insect species (Krüger et al., 2018), sex (Dyck & Hendrichs, 2005), life stage (Zhao et al., 2022), and radiation source (Kaboré et al., 2023; Yamada et al., 2023). For *D. suzukii*, estimates vary: gamma radiation between 50–120 Gy has been shown to be sufficient (Lanouette et al., 2017), while X-ray sterilization often requires around 150 Gy (Kim et al., 2016). However, from the literature reviewed it seems that 200 Gy is generally effective in inducing near-complete sterility without a major fitness trade-off. Additionally, performing irradiation in low-oxygen environments has been found to mitigate negative side effects (Sassù et al., 2019), potentially allowing for even higher doses.

For a more sustainable approach, a lower dosage of X-ray sterilization (40–60 Gy) was evaluated to determine whether factors such as power output influence sterility. It was found that applying the same dosage yielded similar levels of sterility regardless of power output. However, the lowest power setting unexpectedly produced the highest sterilization efficiency, despite requiring the least irradiation time, it also resulted in the shortest insect lifespan. This could potentially be explained by the insect's closer proximity to the radiation source, a factor that might significantly impact sterilization outcomes. Given the small size of *D. suzukii*, which may necessitate higher radiation levels (Krüger et al., 2018), further investigation into such mitigating factors is both relevant and promising. The data obtained suggest that sterilization

can be achieved with lower dosages, and additional factors may contribute to sterilization beyond the overall radiation dose.

3.1.2 Heat sterilization is viable, but needs further work for better mitigation

Following the X-ray sterilization experiments, an investigation was conducted to determine whether heat exposure could serve as a viable sterilization method for use in the SIT. This idea was inspired by literature indicating that the fertility of *D. suzukii* is adversely affected at temperatures exceeding 30 °C (Calabria et al., 2012). Subsequent studies supported this notion, demonstrating that both short- and long-term exposure to elevated temperatures significantly reduce development rates and lifespan (Evans et al., 2018; Green et al., 2019). Initial experiments involved rearing flies at a constant temperature of 30 °C. While pupae successfully developed under these conditions, no offspring were produced. To address this, a temperature cycle was implemented: 18 hours at 30 °C followed by 6 hours at 28 °C. This approach resulted in a high level of sterility; however, it also caused a substantial reduction in lifespan, approximately 65%. To mitigate this loss in fitness, it was tested whether reducing the duration of exposure to 30 °C would preserve longevity while maintaining sterility. The results indicated that decreased exposure to high temperatures significantly improved the flies' lifespan, but also led to partial recovery of fertility. While these findings are consistent with previous research on the effects of heat stress in *D. suzukii* (Evans et al., 2018; Green et al., 2019), they differ in the implementation of a cyclical temperature regime rather than a fixed short-term heat exposure. These results suggest that heat-induced sterilization may be a viable supplementary method in SIT and could potentially be combined with irradiation to reduce the required radiation dose. However, further investigation is needed to refine this technique and assess its practical applicability.

3.1.3 Sexual dimorphism in the pupal stage can potentially be exploited for early sorting

An investigation was also conducted to determine whether pupal weight could serve as a predictor of adult sex. The results demonstrated that female pupae are significantly heavier than their male counterparts. This finding suggests that pupal weight could be used as a non-invasive method for sex separation, potentially allowing for the reduction of females in sterile release batches. However, further research is needed to evaluate the fitness and mating performance of lighter males, to ensure their effectiveness in sterile insect release programs.

3.2.1 La Jolla Virus is a suitable candidate for *Drosophila suzukii* control

The second part of this dissertation aimed to further characterize the LJV. While previous research has shed light on many aspects of LJV (Carru et al., 2018; Carru et al., 2021; Linscheid et al., 2022), its transmission routes remain poorly understood. Entomopathogenic viruses can serve as powerful tools in IPM, but it is crucial to fully characterize them before implementation. Doing so enhances their efficiency and helps prevent unforeseen consequences. LJV belongs to the family Iflaviridae, which includes several well-known arthropod viruses (Valles et al., 2017). Therefore, understanding its transmission routes is important, particularly to assess the risk of off-target infections. Additionally, knowing how the virus is transmitted enables more effective application strategies by informing when, where, and what to target (Belevitch et al., 2024). An investigation was conducted to examine the impact of LJV on the feeding behavior of infected flies. Understanding these behavioral changes may contribute to more refined and effective virus deployment strategies.

3.2.2 It is easily transmitted in a population

The experiments commenced by investigating four potential transmission routes of LJV: airborne, fecal, oral, and venereal. The results indicate that the fecal-oral route is likely the primary mode of infection, as consistent infection was observed in these experiments. Notably, the fecal transmission experiments yielded more conclusive results, largely due to the ability to better isolate and control exposure to fecal matter. In the oral transmission experiments, although the environment was sterilized to the greatest extent possible, contamination with fecal matter could not be fully excluded. This supports the likelihood of a fecal-oral transmission route. No infection was observed in the airborne transmission experiments, and while infection occurred in the venereal group, similar levels of infection were also observed in control groups. This made it difficult to distinguish true venereal transmission from environmental (i.e., fecal-oral) exposure. This also included same-sex control groups, which showed similar infection levels, suggesting that environmental contamination, rather than mating, was the likely source of infection. Therefore, venereal transmission could not be confirmed, and environmental uptake remains the most plausible explanation.

These findings are consistent with previous observations of transmission dynamics in other insect viruses (Cory et al., 2015). Insect viruses are generally not transmitted through the air and are more commonly spread via fecal-oral routes when vertical transmission does not

occur. This pattern is well-documented among members of the Iflaviridae family (Ottati et al., 2020; Habayeb et al., 2009; de Miranda & Genersch, 2010). In the case of *D. suzukii*, this mode of transmission is further supported by the species' biology, as larvae are known to acquire part of their microbiota from the fecal matter in their environment (Blum et al., 2013).

3.2.3 It leads to population decline

This dissertation also builds on the previous work of colleagues by further characterizing how LJV can potentially alter host population dynamics. Prior studies have shown that the virus impairs the completion of the *D. suzukii* life cycle (Linscheid et al., 2022). The results corroborate these findings and further demonstrate the extent to which an infected population can be affected. Specifically, while egg hatching does not appear to be impacted by viral infection, all subsequent life stages show a significant reduction in comparison to non-infected controls (Figure 15).

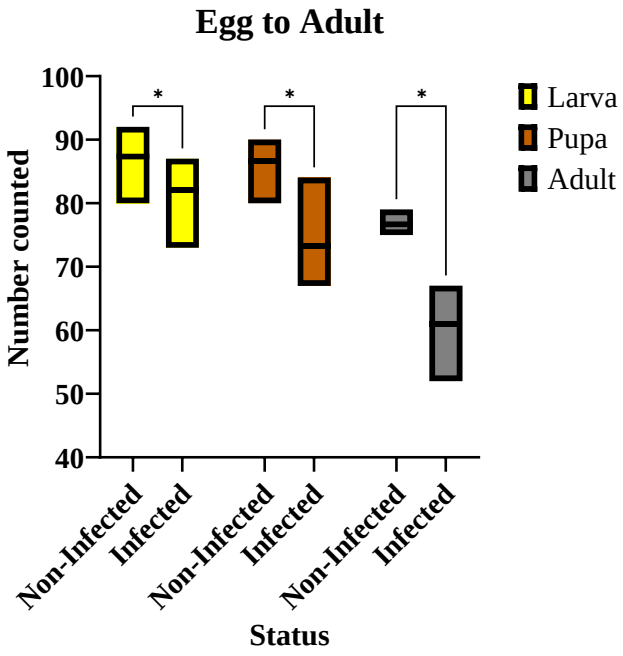


Figure 15. The difference between the non-infected and infected flies, where the infected ones show reduced numbers in all life-stages after all eggs successfully hatched

3.2.4 It alters the feeding behavior of the flies

Finally, the impact of LJV infection on the feeding behavior of *D. suzukii* was investigated. The results indicate that the virus alters female behavior, while no significant effect was observed in males. Infected female flies showed reduced motivation to forage for food compared to their

non-infected counterparts. However, once they located a food source, the time spent interacting with it did not differ significantly between the two groups. This increased engagement upon food contact may partially compensate for reduced foraging activity. Nevertheless, the broader ecological implications of this behavioral shift remain unclear and warrant further investigation. In natural environments where food sources are far less abundant, it is assumed such alterations in behavior could have important consequences for survival and population dynamics.

A comprehensive understanding of the various aspects of LJV biology enhances the potential for its application as a biocontrol agent. The virus demonstrates a relatively rapid mode of action (Linscheid et al., 2022), and has shown the ability to persist within host populations over time (Abdelhafiz et al., 2025). These characteristics are crucial for an effective and environmentally sustainable biopesticide (Ayilara et al., 2023).

Formulating LJV as a biopesticide could significantly improve its stability under natural conditions, particularly by enhancing its resistance to environmental stressors such as ultraviolet (UV) radiation, which is known to degrade viral particles (Wilson et al., 2020). By increasing the virus's environmental resilience, formulation strategies could help maintain infectivity and efficacy following field application, thereby improving the reliability and practicality of LJV as a tool in IPM programs (Šunjka et al., 2022).

3.3.1 RNAi can potentially be a powerful tool for SIT

The use of RNAi could have significantly increased the efficiency of SIT by enabling the production of non-transgenic populations. Targeting genes involved in reproduction and sexual differentiation would offer a straightforward method for early-stage sex sorting and sterilization (Darrington et al., 2017). Oral induction during the larval stage, in particular, would have been advantageous, as it allows for dsRNA delivery through food or genetically modified yeast expressing the target sequences (Murphy et al., 2016; Abrieux & Chiu, 2016). Previous studies support this approach. For example, oral RNAi targeting the female-specific *doublesex* (*dsx*) gene in mosquitoes led to a 20% decrease in female emergence compared to controls (Taracena et al., 2019; Kojin et al., 2022). Injections of dsRNA targeting *tra* in *Drosophila melanogaster* and *Ceratitis capitata* resulted in the development of intersex males (Dietzl et al., 2007; Pane et al., 2002), while *dsx* knockdown in *Tribolium castaneum* reduced female traits and reproductive capacity (Shukla & Palli, 2012). Notably, RNAi has also been used to produce sterile males in *Bactrocera dorsalis* and *C. capitata* (Dong et al., 2016; Gabrieli et al., 2016). In the conducted experiments, dsRNA targeting regulatory genes for sex determination was

delivered through adult injection and larval feeding. The presence or absence of effects was then assessed in the emerged F1 and F2 of the injected adults, as well as in the emerged adults and F1 of the fed larvae. The offspring were investigated because, in some insects, RNAi effects can be heritable or skip a generation (Horn & Panfilio, 2022).

Unfortunately, all results were negative (Figures 7-10), with no apparent effect in the adults or their offspring. In the sex modification experiments, no skewing towards one sex was observed. The sex determination genes *sxl*, *tra*, and *msl-2* were targeted in our approach. The *sxl* gene acts as the master regulatory gene in the somatic sex determination pathway of *Drosophila*. Female development is initiated when *sxl* is activated; when *sxl* remains inactive, individuals develop to male by default process (Meise et al., 1998). *tra* plays a crucial role in somatic sexual development and is functional only when activated by *sxl*. Without functional *tra*, downstream genes such as *dsx* are spliced in the male-specific mode, even in genetic females, resulting in male-like development (Baker & Wolfner, 1988).

In *Drosophila melanogaster*, *tra-2* knockdown using RNAi was shown to shift the sex ratio towards males (Dietzl et al., 2007). Similarly, in *D. suzukii*, a temperature-sensitive mutation introduced via CRISPR led to a bias toward male development by functionally disrupting *tra-2* at elevated temperatures (Li & Handler, 2017). Thus, simultaneous disruption of *sxl* and *tra* was expected to result in a male-biased population. However, no observable effect on sex ratio was detected. *msl-2* is essential for dosage compensation in males by promoting hyper-transcription of the single X chromosome. In theory, knocking down msl-2 should selectively affect males and skew the sex ratio toward females (Kelley et al., 1995). Its use in this experiment was intended to serve as a positive control for RNAi functionality. Despite targeting all three genes, none showed evidence of successful gene silencing, indicating either insufficient knockdown efficiency or potential species-specific resistance to RNAi. Future work should validate silencing at the transcript level using real-time PCR to quantify gene expression following dsRNA treatment.

The genes *soti*, *can*, *sa*, *CG15189*, and *CG5062* were selected for RNAi targeting due to their known or predicted roles in testis development and spermatogenesis. These genes have been previously associated with male fertility in *Drosophila*, and were therefore strong candidates for inducing sterility through gene silencing. It was hypothesized that disruption of these genes would impair spermatogenesis and lead to male sterility (Barreau et al., 2008; White-Cooper, 2010). However, no observable effects were detected in any of the experimental groups (Figure 11-13). The treated males exhibited normal reproductive behavior and produced

viable offspring at rates comparable to controls. This outcome could be due to several reasons, such as the experiments conducted in the later life stages, which makes inducing RNAi more challenging (Taning et al., 2016). Another reason can be attributed to the fact that RNAi in *Drosophila* is not systemic (Roignant et al., 2003). This is why transgenic driver lines are typically designed for RNAi studies in *Drosophila* (Roignant et al., 2003; Tomoyasu et al., 2008). Another factor that could have hindered the RNAi from functioning was oral administration, making the dsRNA vulnerable to degradation by RNases. Finally, the failure could be explained by poor dsRNA design, as the target genes may have been either too general or too specific to elicit an effect. This could have been confirmed by verifying whether the genes were silenced or not via real-time PCR, but the experimentation was stopped after repeated failures.

3.3.2 Further work needed to establish RNAi as a viable control method against *Drosophila suzukii*

This same reasoning could apply to the second portion of the experiments, where there was no significant reduction in the lifespan of the flies (Figure 14). GFP was used as a negative control, and two genes were used as a positive control. The first gene, *vha26*, is essential for the assembly and function of the vacuolar-type H+-ATPase (V-ATPase) complex, which plays a critical role in numerous cellular processes, including pH regulation, protein trafficking, and organelle function (Baum et al., 2007). The second target, *yTub23C*, encodes a gamma-tubulin protein that is vital for embryonic development, cell division, neuroblast spindle orientation, and both male and female germline development (Whyard et al., 2009). Previous research by Murphy et al. (2016) demonstrated that dsRNA targeting these genes in *D. suzukii* had detrimental effects, including increased mortality in larvae and adults, as well as a reduction in adult locomotor ability. However, the dsRNA was delivered via genetically modified yeast expressing the genetic material. It is also important to note that the difference in survivorship reported in the paper is a 20-30% difference at best (Murphy et al., 2016). Another study employed liposome formulations to administer dsRNA to *D. suzukii* adults and larvae, resulting in high larval mortality and efficient silencing of target genes (Taning et al., 2016). However, adult mortality ranged from 10-23%, indicating the necessity for further optimization and identification of better target genes (Taning et al., 2016). Ahn et al. also investigated an alternative gene for RNAi control of *D. suzukii*, but found that dsRNA injection did not result in significant mortality (Ahn et al., 2019).

Overall, while it is established that RNAi and its associated mechanisms are functional in *D. suzukii* (Taning et al., 2016), current research suggests that RNAi-based control of *D. suzukii* currently shows limited feasibility, primarily due to its limited efficacy in adults (Taning et al., 2016; Murphy et al., 2016), which are the primary targets for RNAi-based pesticides. Although larvae could potentially be targeted by GMO plants expressing the dsRNA (Murphy et al., 2016), widespread acceptance of GMO plants is distant.

3.4 Conclusion and future outlook

The invasion of *D. suzukii* has resulted in substantial economic losses for the billion-dollar fruit industry, through both crop damage and the costs associated with controlling its spread. Furthermore, many control methods have environmental consequences, exacerbating the impact of this invasive species. In light of the detrimental effects of chemical insecticides, alternative solutions have been sought. IPM is advantageous as it does not entirely eliminate chemical control, but employs it in a sustainable and responsible way. Effective biological control, a key component of IPM, offers environmental safety and encompasses a broad range of solutions for the same problem.

Currently, D. *suzukii* management predominantly relies on cultural control practices, supplemented by chemical control (Schöneberg et al., 2021). Biological control, particularly through the use of parasitoid wasps, is under active investigation and has seen limited but increasing implementation (Fellin et al., 2023; Firko, 2010; CABI.org). Continued research is essential to enhance and integrate these approaches effectively, as current results demonstrate variable success. This dissertation investigated three biological control strategies aimed at improving sustainable management of *D. suzukii*. First, it examined the SIT as a control tool, contributing new optimization parameters, proposing a potential sexing system, and introducing a novel sterilization approach. The results were very positive, where all parameters achieved similar sterility in the flies, and found that high sterility can be achieved in a short time with a low energy requirement. This potentially can be attributed to the close proximity to the radiation source. It also introduced a novel sterilization method, and a sexing method. Future research should focus on determining the extent to which distance from the radiation source influences outcomes and whether this can be utilized. Additionally, the combined effects of X-ray and heat-induced sterility should be explored to achieve high efficiency, and to assess how this combination potentially mitigates the adverse effects on fitness. It also evaluated the use of RNAi as an alternative tool for sexing and sterilization by targeting genes responsible for sex

differentiation, and male fertility. Although the results are negative, the findings highlight areas for further development, assert that RNAi remains a promising complementary strategy to SIT. Future experiments should focus on earlier life stages, and trying to confirm whether knockdown happened or not.

Additionally, further characterization of LJV was conducted, providing new insights into its population dynamics and the potential influence of persistent infections on host populations. The results demonstrate and reiterate LJV's promise as a biopesticide, for it can persist and easily be transmitted in a population. Moreover, its persistence and mode of infection could be combined with SIT. The proposed strategy of releasing sterilized, virus-infected male flies may offer an additional and synergistic approach to population suppression. Finally, the potential use of RNAi as a standalone biopesticide was evaluated, which also showed no practical effect under current conditions. These findings emphasize the need for further refinement before RNAi can be reliably implemented in *D. suzukii* control programs. This refinement can be achieved by selecting better target genes and better formulations.

Together, these findings present several novel directions for the integrated and sustainable control of *D. suzukii*, thereby contributing to the expanding body of research aimed at identifying effective alternatives to chemical control.

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V Publications

RESEARCH ARTICLE

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Radioactivity and GMO-Free Sterile Insect Technology for the Sustainable Control of the Invasive Pest *Drosophila suzukii*

*Ibrahim Abdelhafiz, Stefan Gerth, Joelle Claussen, Mareike Weule, Eva Hufnagel, Andreas Vilcinskas, and Kwang-Zin Lee**

***Drosophila suzukii* (*D. suzukii*)**, **commonly known as the spotted wing drosophila, is a highly invasive crop pest that is difficult to control using chemical insecticides. To address the urgent need for alternative and more sustainable control strategies, the sterile insect technique (SIT) is improved, which involves the release of sterilized male insects to mate with fertile conspecifics, thereby reducing the size of the pest population in the subsequent generation. The three critical aspects that influence the success of SIT programs in *D. suzukii* are addressed. First, an accurate and nondestructive method is established to determine the sex of individual insects based on the differential weight of male and female pupae. Second, conditions for X-ray sterilization are systematically tested and an optimal dose (90 kV/40 Gy) is identified that ensures the efficient production of sterile *D. suzukii* for release. Finally, the inherent thermosensitivity of *D. suzukii* males is exploited to develop a temperature-based sterilization technique, offering an alternative or additional SIT method for this pest. These advances will contribute to the development of a comprehensive and effective strategy for the management of *D. suzukii* populations, reducing their impact on agriculture and helping to safeguard crop yields.**

1. Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), also known as the spotted wing drosophila, is an invasive pest that causes extensive damage to soft-skinned fruits, resulting in yield losses and spoilage costing millions of dollars every year.[1,2] The species has proliferated globally[3,4] due to its short generation time, extensive dispersal, high fecundity, and ability to thrive across diverse environmental conditions.[5,6] Accordingly, it is now found in Asia, Europe, the Americas, Africa and Oceania.[7] Unlike other drosophilids, which lay eggs in decaying fruit, *D. suzukii* selectively reproduces in the fresh and ripening soft-skinned fruit of more than 70 plant species.[8,9] The females use their serrated ovipositor to lay eggs inside the fruit, wherein the larvae cause extensive direct damage by feeding. This also promotes secondary infections when bacteria, yeast, and other fungi gain access via surface lesions, rendering the infested fruit unpalatable and unsuitable for the market.[10]

Several methods have been evaluated to control *D. suzukii*, including cultural,[11] biological,[12] and behavioral strategies,[13] but most growers still rely on broad-spectrum chemical pesticides.[14] However, such chemicals have a detrimental impact on non-target and beneficial organisms, including pollinators.[15,16] Furthermore, *D. suzukii* has rapidly evolved resistance to available pesticides, and the laying of eggs inside fruit protects the larvae from topical formulations.[17] The reliance on chemical control methods is therefore unsustainable, creating a demand for alternative approaches.[18,19]

The sterile insect technique (SIT) is a promising, environmentally friendly method for pest control involving the mass production of male pest insects that are sterilized either by irradiation or genetic modification, and subsequently released into the environment to mate with wild females.[20] This strategy leads to population suppression or, in the best-case scenario, eradication of the pest in the treated area.[21] SIT has been deployed successfully against mosquitoes, moths, and fruit flies.[22] Single-sex releases (predominantly males) are preferred[23] but dual-sex releases are

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possible, albeit less efficient due to the sterilized insects mating with each other. Successful SIT must include three key components: a mass-rearing system, an efficient sexing mechanism, and an optimized sterilization method.[24] Several mass-rearing systems for *D. suzukii* have already been described[25,26] and various sterilization methods have been proposed and tested[27,28]) and are currently part of FAO/IAEA guidelines for *D. suzukii* mass rearing and SIT. Additionally, genetically modified flies have been assessed for both sterilization and sexing,[29–31] but EU regulations do not allow the mass release of genetically modified organisms (GMOs) into the environment. Ionizing radiation can also be used for sterilization[32] but it generates radioactive waste and is strictly regulated by the International Atomic Energy Agency (IAEA). Therefore, we investigated novel methods to sterilize male insects using X-ray irradiation which is less harmful to the environment, does not produce radioactive waste, and allows the global shipping of treated insects.

X-ray sterilization as a SIT method has been tested in *D. suzukii*.[28,33–35] SIT can also be combined with the incompatible insect technique (IIT), such as the use of endosymbiotic *Wolbachia* to induce feminization, male killing, and cytoplasmic incompatibility ([36,37]; reviewed by[38]). A recent field trial involving the release of sterile males within open polytunnels in strawberry orchards in the UK triggered a significant decline in the target population demonstrating proof of concept in the field.[34] However, the results have proven difficult to replicate in a semi-field investigation.[35]

Here we determined the optimal X-ray conditions and energy parameters for sterilization to develop a more sustainable mode of sterilization. We also tested heat sterilization, given that raising *D. suzukii* at 30 °C has been shown to influence fertility, adult lifespan, egg-laying rates, and egg-to-adult emergence.[39–41] We also developed an easy and scalable method to determine the sex of *D. suzukii* at the pupal stage. Our results provide valuable insights into the efficiency and sustainability of SIT as a means to control *D. suzukii* populations and will help to reduce the impact of this pest on the agricultural industry.

2. Experimental Section

2.1. Maintenance of Insects

The *D. suzukii* (Ontario, Canada) strain used in this study was derived from a laboratory colony established in Ontario, Canada, during the summer of 2012.[42] The flies were maintained in a climate chamber (65% humidity, 26 °C, 12-h photoperiod) on a diet of soybean and cornmeal (60 g L^{-1} cornmeal, 8 g L^{-1} soybean flour, 18 g L^{-1} baker's yeast, 60 g L^{-1} malt, 22 g/L molasses, 6.25 mL L^{-1} propionic acid, and 2 × 0.8 g nipagin) in 50-mL Drosophila vials. Experiments were carried out using flies 3–7 days post-eclosure.

2.2. Sexing of Insects

The mass of 200 individual pupae in three biological replicates was determined (600 pupae in total) using an XA105DU precision balance (Mettler Toledo) to determine if there was any correlation between weight and sex, before transfer to 96-well plates containing ≈500 µL per well of the soybean/cornmeal medium (see above). The openings of the well plate were closed with cotton wool to prevent escape after hatching. When the adult flies emerged, they were sexed by phenotypic analysis to determine whether the pupal weight correlated with sex. If flies did not emerge (25–40%), the closed pupae were dissected and the occupant was sexed as above.

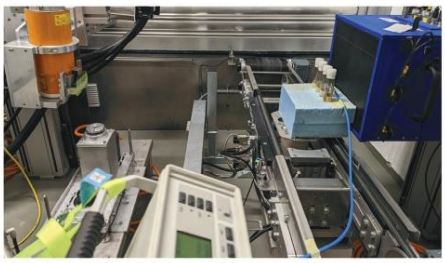

Figure 1. The PhenoCT X-ray machine (Fraunhofer IIS, Fuerth, Germany) used for *D. suzukii* X-ray sterilization and the dosimeter used to measure the applied radiation dose.

2.3. X-Ray Sterilization

Adult flies were sterilized using the PhenoCT X-ray machine (Fraunhofer IIS, Fuerth, Germany) with different energy output levels achieved by restricting the maximum acceleration voltage (**Figure 1**). The flies were sterilized in batches of four Drosophila vials. The energy levels and X-ray doses are summarized in **Table 1**. A GE 225HP/11 X-ray source was used for all energy settings except the 90 kV setting and was positioned ≈800 mm away from the flies. For the 90 kV setting, the flies were placed 250 mm away from the radiation source, and thermo Fisher Scientific PXS5-928 microfocus X-ray source was used. The flies were positioned within the cone of the primary beam.

2.4. Heat Sterilization

Heat-induced sterilization was achieved by exposing the flies to a dual temperature cycle, alternating between 30 and 28 °C, and then completing their life cycle. The effect of maintaining the temperature at 30 °C for different durations (6, 10, and 14 h)

Table 1. Summary of energy levels and X-ray dosages.

Energy	Dose	Distance from radiation source	Average time to dosage
225 kV, 8 mA	40 Gy	800 mm	45 min
	60 Gy		68 min
160 kV, 11.3 mA	40 Gy	800 mm	48 min
	60 Gy		62 min
130 kV, 13.8 mA	40 Gy	800 mm	31 min
90 kV, 9 mA	40 Gy	250 mm	18 min

was explored. Fertility was assessed by counting the offspring in small-scale pairing experiments comprising five biological replicates of 20 virgin untreated females paired with 20 treated males subjected to different heat incubation regimes.

2.5. Fitness Experiments

To assess fitness, the life span of 20 male flies was monitored in each treatment group for both sterilization experiments. After treatment (X-ray, heat, or control), the flies were collected, placed in fresh food vials, and observed daily until all succumbed. The flies were transferred to new vials twice per week to provide fresh food media.

2.6. Single Pairing for Sterilization Assessment

All treated males and untreated control males for both sterilization experiments were individually paired with virgin females in Drosophila vials to assess the efficiency of sterilization. Each pairing lasted for 18 days, with a vial change every 6 days. The flies were then removed and the offspring were counted after emergence.

2.7. Statistical Analysis

Sterilization and survival data were analyzed using Prism v9.2.0 (GraphPad Software). The statistical significance of differences between sterilization and control groups ($\alpha = 0.05$) was calculated by one-way analysis of variance (ANOVA). Kaplan-Meier survival analysis ($\alpha = 0.05$) was used to plot survival curves. For pupal weights, R studio v1.2.1335 was used to measure differences between the sexes and to present the data as boxplots. Homogeneity was calculated using Bartlett's test with significance determined by multiple pairwise comparison ANOVA ($\alpha = 0.05$).

3. Results and Discussion

3.1. The Sex of *D. suzukii* Is Correlated with Its Pupal Weight

We observed a statistically significant difference ($p < 0.001$) in the mean weight of individual pupae (**Figure 2**) with females (1.057 mg, SE 0.0123) being more massive than males (0.873 mg, SE 0.0122). This is concordant with the well-established phenomenon of sexual dimorphism in drosophilids and other insects, where females typically have larger bodies than males.[43] Indeed, the size of pupae has already been used to sex various mosquito species.[44] We have now confirmed that *D. suzukii* females are significantly larger than males from the pupal stage onward, although sex distinction becomes more challenging when examining weights that fall within the overlapping region of the bimodal weight distribution. Nevertheless, we were consistently able to predict the sex of the heaviest and lightest pupae, which fell into the regions of the distribution that were exclusively male or female. Selection of the lightest pupae therefore skews the male-to-female ratio in favor of males, which is ideal for SIT applications. Although selecting the lightest pupae theoretically guarantees an all-male population, the fitness and competitive capabilities of these males warrant further investigation because smaller and lighter males are less competitive than larger and heavier conspecifics. The observed weight disparity presents an intriguing opportunity for the development of an automated sorting process based on sieving, buoyancy, or density gradient techniques. Further studies are needed to investigate the potential loss of males, the percentage of females introduced into the sterilization process, and the irradiation effects on females using the optimal parameters for male sterilization. In this case, the small percentage of female escapees would probably also be sterile and their impact on the efficiency of the SIT strategy would be negligible.

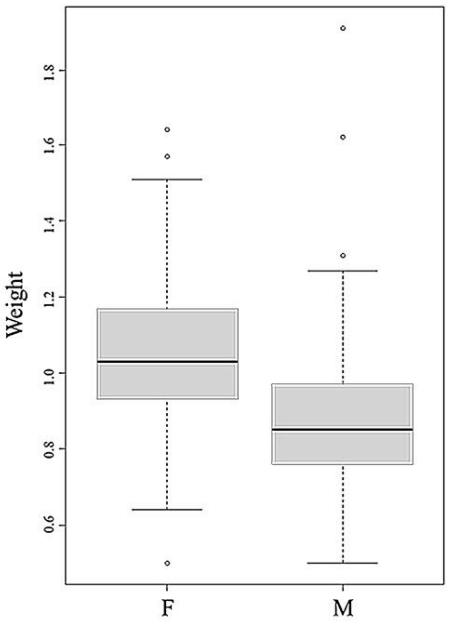

Figure 2. Boxplot showing pupal weights (mean of three replicates) in relation to the observed sex. Statistical significance was calculated by multiple pairwise comparison ($p < 0.001$). Each replicate represents ≈600 sexed pupae. The female (F) mean = 1.057 mg (SE 0.0123) and the male (M) mean = 0.873 mg (SE 0.0122). The results show a significant difference between the weight of the sexes in pupae ($\alpha = 0.05$).

3.2. X-Ray Sterilization Is Efficient and the Fitness Cost Is Minimal

To identify the optimal X-ray sterilization conditions, we tested four energy output settings, each associated with varying radiation dosages (Table 1). We found that the lowest energy setting (90 kV, 9 mA) achieved the most efficient sterilization (**Figure 3**).

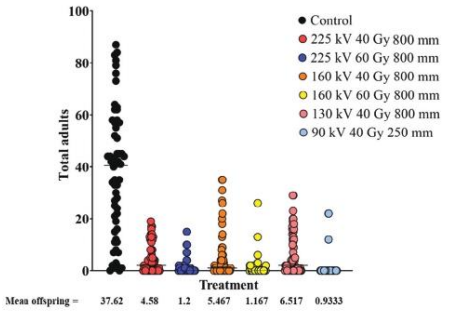

Figure 3. The efficiency of X-ray sterilization at different energy outputs. The experiments involved three biological replicates, each based on 20 single crossings between a virgin female and a treated male. All treatment conditions were found to differ significantly from the control ($p < 0.001$, $\alpha = 0.05$). Each dot represents the total number of offspring of a single pair crossing. The horizontal line represents the median number of offspring from all single pair crossings.

Notably, our study extended beyond the exploration of radiation dosage as the sole determinant of sterilization efficiency due to the nuanced effects reported in earlier investigations. For example, a dose of 80 Gy was sufficient to completely abolish F1 emergence in one study[45] whereas a dosage range of 50–300 Gy in another study still resulted in an F1 hatch rate of 25.8%.[28] These studies also revealed a positive correlation between insect age and resistance to radiation-induced side effects. Our findings reaffirm the principle that the efficiency of sterilization correlates positively with increasing radiation dosage. We found that a dose of 60–80 Gy was sufficient for complete sterilization. However, even our lowest tested dose of 40 Gy achieved ≈97% sterility.

The efficiency of the lowest energy setting not only significantly reduces energy consumption but also shortens the time required to attain the requisite 40 Gy dosage by 10–40 min compared to alternative sterilization methods. We attribute this robust sterilization effect to the closer proximity of the flies to the radiation source. Unlike other conditions, where the distance between source and target was 800 mm, the distance at the 90 kV setting was only 250 mm, thus enhancing the sterilization efficiency.

The success of SIT programs hinges on the ability of mass-released males to compete effectively with their wild conspecifics, so a sterilization method that reduces the lifespan of treated insects will by definition reduce their competitiveness. We assessed the effects of our X-ray sterilization method and found the impact on longevity was minimal (**Figure 4**). Even so, we observed a correlation between the energy output settings and the lifespan of adult flies, with the most efficient sterilization setting (90 kV) reducing the lifespan by ≈3 days compared to untreated controls (**Table 2**). This may reflect the close proximity of the flies to the radiation source. We propose that fine-tuning the sterilization process either by increasing the distance from the radiation source or further lowering the energy output would mitigate the adverse effects on lifespan. This optimization would not only enhance the competitiveness of the sterilized insects but also contribute to the overall success of SIT programs. An alternative to gamma radiation for SIT in the codling moth *Cydia pomonella* using a newly designed X-ray irradiator was recently shown to be applicable in a range of insect pests.[46] Notably, the effective dose was significantly higher than those used in the current study, and age was an important factor influencing the effects of radiation on the fitness of the moths.

3.3. Development at 30 °C Induces Sterility in *D. suzukii* with Variable Effects on Fitness

Next, we explored a novel sterilization method involving heat treatment. Rearing *D. suzukii* at a constant temperature of 30 °C caused complete sterility (data not shown). However, a previous study reported that eclosion is also compromised at this temperature.[40] To address this challenge, we applied alternating temperature cycles of 28 and 30 °C. This achieved almost complete sterility but still triggered a substantial decline in overall fitness, including a remarkable 65% reduction in the lifespan of treated flies compared to the untreated control group (**Figure 5I**).

When we reduced the duration of the exposure period to 30 °C during the coordinated temperature shifts (**Table 3**), the lifespan of the flies increased while the fertility remained low, albeit not completely abolished (Figure 5II). The increase in fertility may be slightly skewed due to the difference in experimental setup (single vs group pairing), but this method was selected to allow the rapid confirmation of effects on fertility at different temperatures. This approach also provides insights into potential field performance, particularly how the treated flies could overwhelm the wild population and reduce the number of offspring. Among the time intervals we tested, the lifespan of flies exposed to 30 °C for 14 h was the shortest (LT_{50} = 7.5 days), confirming the rapid deterioration of fitness, whereas reducing the duration of exposure to 6 h extended the lifespan significantly (LT_{50} = 14 days). This provides a clear opportunity for further optimization. By refining the temperature cycle and exploring lower temperature thresholds, we may uncover more favorable conditions that extend the lifespan of adult flies while ensuring near-complete sterility.

The lifespan of sterilized insects not only influences the effectiveness of competition following mass release but also determines the frequency of repeated releases necessary for effective control, thus influencing costs and labor requirements. We found that X-ray sterilization did not reduce the lifespan of *D. suzukii* adults substantially (a maximum 4-day reduction), although there was a significant reduction at the most effective sterilization dose. This will contribute to the feasibility of SIT programs, particularly following the optimization of X-ray exposure by defining clear and repeatable parameters. In the wild, the lifespan of *D. suzukii* summer morphs ranges from 33 to 60 days, whereas winter morphs can live for up to 160 days.[47] Notably, the frequency of mating in *D. suzukii* remains relatively low, with extended intervals between initial and subsequent matings.[48] Released males must therefore cover the mating windows of females, thereby preventing a population rebound. Other key fitness parameters, such as sexual activity and flight capability, are also relevant for the success of SIT programs. *D. suzukii* tolerates remarkably high exposure to X-rays, with even doses as high

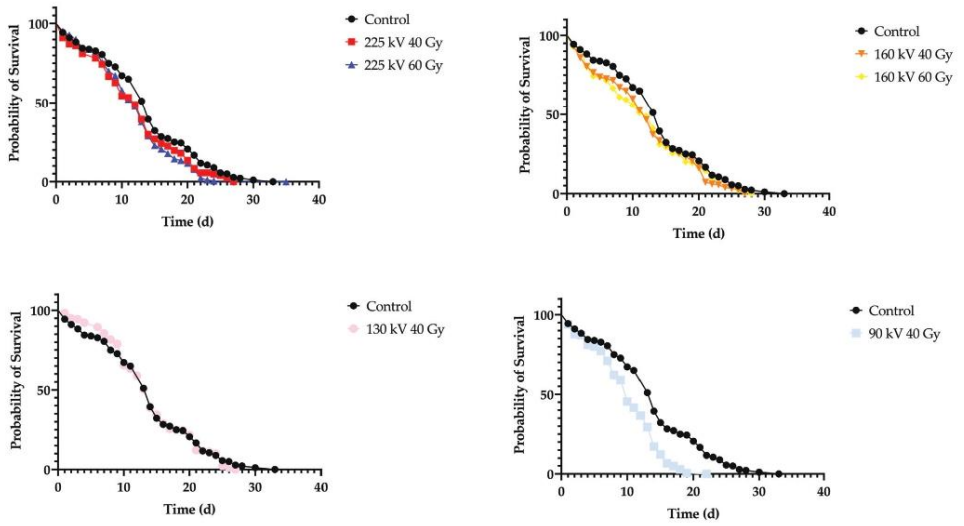

Figure 4. Survival graphs showing the lifespan of flies sterilized by X-ray irradiation. All graphs represent three biological and technical replicates, each comprising 20 individual flies. a) Doses at 225 kV. b) Doses at 160 kV. c) Dose at 130 kV. d) Dose at 90 kV. This experiment revealed a significant difference in lifespan between untreated controls and flies exposed to 90 kV/40 Gy, the settings that achieve the most efficient sterilization ($\alpha = 0.05$).

as 300 Gy primarily affecting fertility and fecundity.[49,50] Importantly, our X-ray sterilization approach appears to be much less aggressive than the heat treatment method, with the lifespan of treated flies hardly diverging from the untreated control group at 130 kV.

4. Conclusion

SIT is a potent and effective pest control method with promising applications against *D. suzukii*. Our research contributes to ongoing control efforts by offering complementary approaches to established methods. The recent field trial, which released sterile males within open polytunnels in strawberry orchards in the UK, triggered a significant decline in the target population, another semi-field investigation resorted to a dual-sex release strategy due to the lack of a sexing system.[35] This achieved a reduction in progeny numbers on infested fruit, yet emphasized the need for a higher sterile-to-wild ratio to optimize the effects. Notably, the releases did not diminish the number of infested fruit, a factor potentially influenced by the dual-sex release approach.[35] Both studies underscore the potential efficiency of SIT against *D. suzukii*, emphasizing the need for a larger number of released insects to compete with the wild population for maximum efficiency. Distinguishing between female and male *D. suzukii* is relatively straightforward during adulthood, but earlier sexing has the advantages of reducing overall costs, streamlining packaging and shipping logistics, and providing the insects with an extended field presence. We have now shown that sex can be accurately determined at the pupal stage.

Compared to chemical pesticides,[51] GMOs,[29] and ionizing radiation,[49] X-ray irradiation offers a safer and more convenient approach, with no risk of environmental contamination or the evolution of resistance in pests.[52] We have also refined the parameters for insect sterilization, showing that X-ray sterilization can be achieved at lower dosages, and may be suitable for combination with other sterilization techniques, including the novel heat-treatment method. In future studies, more extensive experiments are required to determine the fitness of the flies (mating frequency, flight ability) to assess the ability of the sterilized cohort to compete with the wild population. Afterward, semi-field trials using flies sterilized by X-ray or heat exposure will provide efficacy data under field-like conditions.

The efficiency of SIT programs against *D. suzukii* must be optimized given the high fecundity of this pest and its frequent

Table 2. Summary of LT_{50} values for all X-ray sterilization settings.

Energy	Dose used	LT_{50} (days)	P value compared to control
Control	–	14.1	–
225 kV	40 Gy	12	0.0093
	60 Gy	11.8	0.0010
160 kV	40 Gy	11.3	0.0174
	60 Gy	11.6	0.0339
130 kV	40 Gy	13.6	0.6433
90 kV	40 Gy	10.5	<0.0001

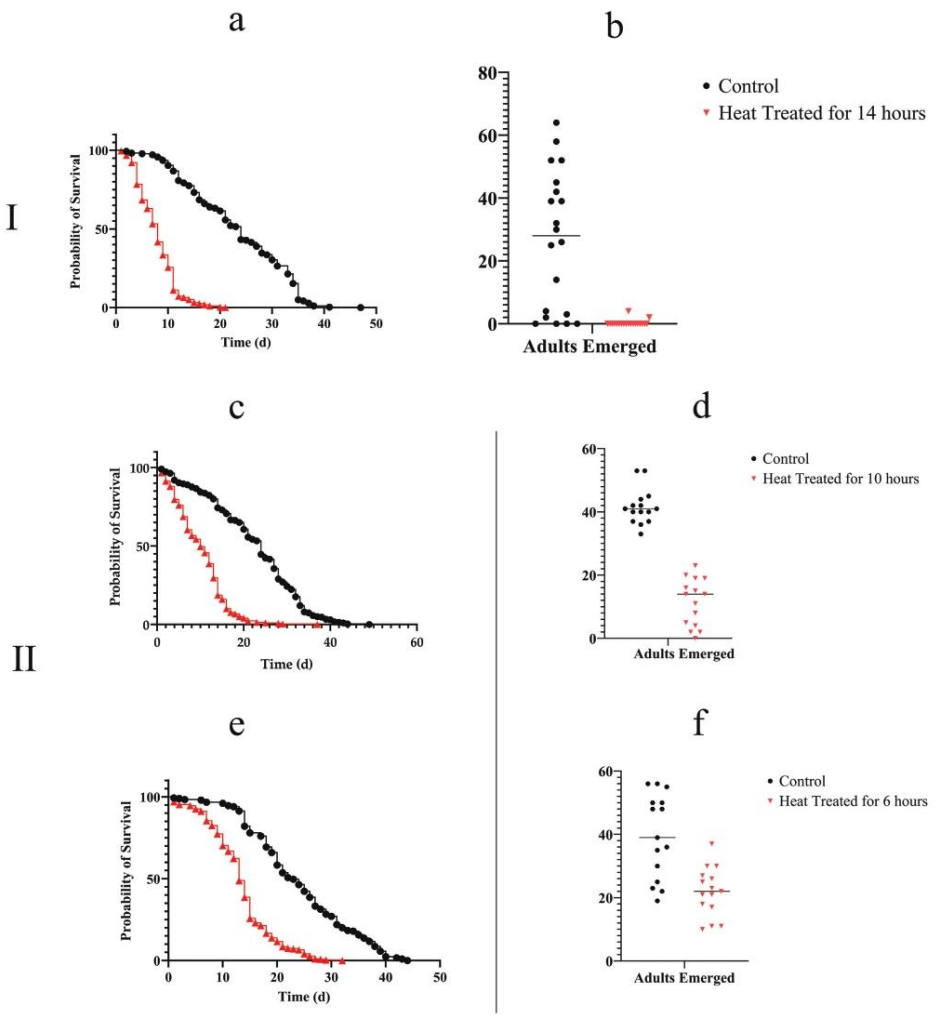

Figure 5. Analysis of heat sterilization parameters. (I) Sterilization of *D. suzukii* by applying alternating temperature cycles at 28 °C for 10 h and 30 °C for 14 h causes a significant reduction in lifespan as well as fertility. a) Probability of survival over time. All graphs represent three biological and technical replicas, each featuring 20 flies ($p < 0.01$). b) Number of offspring per single cross with an untreated virgin female (mean control = 26.35, mean treated = 0.3). (II) The lifespan and fertility of flies improves at 30 °C depending on the incubation time. c) Probability of survival over time after heat treatment for 10 h. All graphs represent three biological and technical replicas, each featuring 20 flies ($p < 0.01$). d) Number of offspring per crossing (20 males × 20 untreated virgin females) after heat treatment for 10 h (mean control = 41.60, mean treated = 11.4). e) Probability of survival over time after heat treatment for 6 h. All graphs represent three biological and technical replicas, each featuring 20 flies ($p < 0.01$). f) Number of offspring per crossing (20 males × 20 untreated virgin females) after heat treatment for 6 h (mean of control = 39.47, mean of treated = 21.93).

Table 3. Summary of LT_{50} values for all heat-based sterilization settings.

Time at 30 °C	Control LT_{50}	LT_{50} (days)
6 h	23 days	14
10 h	22 days	10.5
14 h	19 days	7.5

migration into unconfined crops. Its short generation time heightens concerns of rapid population recovery, requiring frequent and intensive releases.[38] A successful SIT program must therefore feature concerted and well-coordinated releases over a large region involving the inclusion of growers associations or large farms. By establishing cost-effective and energy-efficient methods for sterilization, we can make a significant contribution to the development of SIT methods as promising tools for the control of *D. suzukii*.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

K.-Z.L., S.G., and A.V. performed conceptualization. K.-Z.L., S.G., J.C., and A.V. performed validation. I.A., M.W., E.H., J.C., S.G., K.-Z.L., and A.V. performed investigation. S.G. and K.Z.L. wrote the original Draft. I.A., S.G., K.-Z.L., and A.V. Reviewed and Edited the final manuscript. K.-Z.L., S.G., J.C., and A.V. perfomed supervision. K.-Z.L. acquired funding.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

Drosophila suzukii, high-temperature sterilization, SIT, X-ray irradiation

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La Jolla Virus: The Pathology and Transmission in Its Host *Drosophila suzukii*

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Abstract: *Drosophila suzukii*, commonly known as spotted-wing drosophila, has emerged as a highly destructive pest in global fruit and wine production. The effectiveness of chemical control is significantly compromised by rapid resistance development and a limited range of insecticide options. Biological control presents a promising sustainable alternative. Our previous work suggested the La Jolla Virus (LJV) as a suitable candidate for the development of an insect virus-based control option. Here, we characterized the natural transmission and pathology of the virus. We tested various modes of horizontal transmission, including airborne, venereal and oral, and fecal routes. To understand LJV pathology in infected flies, we studied feeding behavior and demonstrated changes in food absorption compared to non-infected flies. We also investigated the impact on fecundity and egg-to-adult success rate. Altogether, these results collectively improve our understanding of LJV transmission in natural populations and the implication of infected flies in food ingestion and overall fitness.

Keywords: *Drosophila suzukii*; biological pest control; La Jolla virus; iflavirus; transmission; food intake

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1. Introduction

Since the beginning of its invasion more than a decade ago in California and Europe [1], *Drosophila suzukii* has spread across much of the globe [2,3], resulting in severe yield losses in many crops, such as cherries, berries, and grapes. Originally described in Japan and endemic to East Asia, this pest is now found on nearly every continent, causing annual damage in the millions [4,5]. Whereas most other Drosophila species feed and oviposit on decaying fruit, *D. suzukii* females have a preference to lay eggs in intact, ripening, and ripe fruit with their specialized serrated ovipositor. *D. suzukii* has an exceptionally broad range of host plants, targeting soft and ripening fruit across cultivated and wild environments [6,7]. With a high reproduction rate of ~400 eggs per female and a rapid life cycle of 8 days, the species demonstrates extraordinary adaptability. Its ability to tolerate diverse environmental factors and adjust reproductive strategies based on temperature and photoperiod makes it a highly resilient pest capable of causing significant agricultural devastation if left unchecked [8].

Traditional control methods for *D. suzukii* have relied heavily on conventional insecticides, including synthetic pyrethroids, organophosphates, spinosyns, and neonicotinoids.

However, the application of synthetic insecticides comes with many disadvantages, including a high risk of insecticide resistance development [9] and harmful effect on the environment and non-target organisms [10–13]. Moreover, the *D. suzukii* larvae hatching inside the fruit are well protected from the environment and, due to the timing of application prior to harvesting, are difficult to treat. Thus, new and sustainable methods to control *D. suzukii* are urgently needed.

A promising strategy is the use of insect-specific viruses to control insect pests due to their generally high host specificity [13–15]. In previous studies, we identified a potential virus candidate, the La Jolla Virus (LJV), a positive-sense single-stranded RNA virus and member of the Iflaviridae family [16–18]. Initially discovered in a metagenomic approach in *Drosophila melanogaster* [16]. LJV was subsequently isolated from moribund *D. suzukii* specimens in Germany. Intrathoracic injection of LJV caused significant mortality among adult *D. suzukii* flies [17]. Similarly, oral administration of LJV also led to significant mortality in a concentration-dependent manner [19]. LJV demonstrates superior efficacy as an RNA virus against Drosophilids compared to Drosophila C Virus (DCV), particularly when administered orally. While DCV causes only 10–25% mortality in *Drosophila melanogaster* via oral infection, even at high titers [20,21], LJV significantly reduces the lifespan of infected flies, with all LJV-exposed flies succumbing earlier than controls.

Our research aimed to elucidate the transmission dynamics of LJV in *D. suzukii*. Building on previous studies that demonstrated oral infection and vertical transmission [19], we conducted experiments to further characterize horizontal, vertical, and potential vector-mediated transmission routes [22]. We investigated the impact of LJV epidemics on *D. suzukii* populations on lab scale and examined how viral infection affects the pest's feeding behavior. These findings contribute to a more comprehensive understanding of LJV–host interactions, which is crucial for developing effective biocontrol strategies. A key question remains: Can a chronic or natural LJV infection significantly impact wild populations of *D. suzukii*? This study aims to address this critical question by investigating whether persistent viral infections can influence population dynamics, behavior, and the overall fitness of *D. suzukii* in natural settings, providing valuable insights into the potential of LJV as a biocontrol agent.

2. Materials and Methods

2.1. Drosophila suzukii Cultures Maintenance

The *D. suzukii* fly strain was derived from a laboratory colony established in the summer of 2012 in Ontario, Canada [23]. From this line, we produced one LJV negative and one LJV positive subline by adding orally infected flies to a negative line, as described previously [19]. The LJV negative line was maintained in a climate chamber (26 °C, 60% relative humidity, 12 h photoperiod) and checked regularly for LJV as described in 2.2. In contrast, the infected flies were kept in an incubator (model KBWF 240, Binder, Neckarsulm, Germany) under identical environmental conditions, with the addition of air circulation provided by a fan operating at 50% capacity (75 cm height × 60 cm width × 45 cm depth) and checked for LJV as described in 2.2 at the start of the performed experiments. All flies were maintained on a standard fly diet composed of 60 g/L cornmeal, 8 g/L soybean flour, 18 g/L baker's yeast, 60 g/L malt, 22 g/L molasses, 6.25 mL/L propionic acid, and 2 × 0.8 g nipagin, which is poured in 50 mL drosophila vials. All experiments were carried out on flies aged 3–7 days post-eclosion. All experiments were conducted as 3 biological and technical replicates with the exception of the airborne transmission, which was carried out with 3 biological replicates and 4 technical replicates.

2.2. Extraction and Quantification of La Jolla Virus in Flies

RNA was extracted using a TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) from the collected flies, and 50 ng/µL aliquots were then prepared for quantification. The quantification was performed to assess the degree and progress of the infection in flies. Quantification was performed via StepOne real-time PCR system (Applied Biosystems, Waltham, MA, USA) with the Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA, USA). Amplification was performed using the probes and primers described in Table 1. The real-time PCR was performed with the following steps: an initial heating to 55 °C for 10 min, followed by 95 °C for 1 min. This was followed by 40 cycles of 95 °C for 10 s for denaturation and 60 °C for 30 s for annealing, targeting positions 64–95 bp within the 128 bp product.

Table 1. Shows the sequence of the primers and probes used in this study.

Description	Sequence	Product Length (bp)
LJV specific probe *	5′-ACTCGGCGTTATCGTTACAACCGCACATATC-3′	
LJV forward primer	5′-CAACACGTTGTGCTGCCTGA-3′	128
LJV reverse primer	5′-TCCATCCAAACTCCACCTCC-3′	128

* Labeled on 5′ with fluorescent reporter dye FAM, on 3′ with fluorescent quencher TAMRA. The probe is at position 64–95 bp within the 128 bp product.

Viral infection levels were quantified using real-time PCR, with cycle threshold (Ct) values serving as the primary metric. Uninfected control flies consistently exhibited Ct values of approximately 30, while the highest infection levels observed corresponded to Ct values of around 10. This range of Ct values provided a robust measure of infection intensity across experimental conditions.

2.3. Transmission Assays

We arranged several experiments to determine if the transmission of the virus in a population of flies is airborne, venereal, oral, or fecal. For the airborne infection assay, we placed two vials of uninfected flies on the shelf at the top of the incubator, 5 cm from the infected fly culture, and two vials at the very bottom of the infected fly culture, 40 cm from the infected fly culture. Flies were regularly collected every 3 days (5 flies per interval and location). To test the hypothesis of venereal transmission of LJV within a population, different combinations of 10 virgin females were paired with 10 males. The combinations were as follows: As controls, we paired infected males and females (IVF × IM) and non-infected males and females (NIM × NIVF) as positive and negative baselines. The test conditions were Non-Infected Virgin Female and Infected Male (IM × NIVF), Infected Virgin Female and Non-Infected Male (NIM × IVF). As internal control, we set up pairings with the same-sex conditions to check for the requirement of the mating itself, or if factors such as close contact, including oral or fecal routes, are sufficient for transmission. The pairings went as follows: Non-Infected Virgin Female × Infected Virgin Female (NIVF × IVF) and Non-Infected Male × Infected Male (NIM × IM). All flies were allowed to pair for 3 days before collecting 5 of each sex. To differentiate the LJV negative flies from the infected, we cut off the wings of the infected flies. For the oral transmission, we placed 50 infected males on a vial of food and allowed them to feed for 3 days. Then we removed the flies, flooded the vial with CO_2, and used a UV cross-linker (Stratagene Stratalinker 2400, La Jolla, CA, USA) to surface sterilize the vial in order to minimize fecal transmission. After this, 50 females and 50 males from the LJV negative flies were introduced and allowed to feed on the fly food. We then proceeded to collect 5 flies from both sexes every 3 days. For the fecal transmission, we placed a cotton ball saturated with 2 mL of 100 mM sucrose

solution on parafilm in a completely empty drosophila vial. We then introduced 50 infected males and allowed them to feed and defecate on the walls of the vial for 3 days. After this, we removed the flies, the cotton ball, and the parafilm. Then, we thoroughly flooded the vial with CO_2, added a fresh cotton ball on parafilm, introduced 50 LJV negative females and males to the vial, and allowed them to feed. Flies were collected every 3 days, and the cotton ball was replaced as necessary.

2.4. Egg to Adult Viability

To determine the influence of the virus on a fly population, we monitored the egg-to-adult viability of LJV-positive flies compared to LJV-negative flies. In short, we placed flies on grape juice agar plates (30% grape juice, 1% agar), transferring 100 eggs to a new agar plate. We then transferred the agar and placed it in a drosophila vial with food, then counted the larvae after 6 days, the number of pupae, and the number of emerged adults.

2.5. The Feeding Behavior of Infected Flies

To assess the impact of viral infection on feeding behavior, we employed the flyPAD (fly Proboscis and Activity Detector) system, an automated high-resolution behavioral monitoring tool that uses capacitive sensors to detect and analyze feeding behavior in Drosophila [24]. The flyPAD system allows for the extraction of various feeding metrics, including the number of activity bouts, the total duration of activity bouts, and the number of sips, all of which correlate with food intake. The system's sensitivity enables the estimation of the volume of food consumed per sip, providing a comprehensive view of feeding dynamics in individual flies.

We adopted a slightly modified protocol based on previous work [24] The experiment consisted of three runs each for females and males, comparing 24 La Jolla Virus (LJV)-infected flies to 24 uninfected control flies per run. Prior to the experiment, flies were wet starved (females for 24 h and males for 18 h) to standardize hunger levels. Individual flies were then placed in the feeding arena of the flyPAD system. For each assay, we added 4 µL of either 5 mM or 50 mM sucrose solution to the reading electrode. Flies were allowed to feed for 1 h, during which their interactions with the food were continuously monitored by the flyPAD system. The system recorded multiple behavioral metrics, including the number of activity bouts (approaches to food), the total duration of activity bouts, and the number of sips. These parameters were used to estimate food intake and analyze feeding dynamics.

2.6. Statistical Analysis and Graph Design

All analyses were carried out via GraphPad Prism v9.1.2 for Windows (GraphPad Software, San Diego, CA, USA). For all the transmission experiments, a 2-way ANOVA test was run with multiple comparisons against an α of 0.05. The life stage experiment was performed via 2-way ANOVA, and the comparison was performed via Šídák's multiple comparisons and compared to alphas of 0.05. The results and graphs of the behavior test are all calculated and plotted via the flyPAD program.

3. Results

3.1. Airborne Transmission

To investigate whether LJV is airborne transmitted, we raised non-infected flies close to LJV-infected flies in a closed environment, i.e., an incubator with constant temperature and humidity settings. Our findings revealed that infected flies exhibited high titers of LJV, with a baseline mean infection Ct value of 9.9. In contrast, the adjacent non-infected flies maintained their uninfected status throughout the observation period, from day 3 to day 12 post-exposure. The Ct values for these non-infected flies remained relatively stable and

significantly higher than the established baseline threshold, confirming their uninfected state (Figure 1).

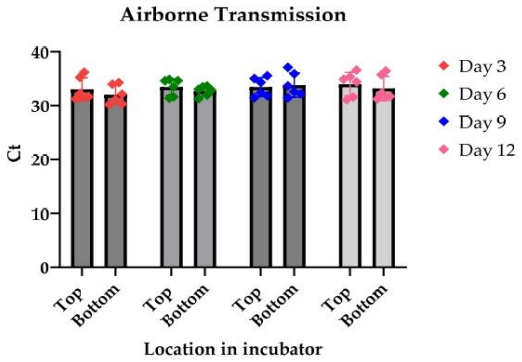

Figure 1. The results of the real-time PCR after several days of the flies being in the vicinity of an infected fly culture. The graph demonstrates no significant change in the Ct of the extracted samples, concluding there was no infection. Results show no significant difference in the infection levels as the days went on. SD of day followed by top/bottom: Day 3: 2.136/1.788; Day 6: 1.567/0.925; Day 9: 1.755/2.241; Day 12: 2.183/2.277. The error bars represent the standard error mean (SEM). *p*-value = 0.38 alpha = 0.05.

3.2. Venereal Transmission

Pairing experiments revealed distinct viral transmission patterns (Figure 2). Non-infected fly pairs maintained their uninfected status, as evidenced by high Ct values (approximately 30) in RT-qPCR analysis. In contrast, pairings involving infected flies resulted in high levels of LJV transmission, indicated by low Ct values (around 10). Notably, same-sex pairings showed transmission rates comparable to female–male pairings. This similarity does not exclude the possibility of venereal transmission but suggests that virus transmission occurs primarily through contact, likely via fecal or oral routes, rather than through venereal transmission.

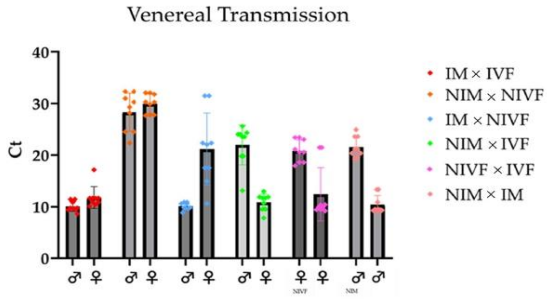

Figure 2. The infection levels after the flies were allowed to mate for 3 days. The first two sets are the positive (IM × IVF, red dots) and negative controls (NIM × NIVF, orange dots), followed by the different combinations tested. All infection levels are significant compared to the negative control (NIVF + NIM) alpha = 0.05.

3.3. The Transmission of the Virus Occurs by Oral and Fecal Contamination

The experimental outcomes for fecal and oral transmission routes showed striking similarities when assessing infection rates after several days of exposure (Figure 3A,B). Control flies maintained their non-infected status, as evidenced by high Ct values (approximately 30) in RT-qPCR analysis. In contrast, flies exposed to either feces or food from LJV-infected individuals exhibited significantly lower Ct values (approximately 20), indicating successful virus transmission through these routes. Notably, the transmission efficiency appeared to follow a time-dependent trend, with higher transmission rates observed after longer incubation periods. This temporal pattern suggests a gradual accumulation of viral particles in the exposed flies, potentially leading to more robust infections over time. These results collectively suggest the effectiveness of both fecal and oral routes in LJV transmission among *D. suzukii* populations, highlighting the virus's capacity for efficient horizontal spread through environmental contamination.

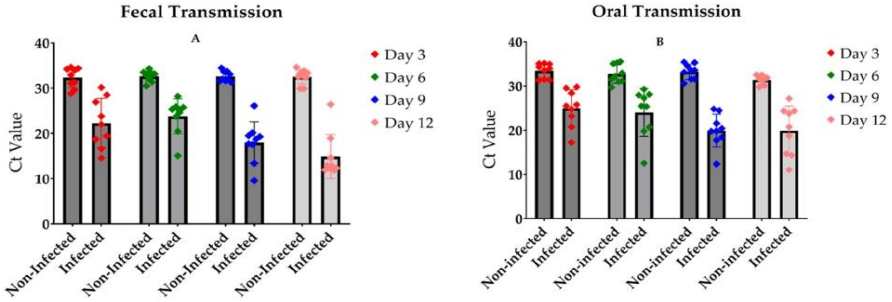

Figure 3. The infection levels in the flies ((**A**) Fecal transmission; (**B**) Oral Transmission) after several days of infection. The figure shows a steady infection rate in both experiments. Infected flies showed a significant difference to the non-infected control (p-value < 0.0001 in **A**,**B**).

3.4. Chronic LJV Infection Decreases Egg-to-Adult Success Rate

Our results demonstrate a significant reduction in the percentage of emerged adults due to the impact of the virus (Figure 4). While all eggs successfully hatched, differences began to emerge at the larval stage, particularly when larvae reached the L3 stage. The virus seems to disrupt normal larval growth, resulting in a gradually widening gap in survival rates when compared to uninfected controls. Although significant differences were observed across all developmental stages, the most pronounced effect was seen during adult emergence. At this stage, the virus caused a substantial decline of around 40% in the number of adults successfully completing pupation and emerging.

3.5. LJV Affects the Feeding Behavior of Female Flies

We employed the flyPAD (fly Proboscis and Activity Detector) system to assess whether LJV infection alters the feeding behavior of *D. suzukii*. Our results reveal significant changes in the feeding patterns of infected females compared to uninfected controls.

The feeding behavior of non-infected and infected flies was evaluated for both genders (Figure 5A,B) using the cumulative duration of activity bouts. This metric, representing the total time engaged in physical activity over multiple bouts during a one-hour period (x-axis in Figure 5A,B), represents in the flyPAD system the sum of all individual activity bout durations recorded for a fly during the observation period. It is calculated in the flyPAD system by summing all individual activity bout durations for each fly. This measure effectively captures the full spectrum of fly movement patterns, from brief exploratory actions

to extended locomotion periods, making it valuable for assessing overall activity levels. While female flies exhibited a notable difference, males showed no significant variation.

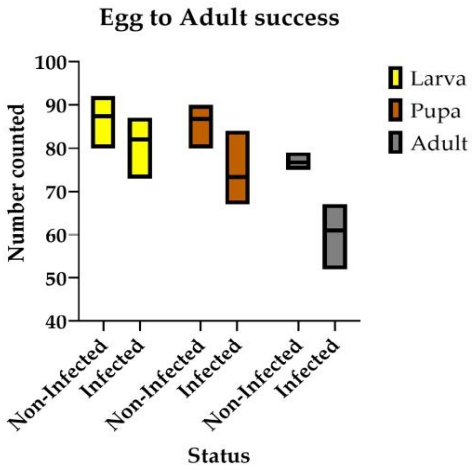

Figure 4. Egg-to-adult success rate of non-infected versus infected flies for larvae (yellow bars), pupa (orange bars), and adults (gray bars). All differences are significant when $\alpha = 0.05$. SE of difference = 3.274.

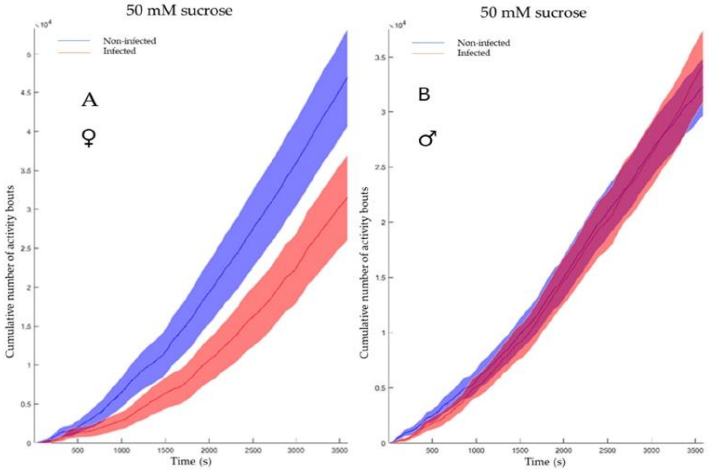

Figure 5. Cumulative duration of activity bouts. (**A**) The feeding activity of the non-infected females (blue) vs. the infected females (red). The software conducted a Mann–Whitney test and shows a *p*-value of 0.036. (**B**) The feeding activity of the non-infected males (blue) vs. the infected males (red) The software conducted a Mann–Whitney test and shows a *p*-value of 0.93.

In contrast, the cumulative number of feeding bursts represents the total count of discrete feeding events recorded by the flyPAD system over a specified observation period.

In Drosophila studies using flyPAD technology, a feeding burst is typically defined as a short, intense period of proboscis extension and food interaction. This metric provides a quantitative measure of feeding frequency, offering insights into the flies' overall food consumption patterns and feeding behavior. By summing these individual feeding events, the total feeding activity can be assessed, which may be influenced by factors such as the LJV infection status. The cumulative number of feeding bursts serves as an indicator of feeding drive and can be used to compare feeding behaviors across different experimental conditions. In our investigation, no significant difference (p-value 0.068 for females; p-value 0.12 for males) was observed between noninfected and infected flies, suggesting no substantial variation in feeding uptake (Figure 6A,B).

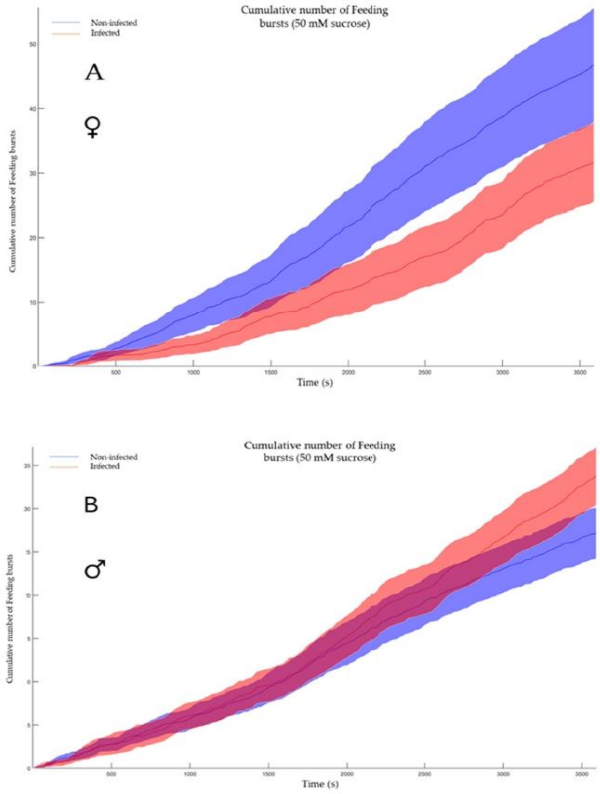

Figure 6. Shows the cumulative number of feeding bursts. (**A**) The feeding activity of the non-infected females (blue) vs. the infected females (red). The software conducted a Mann–Whitney test and shows a p-value of 0.068. (**B**) The feeding activity of the non-infected males (blue) vs. the infected males (red) The software conducted a Mann–Whitney test and shows a p-value of 0.12.

4. Discussion

We studied the transmission routes and pathologies of the La Jolla Virus (LJV) in its host, *Drosophila suzukii*. Although insect viruses typically do not spread through the air, it

is worth noting that certain human respiratory viruses, such as coronaviruses, influenza viruses, and rhinoviruses [25,26], can be transmitted via airborne routes. Our research suggests that airborne transmission does not occur, at least under the specific laboratory conditions we used. The study's experimental findings validate that LJV primarily spreads through the oral–fecal route, with venereal transmission likely playing a secondary role. This finding is consistent with prior studies on iflaviruses, which have demonstrated diverse transmission strategies, including horizontal (oral–fecal) and vertical routes, depending on the host species and environmental conditions. For example, *Euscelidius variegatus iflavirus* 1 (EVV-1) primarily spreads via fecal–oral transmission, as observed in *Nilaparvata lugens* honeydew virus-1 and Nora Virus in other insect species [27,28]. Deformed Wing Virus (DWV), an Iflaviridae family member akin to LJV, illustrates the complex transmission dynamics and infection effects seen in insect RNA viruses [29]. The transmission routes and infection outcomes of DWV are among the most extensively studied within the Iflaviridae family, offering valuable insights into the ecology of these viruses. The entoparasitic mite *Varroa destructor* serves as a crucial vector for DWV, substantially influencing its virulence. The concurrent presence of DWV and Varroa mites results in symptoms like pupal mortality and the characteristic deformed wings of worker bees [30]. Although LJV appears to infect without a vector, similar symptoms, such as pupal death, can be observed in *D. suzukii* LJV infections. A common effect is the severely shortened adult life span in an acute DWV infection. In mite-free conditions, DWV develops into a persistent, covert (chronic-asymptomatic) infection. In contrast, LJV seems to establish a persistent and chronic symptomatic in the absence of a vector, significantly impacting population dynamics by reducing offspring numbers and the fitness of the emerging flies. This outcome is very important since an application of LJV as a biocontrol agent would also be able to spread chronically within a population and maintain their virulence.

Next, we explored the possibility of venereal transmission of the virus. Venereal transmission of arboviruses has been documented in several cases [31–34]. To test this hypothesis, we paired infected flies with their mates and monitored them for infections. All flies paired with infected partners showed initial signs of infection, which was a promising indicator. However, infection was also observed in same-sex pairings, suggesting potential environmental transmission rather than direct sexual contact. To confirm sexual transmission conclusively, future studies should involve dissecting and examining sexual organs for viral presence. If confirmed, sexual transmission could be a valuable mechanism, especially when combined with other techniques, such as the Sterile Insect Technique (SIT). Sterile infected males that have been sterilized with eco-friendly methods [35], could be released as viral vectors to disseminate the infection throughout the population. While venereal transmission cannot be excluded for LJV, its significance appears to be secondary to the oral–fecal route. Studies on other insect-specific viruses, such as Culex flavivirus (CxFV), have shown that venereal transmission plays a minor role compared to vertical and horizontal routes [36]. Similarly, recent research on medfly associated iflaviruses indicates that while vertical transmission via females is predominant, male-mediated venereal transmission is possible but less efficient [37]. Studies comparing iflaviruses have revealed diverse transmission methods within this viral family. While some iflaviruses, like the *Antheraea pernyi* Vomit Disease virus [38] and *Spodoptera exigua* iflavirus [39], primarily transmit vertically through eggs or larvae, this study did not focus on vertical transmission. However, its potential role in LJV epidemiology deserves further exploration.

The predominance of oral–fecal transmission has significant implications for understanding host–virus interactions and population-level dynamics. Insects with gregarious feeding behaviors or those inhabiting densely populated environments are particularly susceptible to rapid viral spread via contaminated food or substrates. The systemic nature

of infection ensures that all life stages contribute to environmental contamination. This continuous viral shedding increases the chances of sustained viral circulation within host populations, even in the absence of overt disease symptoms. Additionally, investigating how environmental factors like temperature or humidity affect fecal–oral transmission efficiency could provide valuable context for understanding seasonal variations in infection rates. Future research should also investigate potential interactions between LJV and other pathogens or microbiota within the host gut. As demonstrated by SeIV1's interaction with baculoviruses [40], such associations may modulate viral infectivity and persistence.

We subsequently evaluated the complete life cycle of both uninfected and infected flies to determine their egg-to-adult survival rate. This investigation builds upon a previous study [19], which demonstrated that oral infection with LJV negatively affects fly eclosion. Our aim was to verify these findings and explore the potential impact on a chronically infected fly population, thereby demonstrating the potential of LJV as a biocontrol agent. Our findings revealed that LJV significantly impairs the adult emergence rate in fly populations. This is in contrast to the picorna-like Nora virus, which can establish infections in laboratory fly strains and persist for several years without causing any significant pathological effects [41]. The persistent presence of LJV in the environment could serve as a continuous control measure, which, when combined with other complementary methods, may lead to population suppression. The results indicate that the virus has its most significant effect during the later developmental stages, possibly affecting essential physiological or metabolic functions required for pupation and adult eclosion. This underscores the virus's capacity to influence population dynamics by decreasing reproductive success and adult survival rates in *D. suzukii*, even in cases of long-term, chronic infection within the population. Nevertheless, we are unable to distinguish between potential maternal influences, where the chronic infection may have resulted in reduced yolk or protein deposition in the eggs, and developmental effects, where the infected larvae's food consumption is impacted. Additional research is necessary to address this question.

Finally, we examined the virus's effect on fly feeding behavior. Our findings indicate that the virus has a more pronounced impact on female flies, with infected females showing reduced feeding-relevant activities compared to their uninfected counterparts. This suggests a reduced drive or motivation to seek out food sources among infected females. This observation is particularly relevant in natural environments where food is likely to be more dispersed and limited, potentially affecting fly survival rates. Interestingly, male flies do not exhibit the same response to the virus, showing no significant differences in feeding behavior.

Our study reveals that while infected flies spend more time locating food, their overall food consumption remains unaffected. Although the statistical difference was marginal (p-value slightly exceeding the 0.05 α threshold), these findings could have significant implications in natural settings, especially in areas with scarce food resources. In field conditions with regular maintenance and fewer hiding spots for flies, we anticipate observable effects, particularly given the infected flies' reduced motivation to search for food. Our research also highlights opportunities for further investigation into how the virus influences *D. suzukii*'s feeding behavior. Future studies could explore whether the virus affects specific receptors and, if so, which ones. Additionally, research could examine if the virus impacts fly mobility and whether different food types alter the foraging behavior of infected flies.

Viruses offer a promising alternative due to their specificity and effectiveness, and LJV has shown significant antagonistic effects against *D. suzukii*. However, like other insect viruses, the application of LJV presents its own set of challenges. Previous research has demonstrated that, once infected, the virus can kill the insect within 5–8 days [19].

This relatively slow action may limit its immediate impact in controlling infestations. To address these challenges, studying the transmission dynamics of LJV within *D. suzukii* populations is a prerequisite for a controlled release in natural environments. Conducting field trials is crucial for gaining insights in natural settings, as laboratory conditions often fail to capture the complexities of real-world environments. Furthermore, developing viral formulations to improve stability and delivery, or employing strategies like continuously infecting food sources with viral particles, could strengthen the practical application of LJV as a biocontrol agent. The mass production of LJV for biological pest control would require the development of cost-effective, large-scale fermentation processes involving cell culture-based methods. Engineering could potentially enhance the speed and effectiveness of LJV's lethal capabilities. Nevertheless, the public generally disfavors genetically modified organisms (GMOs). As a result, approaches that utilize natural selection processes are more widely accepted as alternatives. Additionally, care must be taken to minimize off-target effects, as members of the Iflaviridae [42] are known to be antagonists of bees, raising ecological concerns. Combining LJV with other management techniques, such as SIT, could maximize its efficacy and reduce the likelihood of resistance developing in the *D. suzukii* population.

In summary, our study highlights the critical importance of the oral–fecal route as the primary mechanism for transmitting LJV and the long-term effects of chronic infection, including feeding behavior change on a population level. These findings align with the existing literature on iflaviruses and provide a foundation for further exploration into host–virus dynamics and ecological implications. Understanding these transmission pathways is essential for developing targeted strategies to manage infections caused by LJV and related viruses in insect populations.

Author Contributions: Conceptualization, K.-Z.L. and A.V.; methodology, K.-Z.L., I.A. and T.K.; validation, K.-Z.L. and I.A.; formal analysis, I.A.; investigation, I.A.; writing—original draft preparation, I.A.; writing—review and editing, I.A., K.-Z.L. and A.V.; visualization, I.A.; supervision, K.-Z.L. and A.V.; project administration, K.-Z.L.; funding acquisition, K.-Z.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

LJV	La Jolla Virus
DWV	Deformed Wing Virus
DCV	Drosophila C Virus

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