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Characterization and *in vivo*-studies of entomopathogenic viruses for biocontrol of the invasive pest *Drosophila suzukii*

Dissertation

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"Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar (...)"

Antonio Machado (1875 - 1939)

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Summary

In the past ten years, *Drosophila suzukii*, a fruit fly native to Eastern and Southeastern Asia, has developed into a serious economically relevant pest of soft and stone fruits. This notorious pest has an economic impact on the agricultural industry that reaches to millions of euros in crop losses. For instance, an estimate of 3 million € in losses in cultivated fruit crops, i.e. cherries, raspberries and blueberries, was recently recorded only for the Autonomous Province of Trento. This, combined with the rapid global spread of this pest insect, makes SWD one of the most serious threats for modern horticulture.

There are several features that allowed *D. suzukii* to become a pest: (*i*) its ovipositor that possesses the ability to penetrate the skins of a broad range of fruit; (*ii*) the pest's high reproduction rate and rapid lifecycle; (*iii*) and the ability of the three larval instars to develop inside the fruit. Taken together, chemical control measures cannot be used to effectively fight SWD. Consequently, effective, ecologically safe, and sustainable strategies for its control are urgently required.

To the best of the author's knowledge, no chemical, biotechnological, or biologically effective measures have been yet described for the control of this invasive pest species. However, it is known from literature that viruses have proven for more than fifty years to be host-specific and environmentally friendly biocontrol agents. Consequently, the main goal of this thesis was the study of host-specific viruses identified from naturally infected *D. suzukii*. This was achieved by sampling larvae from orchards and estimating their health status. Subsequently, viruses obtained from collected larva were screened. Finally, four viruses were identified: *Drosophila* A virus (DAV), La Jolla virus (LJV), Mots Mills-like virus (MMlV) and Teise virus. Of those, DAV and LJV were isolated, functionally studied and compared by using *D. suzukii* as model insect for the first time.

In this thesis, LJV was chosen due to its relatively high virus-host adaptation, which seemed to significantly impact the lifespan of *D. suzukii*. Subsequently, a virus-specific ultra-purification protocol was established, followed by LJV genomic structural characterization. Thereafter, the fundamental biological features of this Iflavirus were studied such as (*i*) infection across different developmental stages and organs, (*ii*) a probable transmission route and (*iii*) host-virus adaptation experiments *in vivo*. However, formulation and details of the up-scale have to be investigated further on the way towards a sustainable, virus-based insecticide for effective biocontrol of *D. suzukii.*

Zusammenfassung

In den letzten 10 Jahren hat sich *Drosophila suzukii*, eine aus Ost- und Südostasien stammende Fruchtfliegenart, zu einem wirtschaftlichen relevanten Schädling von Weich- und Steinobst entwickelt. Die ökonomischen Folgen dieses Schädlings für die Agrarindustrie sind in Millionenhöhe zu beziffern. Allein für das Trentino wurden die monetären Verluste im Obstbau (Kirschen, Himbeeren und Blaubeeren) kürzlich auf etwa drei Millionen Euro geschätzt. Durch ihre rasche globale Verbreitung ist *D. suzukii* in den vergangenen zehn Jahren zu einer ernsthaften Bedrohung für den modernen Obstbau geworden.

Bedingt durch mehrere artspezifische Eigenheiten, ist es *D. suzukii* gelungen, sich zu einem weltweit bedeutsamen gartenbaulichen Schädling zu entwickeln: (*i*) Der gezähnte Ovipositor, der ermöglicht es ihr, verschiedenste Fruchtschalen zu durchdringen. (*ii*) Die hohe Reproduktionsrate und eine kurze Generationszeit ermöglichen Massenvermehrungen. (*iii*) Durch die Entwicklung innerhalb von Früchten sind die drei Larvalstadien gut gegen chemische Pflanzenschutzmittel geschützt. Daher wird es immer wichtiger, wirksame biologische Alternativen zur nachhaltigen Bekämpfung zu finden.

Nach Kenntnis der Autorin wurden noch keine chemischen, biologischen oder biotechnologischen Alternativen zur Bekämpfung dieser invasiven Schädlingsart beschrieben. Nichtsdestotrotz finden sich in der Literatur seit über 50 Jahren etliche Beispiele für die erfolgreiche Bekämpfung von Schadinsekten mittels wirtsspezifischer Viren.

Daher bestand das Hauptziel dieser Dissertation in der Untersuchung wirtsspezifischer Viren, die in natürlich infizierten *D. suzukii* identifiziert wurden. Aus in Obstgärten gesammelten toten und moribunden Larven wurden Viren isoliert und anschließend identifiziert. Schließlich wurden vier Virusarten identifiziert: Drosophila A-Virus (DAV), La Jolla-Virus (LJV), Mots Mills-like-Virus (MMlV) und Teise-Virus. Zwei davon, DAV und LJV, wurden erstmalig aus *D. suzukii* isoliert, funktionell charakterisiert und verglichen. Hierbei diente *D. suzukii* auch erstmals als Modellorganismus.

In dieser Arbeit wurde das LJV aufgrund seiner relativ hohen Virus-Wirt-Adaptation ausgewählt, da es die Lebensdauer von *D. suzukii* signifikant zu beeinflussen schien. Anschließend wurde eine virusspezifische Vorschrift zur Aufreinigung des LJV entwickelt, woran sich Untersuchungen zur genomischen Struktur des Virus anschlossen. Im Folgenden wurden die grundlegenden biologischen Eigenschaften

dieses Iflavirus untersucht, d. h. (*i*) das Vorhandensein in verschiedenen Entwicklungsstadien und Organen, (*ii*) der Übertragungsweg auf andere Fliegen, sowie (*iii*) die Virus-Wirt-Adaptation durch serielle *in-vivo*-Passagen.

Auf dem Wege zur Entwicklung eines virusbasierten und somit nachhaltigen biologischen Insektizids stehen nunmehr Untersuchungen zur Formulierung und zur großtechnischen Herstellung von Präparaten an.

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List of abbreviations

Amino acids: (aa)
Antimicrobial peptides: (AMP)
Base pair (s): (bp)
Biocontrol agents: (BCA)
Budded virions: (BV's)
Cydia pomonella granulovirus: (CpGV)
Deformed Wing Virus: (DWV)
Dithiothreitol: (DTT)
Drosophila A virus: (DAV)
Drosophila C virus: (DCV)
Drosophila melanogaster Sigma virus: (DmelSV)
Drosophila X virus: (DXV)
Edman degradation: (ED)
Embryonic hemocytes: (EH)
Fetal bovine serum: (FBS)
Giessen: (Gi)
Granulosis viruses: (GV's)
Immune deficiency pathway: (IMD)
Integrated Pest Management: (IPM)
Internal ribosome entry site: (IRES)
Isoelectric focusing: (IEF)
Kriftel: (K)
La Jolla Virus: (LJV)
Mass spectrometric: (MS)
Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry: (MALDI-TOF-MS)
Median survival time: (ST50)
Microliter (µl)
Minutes (min)
Motts Mills-like Virus: (MMlV)
Nanoliter (nl)
Next Generation Sequencing: (NGS)
Non-structural proteins: (NSP)
Nuclear Factor-κB: (NF- κB)
Nuclear polyhedrosis viruses: (NPV's)
Nucleotide (nt)
Nucleotide-binding oligomerization domain-like receptors: (NOD-like receptors)
Occlusion bodies: (OB's),
Ockstadt: (OS),
Open Reading Frames: (ORFs)
Oryctes rhinoceros nudivirus: (OrNV)
Oxford Nanopore Technologies's: (ONT)
Paraformaldehyde: (PFA)
Pathogen-Associated Molecular Patterns: (PAMPs)
Pattern Recognition Receptors: (PRRs)
Phenylthiourea: (PTU)
Polyethylene Glycol: (PEG)
Polymerase Chain Reaction: (PCR)
Polyvinylidene difluoride: (PVDF)
Rapid amplification of cDNA ends: (RACE)
Retinoic acid-inducible gene-like receptors: (RIG-I-liker receptors)
Reverse Transcription: (RT)
RNA induced silencing complex: (RISC)
RNA interference: (RNAi)
Sackbrood Virus: (SBV)
Seconds (s)
Single nucleotide polymorphism: (SNP)
Single-stranded RNA: (ssRNA)
Slow Bee Paralysis Virus: (SBPV)
Sodium dodecylsulfate polyacrylamide gel electrophoresis: (SDS-PAGE)
Spotted Wing Drosophila: (SWD)
Sterile distilled water: (dH2O)
Structural protein region: (P1)
Tris(hydroxymethyl)aminomethane: (TRIS)
Tumor Necrosis Factor Receptor: (TNFR)
Vesicular Stomatitis Virus: (VSV)
Viral genome-linked protein: (VPg)
Viral Proteins (VP)
Viral RNA-dependent RNA polymerase: (vRdRp)

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1 Introduction

1.1 Drosophilidae: biodiversity and richness

The family Drosophilidae is well known for the laboratory fruit fly, *Drosophila melanogaster*, probably one of the most widely notorious living species. The family of Drosophilidae also includes more than 4,000 species among which there is a wide range of plant feeders, predators and yeast-grazing species (Marshall, 2013). In its current state, the genus *Drosophila* (around 1,500 species) generally comprises saprophagus filter-feeders attracted to yeast and other microorganisms such as fungi (Marshall, 2013). There have been well-supported proposals to split this large and heterogeneous group into more manageable genera, which would restrict the name *Drosophila* to that group of species most closely related to the type species of the genus, *Drosophila funebris*. However, because the scientifically relevant *D. melanogaster* is in one of those other groups, such a division would land place this most famous one of flies into another genus, *Sophophora* – an undesirable change, which is unlikely to gain widespread acceptance. *Drosophila* is a perfect example of adaptive radiation as well, a perfect known example is the arrival of an ancestral species to the Hawaiian Islands 26 million years ago, which resulted in 1,000 different species showing a wide range of morphologies (O'Grady & DeSalle, 2008) as illustrated in Figure 1.

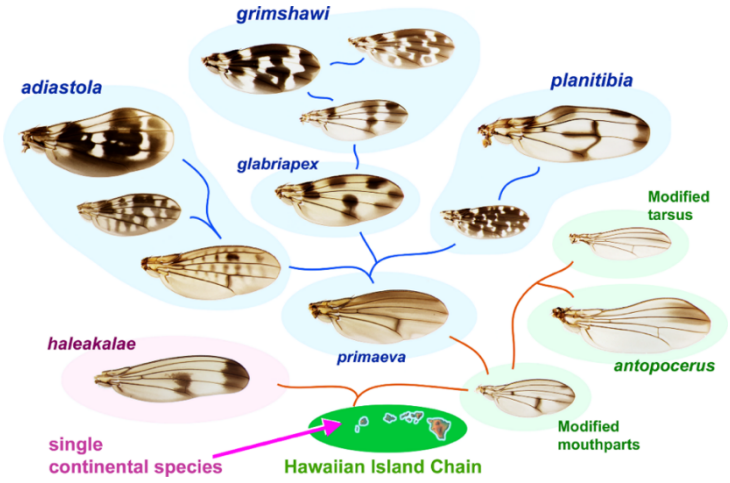


Figure 1. Overview of relationships among major species groups and picture wing subgroups according to Edwards et al. (2007).

Several *Drosophila* species are saprophagous on decaying fruit and can become domestic pests, but only a few species are primary pests that reproduce in fresh fruits. One representative example of the latter

group is *Drosophila suzukii*, the Spotted Wing *Drosophila* (SWD), named after the black maculae on the males' wings (Figure 2).

1.2 *Drosophila suzukii*

Drosophila suzukii is well distinguished from the laboratory model species, *D. melanogaster*, because of the male´s characteristically spotted wing pattern as well as the female´s prominently serrated ovipositor. The latter is a specialized tool that allows oviposition into intact ripe fruit (Lee et al., 2011; Atallah et al., 2014) as illustrated in Figure 2.

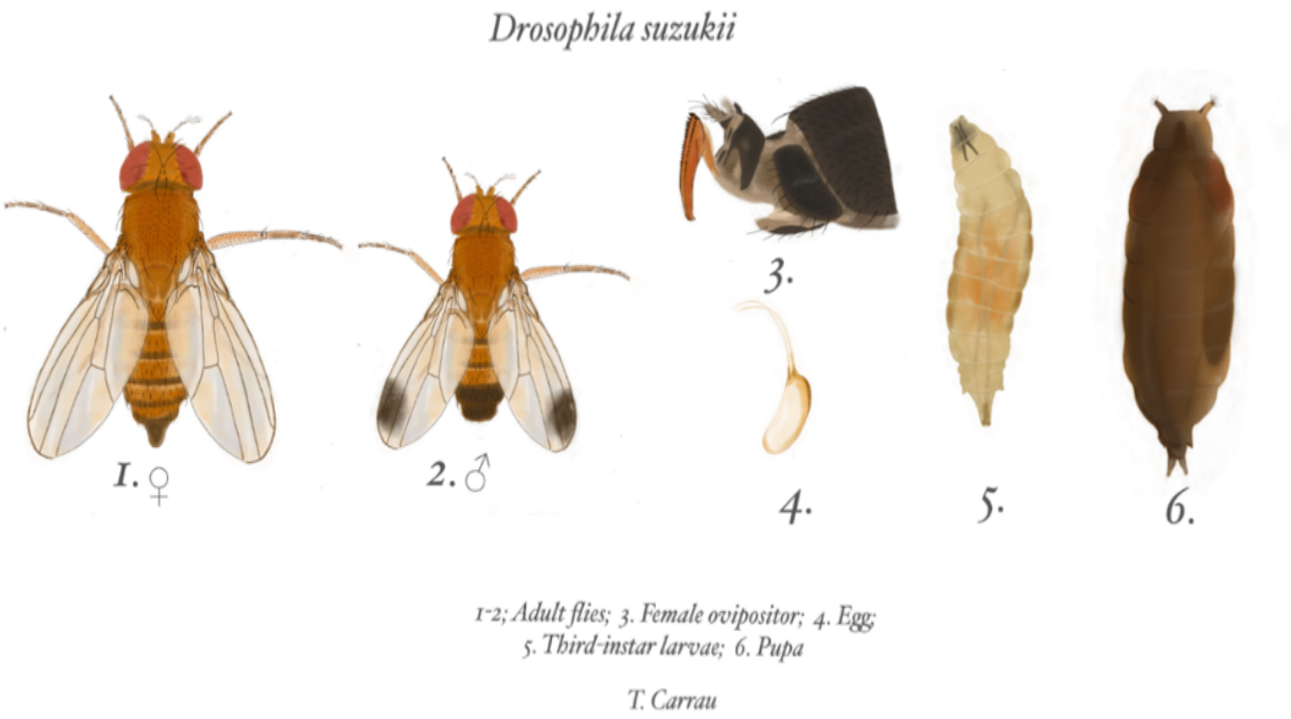


Figure 2. Developmental stages of *Drosophila suzukii*. (**1**) Female and (**2**) male can be differentiated by the two spots in the wings (males) and bigger body size of females. (**3**) The serrated ovipositor is a characteristic feature of this species, used to lay the (**4**) eggs inside of the ripening fruits. Finally, (**5**) larvae develop inside the fruit and rise to the surface to pupate (**6**).

Although this ovipositor is as well shared by a small number of other species from the genus *Drosophila,* only *D. suzukii* has the ability the ability to penetrate a wider range of fruit skins (Atallah et al., 2014). This leads to substantial damage to fruit crops shortly before harvest (Tochen et al., 2014; Wiman et al.,

2014). This situation is even worsened by the pest's high reproduction rate and its rapid lifecycle[1], which includes three larval instars that develop inside the fruit. Newly hatched larvae are well protected under the fruit skin, impeding the pest control with common pesticides. Combined with the rapid acquisition of resistance, chemical insecticides are largely unsuccessful. The development of effective and ecologically safe insecticides against *D. suzukii* is crucial for its control.

Drosophila suzukii was originally an Oriental pest of small fruit crops that was first described as an invasive pest in Hawaii in the 1980´s (Kaneshiro, 1983; Kanzawa, 1939). Following the first record in Spain, *D. suzukii* started spreading northwards through Europe (Asplen et al., 2015; Calabria et al., 2012; Deprá et al., 2014; Walsh et al., 2011). Recently, it has as well been shown to be one of the four most abundant drosophilid species in the Italian region of Apulia (Antonacci et al., 2017).

In parallel, there has been record of the spread of *D. suzukii* across the Neartic and Neo-tropical ecozones with the early detection in 2008 in California (Bolda, 2008). Later its presence was confirmed in the North Eastern states (Maier, 2012). Currently, the spreading of this pest has been recorded from Canada to Argentina as well (Bolda et al., 2010; Deprá et al., 2014; Lue et al., 2017).

The underlying problem associated with this pest is the potential damage that it can cause to commercial soft fruits (Figure 3). Because of the above-mentioned egg laying system and the larval feeding behavior inside, fruits are exposed to opportunistic pathogens (Hiebert et al., 2020). As a result, a substantial crop loss has been reported wherever *D. suzukii* populations have been established (Walsh et al., 2011). As a result, the European horticultural industry has reported up to €8 million of losses in Northern Italy (Wenneker et al., 2015), and $49.8 million in revenue losses accounted for Californian raspberry growers (Farnsworth et al., 2017; Goodhue et al., 2011).

[1]It has a duration of approximately 10 days at 25 ºC and up to 25 days when temperature decreases to 15 ºC (Kanzawa, 1939).

Figure 3. Fungal infection on *Drosophila suzukii*-infected cherries.

1.3 Integrated Pest Management

As the rapid spreading of *D. suzukii* has shown to be a potentially serious economic threat, it is imperative to find an effective and biologically safe control for this pest. Integrated Pest Management (IPM) has arisen in the past years and involves the control of harmful organisms as part as plant protection programs in order to reduce or minimize the risks to human health and the environment (Knipling, 1972). It has been already estimated that the implement of IPM for *D. suzukii* infestations can result in a significant mitigation of economic losses (Del Fava et al., 2017).

The main focus of IPM is to reduce the use of chemical insecticides for pest control, thus lowering possible environmentally associated impacts, managing pesticide resistance and, in general, producing safer insecticides. Strategies such as the introduction of natural predators, entomopathogens and preventive cultural measures, among others, alongside with the responsible use of chemical pesticides are as well part of most IPM programs. Nonetheless, the control of *D. suzukii* appears to be a scientific challenge for the implementation of IPM programs because of its short generation time, wide fruit range and cryptic feeding stages in close-to-harvest fruit combine to hinder conventional control (Medd, 2019).

Currently, IPM solutions for control of SWD are increasing, however including mostly the cultural control, such as applying crop hygiene, increasing the number of weekly harvest or setting commercially available *D. suzukii* traps (Cha et al., 2015; J. C. Lee et al., 2012, 2013; Medd, 2019).

Research on biocontrol of *D. suzukii* has been conducted for more than a decade; however, no breakthrough has been achieved so far (Schetelig et al., 2018). Although biocontrol by natural predators such as parasitoid wasps would basically be an option, preliminary studies under laboratory conditions indicated a high hemocyte load in larvae, pupae, and adults of parasitoid-infected *D. suzukii*. Consequently, these studies have excluded the use of native European parasitoid species such *Leptopilina heterotoma, L. boulardi, Asobara tabida, Pachycrepoideus vindemmiae* and *Trichopria* cf *drosophilae* (Chabert et al., 2012; Kacsoh & Schlenke, 2012; Poyet et al., 2013). However, commercially available hymenopteran species of the genus *Orius*, which consists of small predatory bugs, have already been demonstrated to display some biocontrol potential in SWD-infested strawberry fields (Arnó et al., 2012; Gabarra et al., 2015).

Commercially available microbial biopesticides formulated with entomopathogenic fungi (Woltz et al., 2015) did not lead to any substantial progress in biocontrol of SWD, either. Notably, Hiebert et al. (2020) have recently reported on the susceptibility of *D. suzukii* to isolated bacteria in SWD-infested orchards. However, many insect viruses offer a remarkable potential as host-specific and environmentally friendly biocontrol agents (Hunter-Fujita et al., 1998), thus being excellent candidates for inclusion into existing IPM programs. For most viruses, the titers achieved in the current production process still need to be improved in order to facilitate future commercialization of virus-based insect biocontrol agents (J. B. Carter, 1984; Sun & Peng, 2007).

In spite of the broad diversity of known entomopathogenic viruses, most of the successfully established virus-based insecticides belong to the Baculoviridae (Hunter-Fujita et al., 1998). This family consists of hundreds of species classically divided into two genera: the Nuclear polyhedrosis viruses (NPV's) and the Granulosis viruses (GV's). Both of those genera are well-studied pathogens because of their importance to different fields: they have been established in biocontrol of Lepidopteran pests and as expression vectors in molecular biology (Luckow & Summers, 1988; G. E. Smith et al., 1983; van Regenmortel et al., 2000).

Baculoviridae are double-stranded DNA viruses with a genome between 80 and 200 kb in size. The budded virions (BV's), transmitted from cell to cell, and the occlusion bodies (OB's) are the two extracellular virions that can be found (Granados, 1980). The latter are 0.5 to > 20 μm in diameter and usually visible under a light microscope. Surface lattices protect virions from the environment. A main example of biological control using baculoviruses is the application of *Cydia pomonella* granulovirus (CpGV), first isolated by (Tanada, 1964) for field control of the codling moth, a major pest in apple and pears orchards (Cross et al., 1999).

Additionally, entomopathogenic viruses that are being used in IPM are found in other families. The *Oryctes rhinoceros* nudivirus (OrNV) belonging to the Nudiviridae (Huger, 2005; Wang et al., 2007) has been commonly been used in the Pacific Ocean islands and India for biocontrol of the coconut rhinoceros beetle (*Oryctes rhinoceros*). Although the virus specifically targets the larvae of this beetle, it also exerts a negative impact on the adult beetle's fitness (Gopal et al., 2001; Mohan & Pillai, 1993). Closely related nudiviruses infecting Drosophilidae have been described as well (Webster et al., 2015). The second family, with a promising potential as viral insecticides, is the Parvoviridae. It comprises single stranded DNA viruses, which have first been described by Meynadier (Meynadier, 1964) as entomopathogens of the honeycomb moth (*Galleria mellonella*). Closely related viruses from this family are currently being used for biocontrol of mosquitoes and cockroaches (Carlson et al., 2006; Jiang et al., 2008; Ledermann et al., 2004).

1.4 *Drosophila* natural viruses

Until recently, only eleven drosophilid viruses were known, but only five of them were isolated and sequenced to perform functional assays (*Drosophila melanogaster* Sigma virus [DmelSV], *Drosophila* C virus [DCV], *Drosophila* A virus [DAV], Nora Virus and *Drosophila* X virus [DXV]) (Brun & Plus, 1980; Huszar & Imler, 2008).

The first characterized and studied drosophilid virus was DmelSV from the Rhabdoviridae (Berkaloff et al., 1965; Teninges, 1968; Teninges et al., 1993). It was described more than 80 years ago because of an unusual CO_2 sensitivity of infected *D. melanogaster* flies (L'heritier & Teissier, 1937). This virus

was confirmed for other drosophilid species as well (Longdon et al., 2010). It can be transmitted vertically and *via* injection.

The next virus to be isolated was DCV (Jousset et al., 1972) that is closely related to the Cricket Paralysis virus (CrPV), both belonging to the Dicistroviridae family (Scotti et al., 1981). DCV is known to be transmitted orally, causing a systemic infection that leads to intestinal obstruction (Chtarbanova et al., 2014). In contrast, the CrPV is a lethal virus that was described to cause cytopathic effects in *D. melanogaster* (Scotti, 1975). *Drosophila* X virus (DXV), a member of the family Birnaviridae, was described to infect *D. melanogaster* cell lines and was also found as a virus contaminating other cell lines. Although intensive studied; so far it has not been described to cause natural infections in wild drosophilids (Adams & Bonami, 2017; Dobos et al., 1979).

The P virus of *Drosophila* was first described by Plus & Duthoit (1969). It possesses an icosahedral capsid and seems to be endemic in many *Drosophila* populations. David & Plus (1971) observed that naturally infected flies show little to no effect; however, when injected, female sterility and reduced lifespan are the main consequences. Further characterization was performed by Teninges & Plus (1972) who classified it as a member of the Picornaviridae.

DAV has been described to cause natural infections in drosophilids, being less pathogenic than DCV. It was recently characterized by Ambrose et al. (2009) showing unusual morphological and molecular features that delimitates this virus from morphologically closely related RNA viruses. Finally, the *Drosophila* Nora virus, a picorna-like virus, is a non-enveloped RNA virus. It is transmitted horizontally, causing negative impact on the fitness of infected flies (Habayeb et al., 2009).

Recently established metatranscriptomic techniques remarkably facilitate the search for novel viruses. Webster et al. (2016, 2015) described up to 50 new viruses that are associated with the genus *Drosophila*. These authors used next generation sequencing to perform an epidemiological survey including 2000 individuals of wild drosophilidids. It was found that 30% of the flies carried at least one virus species, and 6% of flies were co-infected with more than one virus species. Using a similar approach, Medd et al. (2018) described another 18 new RNA viruses associated with *D. suzukii*.

So far, very little is known about the host-virus adaptation of *D. suzukii*; and no functional studies have been conducted to examine *in vivo* interactions between the recently described drosophilid viruses and *Drosophila suzukii*. Lee & Vilcinskas (2017) have recently shown that *D. suzukii* is susceptible towards

natural viruses of other drosophilids[2]. Additionally, the molecular mechanisms for virus-host adaptation in *D. suzukii* are still unknown.

Recently, the family Iflaviridae that encompasses viruses found in hemipteran, coleopteran and lepidopteran insects (Liu et al., 2017; Smith et al., 2014; Sparks et al., 2013) has regained attention after identification of novel viruses infecting the notorious pest *D. suzukii* (Webster et al., 2015). Viruses present in this family possess a positive-sense, single-stranded RNA genome. So far, functional studies of Iflaviruses of *D. suzukii* have not yet been reported. RNA viruses share characteristics such as their rapid adaptation to the host, which is a key of their evolution and success. This is mainly because their mutation rate per nucleotide site, is in the range of 10^{-3} to 10^{-5} for a 10-kb genome, ensuring an average of 0.1 to 10 mutations in each RNA molecule (Domingo & Holland, 1997; Drake et al., 1998). Leading to a better adaptability of viruses in the event of environmental changes. Likewise, viruses may be able to respond in a nearly deterministic fashion to some selective pressures (Domingo & Holland, 1997). Resistance to neutralizing antibodies (Borrego et al., 1993; Vandepol et al., 1986) or to antiviral inhibitors are some examples of RNA-virus adaptation.

1.5 Antiviral immunity in *Drosophila*

In insects, the innate immune system is the unique response to pathogens, this differs from the adaptive response solely found in vertebrates. The immune system of *Drosophila* includes an antiviral response, which is highly conserved in a way that is comparable to other vertebrate and invertebrate immune systems. The innate antiviral response is a cluster of different events that can occur altogether when the insect is exposed to a viral pathogen. It includes signal molecules, transcription factors, signal transduction pathways and cascades that are activated through different pathways: Toll pathway, immune deficiency pathway (IMD), JAK/Stat pathway, Toll-7 autophagy pathway, transcriptional pausing pathway and the RNA interference (RNAi) pathway (Sabin et al., 2010).

In order to identify a potential threat, the *Pattern Recognition Receptors* (PRRs) recognize conserved viral components, such as viral glycoproteins and viral genetic material, known as *Pathogen-Associated Molecular Patterns* (PAMPs) (Takeuchi & Akira, 2010; Zambon et al., 2005). The PRRs can be further

[2] Such as DCV, FHV and CrPV.

subdivided into two groups, depending on their location in the cell: the first group comprises membrane-bound sensors, such as Toll-like receptors or C-type lectin receptors; whereas the cytoplasmic sensors, such as retinoic acid-inducible gene-like receptors (RIG-I-liker receptors) or nucleotide-binding oligomerization domain-like receptors (NOD-like receptors) form the second group (Akira et al., 2006).

The PAMPs signals, induced by a pathogen recognition, trigger an activation of the above two pathways. Subsequently, a transcriptional activation starts in order to induce effector molecules to suppress the pathogen. In *Drosophila,* the Toll and IMD are two of the most studied signaling pathways, both relaying on the nuclear factor-κB (NF-κB). Both pathways are associated with the bacterial and fungal defense, which finally lead to the production of antimicrobial peptides (AMPs) to suppress infections. However, the Toll pathway in *Drosophila* has recently been described to be activated by DXV infection (Ramirez & Dimopoulos, 2010; Xi et al., 2008). Likewise, the IMD pathway, sharing several conserved components with the mammalian tumor necrosis factor receptor (TNFR), has also been implicated in antiviral defense against CrPV (Costa et al., 2009; Myllymäki et al., 2014), as illustrated in Figure 4.

The JAK/STAT signaling pathway is another important component of the *Drosophila* antiviral defense. It is composed of ligands (unpaired (*upd1*-3)) that bind to the so-called domeless receptor (*Dome*) and signals triggered by the kinase JAK (*Hopscotch*/*Hop*). The latter transcribes the factor STAT (*STAT92E*/*Marelle*), which results in the production of the antiviral *vir*-1, amongst other antimicrobial effectors, as illustrated in Figure 4 (Xu & Cherry, 2014). In *Drosophila, vir*-1 has been described to be upregulated when flies are infected with DCV and FHV (Dostert et al., 2005; Hedges & Johnson, 2008).

Autophagy has been identified to play a crucial role against Vesicular Stomatitis Virus (VSV) in *Drosophila* (Shelly et al., 2009). Degradation mechanisms are activated in the flies' cells after viral infection to control the replication. The viral recognition for the repression of the Akt signaling still has not been described; however, it is known that this mechanism is activated via attenuation of the PI3K/Akt pathway (Sabin et al., 2010) as shown in Figure 4.

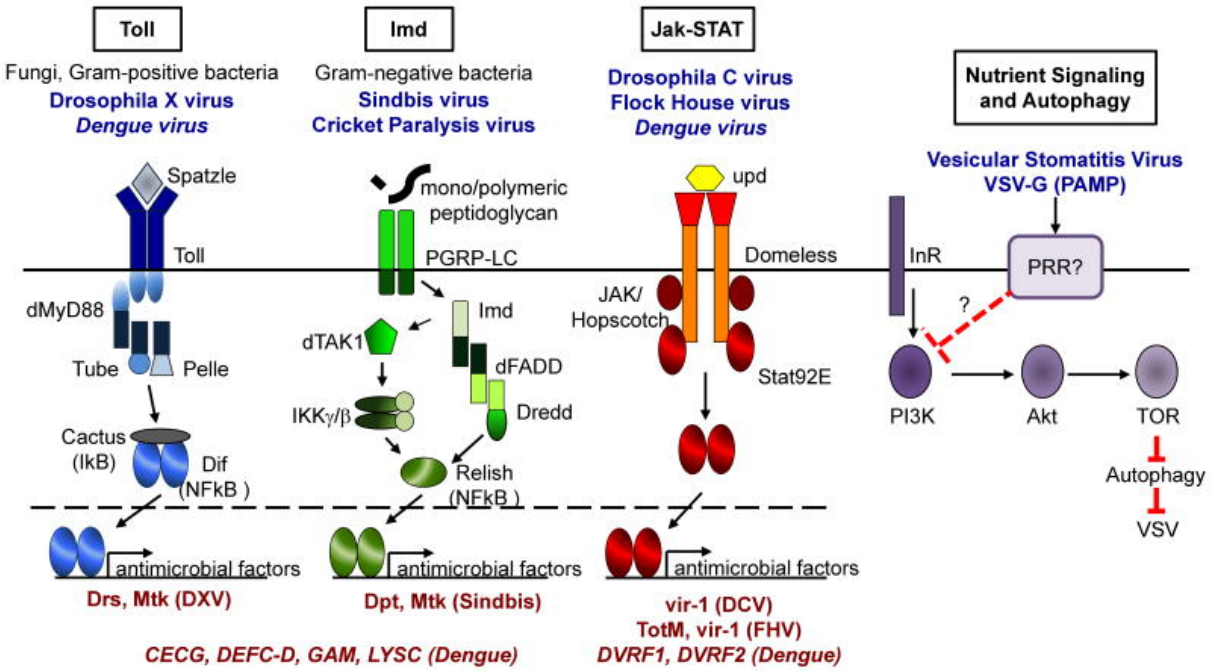


Figure 4. Schematic figure of the antiviral innate immune signaling in insects. Four pathways including three classical immune signaling pathways (Toll, Imd, Jak-STAT) are responsive to infection by different viruses (blue text) based on studies in both *Drosophila* and mosquitoes (italics). The Toll pathway, responsive to fungi and gram-positive bacteria, has been found to be antiviral in response to infection by *Drosophila* X and Dengue viruses. The Imd pathway, responsive to gram-negative bacteria, is anti-viral in response to infection with Sindbis and Cricket Paralysis viruses. The Jak-STAT pathway restricts infection by *Drosophila* C and Dengue viruses. Some downstream effectors were induced by infection (red text, italicized for studies in mosquitoes). In addition, *Drosophila* recognize VSV via the glycoprotein VSV-G (the PAMP), through an unidentified PRR which leads to attenuation of nutrient signaling, likely at the level of PI3K. Repression of this pathway results in the induction of antiviral autophagy which attenuates VSV replication. Figure from Sabin et al. (2010)

Finally, the most important antiviral strategy in *Drosophila* is the RNAi pathway (Obbard et al., 2006; Zambon et al., 2006). The latter includes the small-interfering (si) RNA pathway, the micro (mi) RNA pathway and the piwi-interacting (pi) RNA pathway (Kim et al., 2009). In insects, siRNA pathway has been investigated most thoroughly. It initiates when the cytoplasmatic receptors bind to the viral dsRNA (Ding & Voinnet, 2007; Saleh et al., 2009). Then, the siRNAs are loaded to the *RNA induced silencing complex* (RISC) guiding the Argonaute slicing enzyme to complementary sequences of viral RNA, which are, in turn, cleaved, thus preventing viral replication. This is illustrated in Figure 5 (Attarzadeh-Yazdi et al., 2009; Poirier et al., 2018; Saleh et al., 2009; Tassetto et al., 2017).

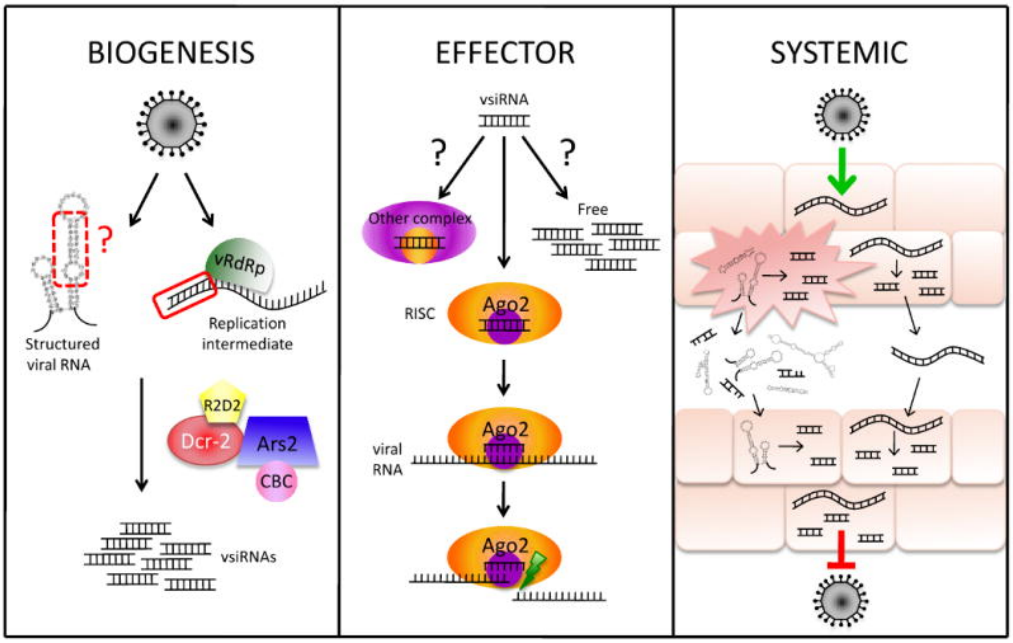


Figure 5. Antiviral Immunity in the Fruit Fly, *Drosophila melanogaster*. The biogenesis of virus-derived siRNAs (vsiRNAs) is mediated by Dcr-2, which recognizes dsRNA produced by the viral RNA-dependent RNA polymerase (vRdRp) or structured regions of single stranded viral RNA (left panel). Dcr-2 complexes with R2D2 and Ars2, which is required for efficient dsRNA processing and interacts with the nuclear *cap*-binding complex (CBC) (left panel). Following vsiRNA biogenesis, Ago2 mediates the effector step of antiviral silencing through RISC-mediated cleavage of viral RNA (middle panel). The vsiRNAs not bound to Ago2 remain stabilized, either in another complex or free in the cytoplasm. A potential model for systemic antiviral RNAi is depicted at right. Viral RNA produced during infection is released extra-cellularly, either by controlled export or by lysis of the infected cell. The RNA is then internalized and processed by uninfected cells, protecting them from subsequent infection. Figure from Sabin et al. (2010)

1.6 Cell culture and viruses

To the best of our knowledge, no cell lines are available for the notorious fruit pest SWD. One strategy to control invasive pests such as *D. suzukii* is to find new ways to target the immune system, rendering the flies or their larvae more vulnerable to pathogen or parasite attack (Schetelig et al., 2018). Due to the high host specificity of insect viruses, the development of host-specific cell culture systems is required for scale-up processes. In addition, the establishment of cell lines such as embryonic permanent lines and the subsequent isolation and study of hemocytes will not only provide a key to the specific virus replication for future insecticide production, but also contribute to the understanding of insect cellular immunity.

1.6.1 The decisive role of hemocytes in the immune response

Hemocytes are an active part of the immune response against viruses in *Drosophila.* Qiu et al. (1998) were the first to describe that the Toll and the JAK/STAT transducer have implications in the activation of these cells (Harrison et al., 1995; Luo et al., 1995; Morin-Poulard et al., 2013). Despite this, the activation of some of these pathways remain unclear. Hemocytes also secrete a variety of opsonins and other AMPs, such as *upd3* that activates JAK/STAT signaling in the fat body (Agaisse et al., 2003). Recently, a new element has been added to this response. In this case, a form of systemic memory is exhibited, in which hemocytes endogenize fragments of RNA virus as DNA copies, and these endogenous copies form a source of secondary viral siRNAs (Tassetto et al., 2017).

Hematopoiesis in *Drosophila* occurs in different ontogenetic stages, generating two hemocyte populations: the first one arises from the head mesoderm during early embryogenesis and the second from the mesodermal lymph glands at a later stage of development (Traver & Zon, 2002). Differentiation of the embryonic hemocytes (EH) occurs during the final stage of embryogenesis, hemocytes known as plasmatocytes, i.e. small spherical cells with phagocytic capacities, originate in the procephalic mesoderm and migrate to colonize the entire embryo, making up the majority of all hemocytes in arthropods. Plasmatocytes act as macrophages by recognition, thus leading to phagocytosis, which eliminates microorganisms and apoptotic cells (Banerjee et al., 2019; Franc et al., 1996, 1999; Lavine & Strand, 2002; Tepass et al., 1994). Crystal cells differentiate at around the same time near the anterior region of the gut (Lebestky et al., 2000). They are involved in the melanization of pathogens and take part in innate immune response, by producing free radicals (Meister & Lagueux, 2003). Finally, the lamellocytes represent a third independent hemocyte lineage, which is present in small amounts in healthy larvae, as illustrated in Figure 6. The lamellocytes are large, flat cells that encapsulate invading cells that are too large to be phagocytosed by plasmatocytes (Meister & Lagueux, 2003). However, they are significantly induced when the insect is infected with pathogens. This occurs, for instance, in response to parasitoid wasp attack (Banerjee et al., 2019).

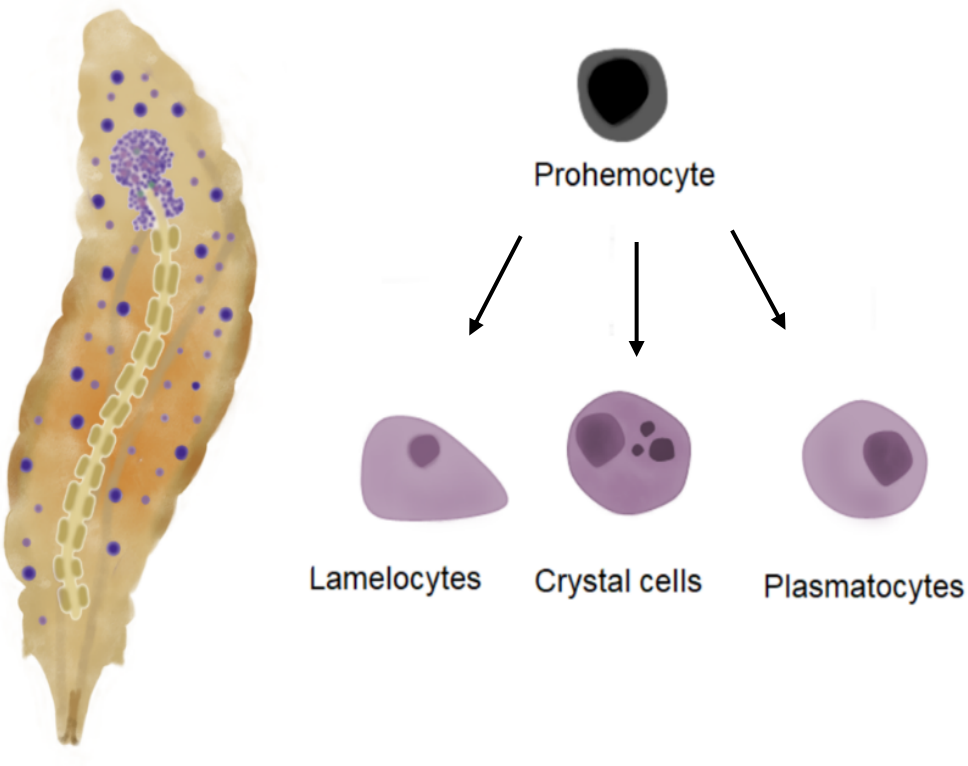


Figure 6. Schematic hematopoiesis in a *Drosophila melanogaster* larva, based on Tepass et al. (1994)

2 Project Goals

Because of the global economic threat *D. suzukii* poses for farmers, it is imperative to find an adequate pest control strategy. Current methods are often based on the use of synthetic organic pesticides that also harm non-target organisms because of their rather unspecific mode(s) of action, thus contributing to the dramatically decreasing biodiversity of beneficial insects. Therefore, the main goals of this thesis are the screening for natural viruses pathogenic to SWD and the development of an eco-friendly, virus-based biological insecticide to control this emerging pest.

2.1 Goal 1: Isolation of naturally infected specimens and virus identification

This part of the thesis is aimed at establishing a protocol for the collection of dead and moribund larvae followed by identification, isolation and storage of natural pathogens that can potentially be used for biocontrol of SWD. In order to ensure a more or less constant supply of larvae for pathogen isolation, field work was necessarily restricted to the seasonal peak in abundance of *D. suzukii* in its natural habitats, such as cherry orchards or strawberry fields. In the case of viruses, each larva will be homogenized, and RT-PCR will be performed for a panel of specific drosophilid viruses.

2.2 Goal 2: Characterization of potential viral candidates for biocontrol of *D. suzukii*

After identification of the viral candidates for the biocontrol of *D. suzukii*, virus production *in vivo* will allow the specific isolation and purification. The use of sucrose and/or CsCl cushion will facilitate the isolation of pure viral particles. These will then be characterized morphologically and genetically. Finally, they will be tested to assess the natural susceptibility of the target organisms.

2.3 Goal 3: Biological characterization of LJV and cell culture

After selection of the viral candidates for biocontrol agents (BCA) that were previously isolated and characterized (see Goal 2), the susceptibility of the target organism will be studied. This is usually carried out by performing biological investigations of the virus such as survival assays, fitness studies, and

estimations of virus reproduction. Additionally, the development of a standardized protocol for the establishment of cell cultures of the desired target organism will be part of the project; hence, it will facilitate working strategies targeting the immune system of SWD as well as virus mass production.

3 Materials

Table 1. List of chemicals

Substance	Supplier	Product number
2-Propanol, Rotisolv®	Carl Roth	AE73.1
2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI)	Sigma Aldrich	5087410001
Agar-Agar, BioScience Grade	Carl Roth	6494.1
Alexa Fluor™ 488 Phalloidin	Thermo Fisher	A12379
Chloroform	Carl Roth	3313.1
Ethanol, Rotipuran	Carl Roth	9065.1
Ethyl 4-hydroxybenzoate, 99%	Alfa Aesar	A13172
Exo-SAP	Thermo Fisher	78205.10.ML
Glycerol	Carl Roth	7530.1
GoTaq® G2 DNA Polymerase	Promega	M7841
Grace's Insect Media	Sigma Aldrich	G9771-1L
iScriptcDNA Synthesis Kit	Bio-Rad	170 8 8 91
Isoamyl Alcohol	Carl Roth	8930.1
JM109 Competent Cells	Promega	L200A
LB-Agar (Luria/Miller)	Carl Roth	X969.3
PEG	Abcam	ab102538
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher	15140122
pGEM®-T Easy Vector Systems	Promega	A1360
Propionic acid	Carl Roth	6062.2
Proteinase K	NEB	P8107S
REDTaq® DNA Polymerase	Merk	D4309-250UN
RNase A	Thermo Fisher	EN0531
RTL Lysis Buffer	Qiagen	79216
Schneider′s Insect Medium	Sigma Aldrich	S0146
Sugar syrup	Goldsaft	01901
Syber ® Safe DANN gel stain	Ivitrogen	S33102
TRI Reagent	Zymo Research	R2050-1-200
Tris(hydroxymethyl)aminomethane (TRIS), PUFF-ERAN®	Carl Roth	5429.1

Table 2. List of consumables

Material	Supplier
Drosophila tubes PS, 50ml, Ø28,5x95 mm,	Nerbe plus
15 mL Cellstar® Polypropylene Tube	Greiner
25 cm cell culture Flask	Thermo Fisher
25 cm cell culture Flask (with filter)	Thermo Fisher
50 ml CELLSTAR® Polypropylene Tube	Greiner
6-well plate	Thermo Fisher
96 PCR plate	VWR
96 qPCR plate	Thermo Fisher
AMPure XP beads	Beckman Coulter
Capto™ Core 700 C	Sigma Aldrich
Ceramic beads	OMNI International
Eppendorf tubes	Sarstedt
Foam stoppers for Drosophila tubes PS, 50ml, Ø28,5x95 mm,	nerbe plus
Micro tube 2 ml	Sarstedt
Nanopore flowcells	Nanopore tech
Nunc™ Lab-Tek™ II Chamber Slide™ System	Thermo Fisher
Parafilm® M	Carl Roth
PCR stripes	VWR
Petri Dishes	Greiner
Rotilabo ® folded filters, type 600P	Carl Roth

Table 3. List of devices

Machine	Model	Company
Autoclave	MediaClave	Integra
Bioinformatics Software for Sequence Data Analysis	Geneious v10.2.251	Geneious
Biosafety cabinet	Safe FAST Elite	Unity Lab
Cell counting chamber	Neubauer chamber	EMS
Cell counting chamber	Thoma chamber	EMS

Centrifuge	5415R	VWR
Disruption instrument	FastPrep® System	Mpbio
Confocal microscope	SP8	Leica
Freezer -20°C	K12023S-3	Miele
Freezer -80°C	FDE50086FV	Thermo Fisher
Fridge 4°C	Refrigerator BioCompact RR210V2A	Fleisch
Gel Documentation Station	Dark hood dh-40/50	Biostep
Gel Electrophoresis Chamber	PowerPac™ Basic Power Supply	Biorad
Inverse Microscopy	FLoid ® Cell Imaging Station	Thermo Fisher
Long-Read sequencing Technology	Oxford Nanopore Technologies's (ONT) MinION	Nanopore tech
Micro-volume spectrophotometer	NanoDrop ND-2000	PEQLAB
PCR cycler	Eppendorf® Mastercycler® Pro Thermal Cyclers	Merk
Pipette	20 µl/ 50µl / 100 µl/ 200 µl / 1000 µl	Eppendorf
Real-time PCR cycler	StepOnePlus ® Real-Time PCR System	Thermo Fisher
Shake Incubator	MaxQ 8000	Thermo Fisher
Swing-bucket rotor	SW42	Beckman
Thermoshaker	Thermomixer comfort	Eppendorf
Vortex	Vortex Genie 2	Scientific industries, Inc.
Water purfication	Milli-Q® HX 7000 SD	Millopore

4 Methods

4.1 *Drosophila suzukii* rearing

During this four-year thesis, six different *D. suzukii* strains were maintained, originating from Italy, USA, Canada (Ontario), and Germany (Ockstadt, Giessen and Kriftel). The latter three were established in the laboratory as described in the Results section 5.1.1 (see page 35). However, for the following experiments, only the Canadian line has been used, which was regularly checked for viral and bacterial contamination such as *Wolbachia,* primers listed in Supplementary Table S1. All *D. suzukii* strains were maintained under a controlled environment at 26°C and 60% humidity with a 12 h photoperiod. The insects were reared on a medium consisting of soybean and cornmeal (10.8% [*w*/*v*] soybean and corn-meal mix, 0.8% [*w*/*v*] agar, 8% [*w*/*v*] malt extract, 2.2% [*w*/*v*] molasses, 1% [*w*/*v*] nipagin, 0.625% propionic acid). Flies feeding on this particular medium are illustrated in Figure 7. All experiments were carried out using only female flies that were staged to be between 3 to 7 days old.

Figure 7. *Drosophila suzukii* food vial containing adult flies.

4.2 Virus screening

4.2.1 Sampling area and fruit collection

To collect natural pathogens, sampling was planned to coincide with the best environmental conditions for *D. suzukii*. Infested fruits with larvae were sampled in Ockstadt (50.322439, 8.72147) at ~11 a.m. on the 18th of August, 2016 (20.2°C and 80% humidity); Kriftel (50.092323, 8.472857) at ~11 a.m. on the 19th of September 2016 (18 °C and 74% humidity) and Giessen (exact location is unavailable because samples were provided by a colleague) on the 26th of September 2016 (17 °C and 80% humidity). A second sampling was performed at the same Ocktstadt site on August 3, 2017. All three sampling sites are located in Hesse, Germany, and are widely known for their high number of cherry and raspberry fields (Figure 8-A).

A total of 270 cherries, blackberries and raspberries were collected in the course of four sampling events. Of all sampled fruit, 172 cherries were collected in Ockstadt; 28 blackberries and 34 raspberries were kindly provided by a colleague from Giessen, and 17 blackberries and 2 raspberries were collected in Kriftel. As soon as they were collected, all samples were stored in individual tubes until further laboratory work. Fruit were carefully inspected under a binocular microscope for *D. suzukii* larvae, their health status was recorded and sorted into categories accordingly. Morbidity was defined as the proportion of individual larvae with melanization, flaccid appearance, intersegmental body extrusions and slow movement. Sampling units were then established of 1-5 larvae, which were then homogenized in 50 µl sterile distilled water (dH_2O) with a pellet pestle. The homogenate was mixed with 450 µl Tris-HCl (10 mM, pH 7.2) and passed through a 0.22 µm membrane to remove bacteria while allowing viruses to pass into the filtrate. The cleared isolates were stored at - 80°C (Figure 8-B).

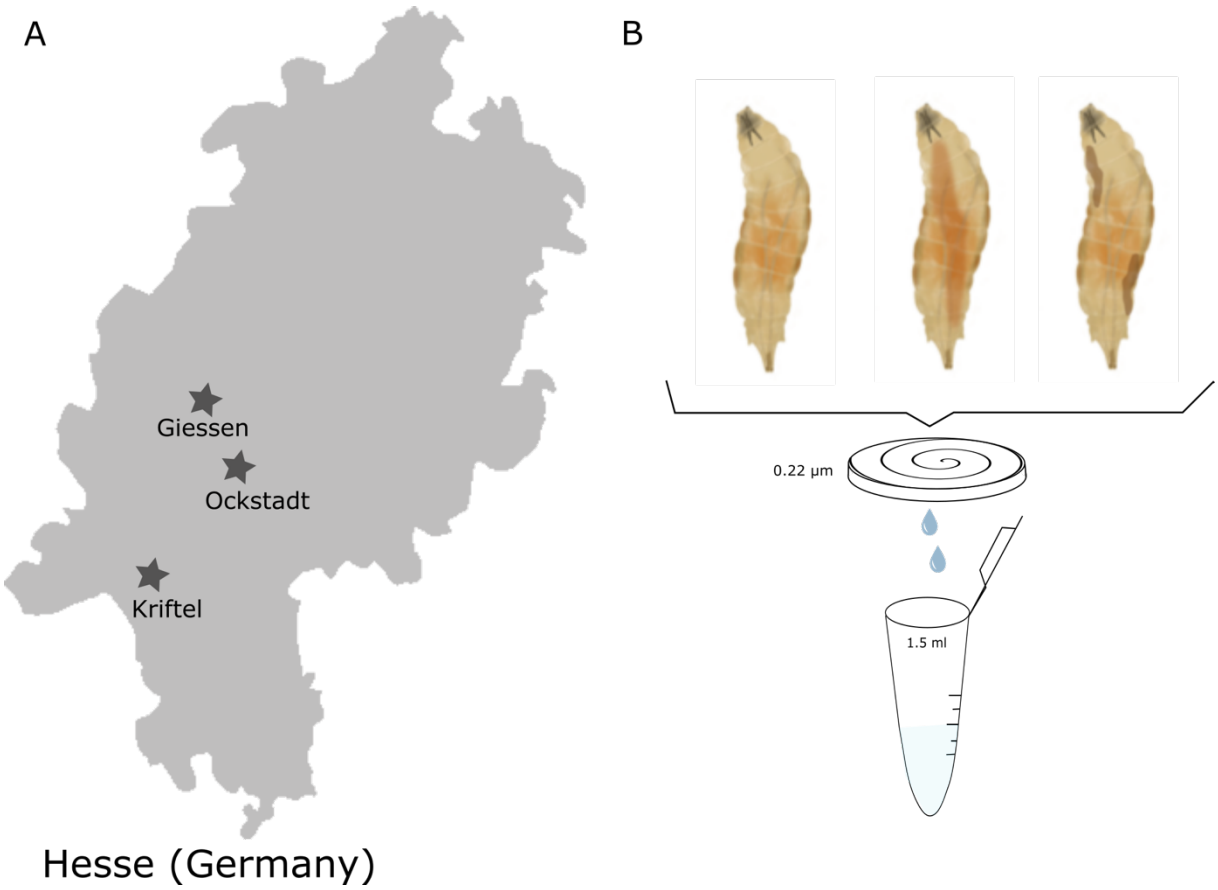


Figure 8. Provenance of *D. suzukii* samples (**A**) and workflow for processing of the isolated larvae from infected cherries (**B**). Larvae were homogenized, and this homogenate was filtered through a 0.2 µm membrane to produce virus-containing isolates. All samples were stored at -80°C until further experiments were performed.

4.2.2 Virus identification

In order to identify *D. suzukii* viruses, an individual sequencing assay was performed for each isolate. For this purpose, 46 nl/fly of each sample (n = 57) was injected in 1 g of female flies (~1,000 insects) and potential viruses were allowed to precipitate using a polyethylene glycol (PEG) precipitation kit (Abcam), following the manufacturer instructions. Subsequently, RNA was isolated from individual pellets, and its quantity and quality were measured with an Agilent 2100 bioanalyzer. In a nutshell, this technique performs a gel electrophoresis through a microfabricated chip, where electrical voltages are applied to individual electrodes, each of which is connected to a separate high voltage power supply. Samples are separated electrophoretically, and the Bioanalyzer software automatically calculates size and concentration of each separated band, displaying the results in real-time (Gottwald et al., 2001).

For long-read sequencing, Oxford Nanopore Technologies's (ONT) MinION (Figure 9) and flow cells were used. To prepare sequencing libraries for the MinION, 250 ng of RNA was reverse-transcribed

using RNA-specific VN primers[3] that were complementary to the 3'-end of the respective genome. Excess primers were removed from the PCR product using AMPure XP beads (Beckman Coulter). Then, the cDNA library was loaded onto a MinION MkIB device equipped with an XX flow cell. The MinKNOW control software was used to select a 48-hour sequencing protocol and was allowed to proceed for at least 4 hours, until high-quality data accumulation ceased.

Figure 9. MiniOn portable, real-time device for sequencing (Nanopore Technologies), used for long-reads sequencing of the collected samples.

Illumina Technologies for short-read sequencing was also performed in order to achieve a wider range coverage, thus gaining a better view of the virus epidemiology. Finally, confirmation with RT-PCR was performed for all samples collected using the primers listed in Supplementary Table S2.

4.3 Genomic virus characterization

4.3.1 Virus purification

Up to 1500 female flies were intrathoracically injected using 46 nl/fly of the viral solution described in the Results section 5.3 (see page 39). The flies were incubated for 3 days under standard rearing conditions as described above. Flies were anesthetized briefly with CO_2, collected in a 50 ml tube and euthanized by freezing at -20°C overnight. The flies were homogenized and this homogenate was cleared by centrifugation (3.000 × g for 20 min at 4°C). The clarified lysate was through a 0.22 μm membrane

[3] Oligo d(T)23 VN is used for the priming and sequencing of mRNA adjacent to the 3'-poly A tail Khan et al. (1991). V = A or G or C; N = A or G or C or T.

and concentrated by ultra-centrifugation through a 20% (*w*/*v*) sucrose cushion. The pellet was resuspended on an orbital shaker overnight and loaded onto a 1.0-1.5 g/ml CsCl density gradient. After centrifugation at 30,000 rpm in SW41Ti rotor (Beckmann) for 20 hours, the vial was inspected with illumination from below to visualize density bands.

4.3.2 La Jolla Virus nucleotide sequence determination

RNA of the LJV virus stocks was analyzed by Illumina sequencing to obtain a consensus sequence and to exclude the presence of contaminating viruses in the stocks. Furthermore, pathogen specific RT-PCRs were used to test for known drosophilid viruses. Total RNA from virus suspensions was extracted using the QIAamp RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was carried out using the OneTaq One-Step RT-PCR Kit (NEB, Ipswich, USA) or the One Step RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Several primer pairs (Supplementary Table S3) that were hybridizing with the LJV sequence MH384278 from GenBank were designed. Resulting PCR amplicons with suitable length were purified using the Monarch PCR purification Kit (NEB, Ipswich, USA), and sequenced by a commercial provider (Eurofins Genomics, Ebersberg, Germany) using the PCR primers. A 5'-Rapid amplification of cDNA ends (RACE) protocol was adapted using the terminal deoxytransferase (TdT; NEB, Ipswich, USA). Briefly, first strand cDNA was synthesized from total RNA of a virus concentrate with the LJV genome specific primer LJV_Part_1 and the Omniscript RT Kit (Qiagen, Hilden, Germany). The cDNA was precipitated with ethanol and poly-A or poly-C tailed using TdT. Another genome specific primer (LJV_RACE_1) was used in conjunction with a primer containing oligo-dt / oligo-dg and adapter sequences (T1/T2) to amplify the LJV 5'-end. Finally, a nested PCR was performed using primers hybridizing with adapter (T22) and 5'-terminal LJV sequences (LJV_RACE_2). DNA fragments belonging to LJV were further sub-cloned in the pGEM-T easy vector (Promega. Madison, USA). The sequences of the LJV-Ds-OS20 strain was submitted to GenBank with the entry number **MW556743**. An alignment was constructed with the sequence from the LJV-ORF and 56 sequences from GenBank corresponding to the species LJV. The sequences were aligned in Geneious v10.2.6 (https://www.geneious.com/), using the ClustalW algorithm, and manually edited to correct possible errors.

4.3.3 LJV phylogenetic analysis

Nucleotide sequences were aligned with Geneious v.10.2.6 (https://www.geneious.com). The phylogenetic tree was constructed using the neighbor-joining method with a ClustalW alignment (Thompson et al., 1994), using Geneious v.10.2.6 (https://www.geneious.com). The nucleic acid sequence of the vRdRp (483 bp) gene of different published full-length LJV sequences from Drosophilids (n = 18) and from *Apis mellifera* (n = 1) and the vRdRp of *Apis mellifera* deformed wing virus (DWV) (n = 1) as outgroup were aligned. The alignments were edited manually where necessary, using the conserved protein domains as a guide. Bootstrap analyses were based on 1,000 replicates.

4.3.4 Morphological characterization

4.3.4.1 Electron Microscopy

For Electron Microscopy (EM) imaging, viral particles were spotted onto carbon-coated copper palladium 400-mesh hexagonal grids and incubated for 1 min. The grids were rinsed twice in drops of water and blotted dry, and were then stained with 2% uranyl acetate. The grids were imaged using a Philips Biotwin 120 kV electron microscope and images were captured using a SIS Olympus Keenview camera.

4.3.4.2 SDS page

Samples were denatured by heating at 95°C for 5 min in the presence of Dithiothreitol (DTT) before separation by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using SDS 4–20% Criterion™ TGX Stain-Free™ protein gels (BioRad). The samples were separated at 250 V for 25 min.

4.3.4.3 *N*-Edman degradation and mass spectrometry

For 1-D gel electrophoresis, the protein samples were directly subjected to 10-12.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. For 2-D gel electrophoresis, the protein samples were solubilized in 6 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and 2% Pharmalyte 3–10. IPG-strips (pH 3–10) were rehydrated, the protein solution added, and the isoelectric focusing (IEF) was performed using 32.05 kVh. After focusing, the IPG-strips were equilibrated for 10 min in 2 ml equilibration stock solution (ESS; 6 M urea, 0.1 mM EDTA, 0.01% bromphenol blue, 50 mM Tris-HCl pH 6.8, 30% glycerol) and subsequently incubated for 15 min in 2 ml ESS I (10 ml ESS containing 200 mg SDS, 100 mg DTT) followed by an incubation for 15 min in ESS II (10 ml ESS containing

200 mg SDS, 480 mg iodacetamide). Second dimension separation was performed by 12.5% SDS-PAGE, the proteins were transferred to a PVDF membrane, and stained using Coomassie brilliant blue R-250. *N*-terminal amino acid sequencing was performed by Edman degradation (ED). The individual protein bands and spots were excised from the membrane and analyzed by an ED device. For matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), the selected spots were digested after reduction and carbamidomethylation with trypsin. MALDI-TOF-MS analysis was performed on an Ultraflex TOF/TOF mass spectrometer equipped with a nitrogen laser and a LIFT-MS/MS facility. The instrument was operated in the positive-ion reflectron mode using 2,5-dihydroxybenzoic acid and methylendiphosphonic acid as matrix. For data processing and instrument control, the Compass 1.4 software package consisting of FlexControl 3.4, FlexAnalysis 3.4 and BioTools 3.2 was used. Proteins were identified by MASCOT peptide mass fingerprint search (http://www.matrixscience.com) using the NCBInr and Uniprot databases as well as the translated open reading frame (ORF) sequence from the LJV genome. For the search, a mass tolerance of 75 ppm was allowed and carbamidomethylation of cysteine as global modification and oxidation of methionine as variable modification were used. A false positive rate of 5% was allowed.

4.4 Biological virus characterization

4.4.1 Survival analysis

Three-day-old to seven-day-old post-eclosed females were infected by the intrathoracic injection of 46 nl/fly of a viral suspension in Tris-HCl (10 mM, pH 7.2) as described above and survival experiments were carried out using three replicate cohorts of 20 females each. Three biological replicates for each condition were performed. Survival curves were plotted and analyzed by log-rank analysis (Kaplan-Meier Method). *P* values lower than 0.05 were considered statistically significant: $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$. All analyses were carried out using RStudio software version 1.2.5033.

4.4.1 LJV *in vivo* replication

For the quantification of virus loads in insect homogenates and virus suspensions, quantitative real-time RT-PCRs were performed on an ABI 7500 cycler (Applied Biosystems, Foster City, USA) using the

LJV specific primers listed in the Supplementary Table S4 together with the Luna universal probe one-step RT-qPCR kit (NEB). A plasmid harboring the cDNA target sequence from LJV behind a SP6-promoter was linearized with MluI, gel-purified and spectrophotometrically quantified. A synthetic RNA-fragment was transcribed from the cDNA template using the HiScribe SP6 RNA synthesis kit (NEB), purified with the peqGOLD total RNA kit including the DNA removal step and quantified spectrometrically. In order to obtain a standard curve, a ten-fold dilution series of the RNA control was included in the qRT-PCR setup.

Genome copies were calculated by 7500 System SDS Software (Applied Biosystems) based on the standard curve. Adult females, pupae and larvae from *D. suzukii*, were injected intrathoracically injected using 46 nl of the diluted LJV isolate containing 10^{10} genome equivalents (GE) per ml (GE/ml). After three days of incubation at 26 °C, the adult flies were anesthetized using CO_2 and euthanized by freezing at −80 °C in Eppendorf tubes containing PBS with 0.05% Triton X-100.

4.4.1 Infection site and organ

To identify the site of infection, female flies were infected with positive isolates and dissected head, thorax and abdomen 3 days later. Subsequently, to identify the infected organ, a second batch of female flies were infected and gut, fat body and ovaries were dissected. The relative amount of genomic viral RNA in the head, thorax, abdomen, gut, fat body and ovaries was determined by quantitative RT-PCR with the primers listed in Supplementary Table S5. Three biological replicates for each condition were performed. The data were analyzed using an unpaired two-tailed Student's t-test. *P* values lower than 0.05 were considered statistically significant: $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$. All analyses were carried out using RStudio software version 1.2.5033.

4.4.2 Oral infection experiments

Oral infection experiments were performed on L3 larvae and on 3–7 days-old adult females. For adults, the protocol described by Ferreira et al. (2014) was followed with some modifications. Four µl of the virus isolate mixed with 50% sucrose solution was added in a piece of Parafilm ($2cm^2$). Thirty flies were placed in each tube after a period of 4h of starvation and left feeding for 24 hours. For mock oral infections, flies were exposed to Tris-HCl (10 mM, pH 7.2) mixed with 50% sucrose solution. After this

infection period, flies were transferred to new vials containing standard diet as described in Methods section 4.1 (see page 19). Five adult flies, were snap-frozen from the 1st to the 5th post-infection day in order to quantify the possible daily viral replication.

L3 larvae were infected by pipetting ~250 µl of virus suspension on top of the rearing media and were allowed to feed on the virus-containing media until pupation, upon which they were also recovered. RT-PCR (primers listed in Supplementary Table S5) was then performed for quantification in both adults and larvae. Three biological replicates for each condition were performed. The data were analyzed using an unpaired two-tailed Student's t-test. *P* values lower than 0.05 were considered statistically significant: $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$. All analyses were carried out using RStudio software version 1.2.5033.

4.4.1 Fitness studies

In order to quantify the influence of the isolated viruses on the fitness of the flies, climbing and recovery assays were performed. Briefly, for the climbing assay, staged female cohorts were intrathoracically injected with viral samples in Tris-HCl (10 mM, pH 7.2). The same buffer solution was used as a mock control. The survival rate was recorded daily. Five days post-injection, synchronized flies were transferred without anesthesia to a 50 ml glass-cylinder and forced to the bottom with a piece of cotton. After an adaptation period of 30 s, the climbing ability of flies was quantified as the number of animals reaching the top of the cylinder (10 cm) in 15 s. Three biological replicates for each condition were performed. The number of flies reaching the top was converted into % value, and the mean % value (±SEM) was calculated for a minimum of 6 experiments. Flies were then exposed for 30 s to CO_2, and the recovery time of each fly for each condition was recorded. The data were analyzed by a Kruskal-Wallis test, and survival curves were plotted and analyzed by log-rank analysis (Kaplan-Meier method). *P* values lower than 0.05 were considered statistically significant: $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$. All analyses were carried out using RStudio software version 1.2.5033.

4.4.2 Analysis of gene expression

Flies were collected after 3 days post-injection for quantitative real-time RT-PCR. Total RNA was extracted the flies using TRI Reagent (Zymo Research) according to the manufacturer's instructions, and

was reverse transcribed using the iScriptcDNA Synthesis Kit (BioRad). The resulting cDNA was amplified with three technical replicates using the gene-specific primers listed in Supplementary Table S6, and Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions.

4.4.3 LJV serial passage experiment

Passage experiments were carried out using cohorts of 20 females. After three days, flies were homogenized, and the resulting solution was filtered through a 0.22 µm membrane. Forty-six nl of this filtrate were intrathoratically injected to the next generation of flies, as schematically drawn in Figure 10. This procedure was repeated up to 15 times for each one of the produced isolates described in Methods section 4.2.1 (see page 20). After every passage round, the survival rate of injected flies was recorded, and five living flies were frozen at -80° for future experiments. Viral proliferation in *D. suzukii* the load post injection was measured by quantitative RT-PCR.

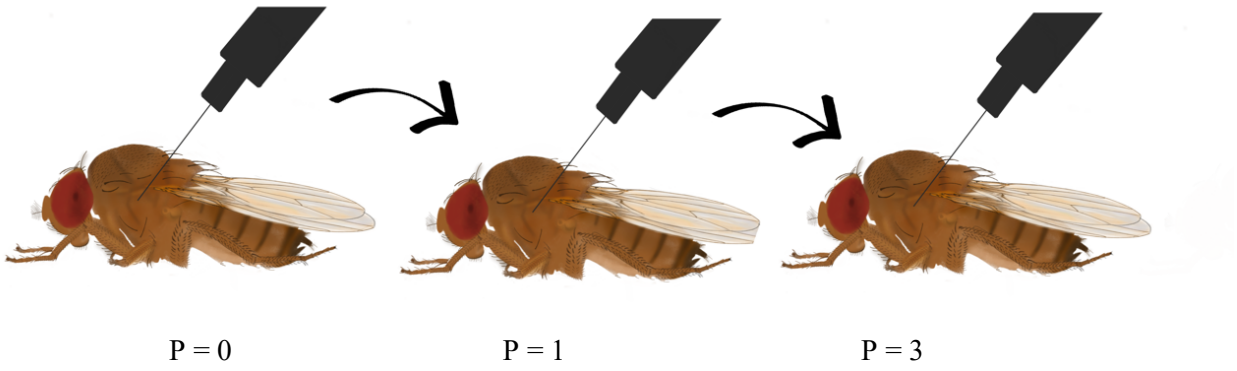


Figure 10. Scheme of the viral serial passage experiment. Twenty *Drosophila suzukii* females were serially infected by intrathoracic injection. The remaining flies were homogenized, and this homogenate was injected in the following generation. For every passage, five flies were frozen at -80°C for further experiments. **P**: passage

4.5 *Drorophila suzukii* cell culture

4.5.1 Hemocyte collection

For hemocyte collection, surface sterilization of the L3 instar larvae was avoided. Up to ten washing steps were performed using dH_2O. Hemocyte isolation was performed using chamber slides. Although different attempts were made, the best protocol for hemocyte isolation was performed in a drop of Grace's Insect Medium supplemented with phenylthiourea (PTU)[4], 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (PS) at 10,000 U/ml. At least 30 to 50 larvae were required to produce enough hemocytes for any experiment. Hemocytes were allowed to attach to the glass surface of the chamber for at least 30 minutes.

4.5.1 Fluorescent microscopy

Isolated hemocytes were allowed to adhere to the bottom of the slide, cells were then fixed with 4% paraformaldehyde (PFA), washed and stained with 2-(4-amidinophenyl)-1*H*-indole-6-carboxamidine (DAPI) for 5 min and with phalloidin for 20 min at room temperature. The slides were analyzed by confocal microscopy (Leica SP8). The identification of plasmatocytes was verified in isolated cells fixed with 4% PFA, blocked with 1% bovine serum albumin (BSA) for 5 min and incubated with the so-called 'anti NimC1 antibody', which consists of two anti-plasmatocyte antibodies, P1a and P1b (Kurucz et al., 2007), for 1 h. Subsequently, the cells were incubated with an anti-mouse Alexa Fluor 555-conjugated antibody (Thermo Fisher), mounted and analyzed by confocal microscopy.

4.5.2 Cell viability assay

To detect living cells after extraction of hemocytes, a combination of Hoechst and SYTOX Green dyes was used. High resolution and non-invasive live cell imaging microscopy (Nanolive) was used to record the lifespan of the cells. Hoechst is a cell-permeable dye that can bind to DNA in living cells. A positive reaction is recorded when blue-cyan fluorescent light is emitted. SYTOX Green was used for labeling

[4] Phenoloxidase is the key enzyme of melanization catalyzing the oxidation of phenols.Phenylthiourea (PTU) is the well-known and widely used competitive inhibitor of phenoloxidase (Ryazanova et al., 2012).

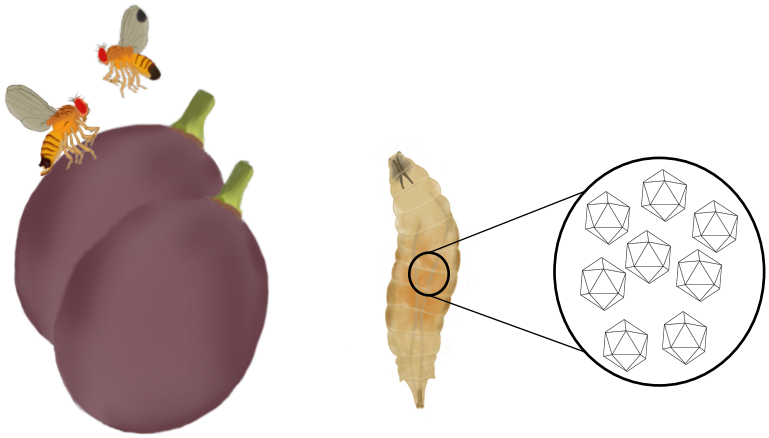
necrotic cells (Roth et al., 1997). Hoechst dye is excited by UV ~350 nm and emit light of ~461 nm wavelength and SYTOX Green dye is excited by ~488 nm and emit light of ~504 nm wavelength.

4.5.3 Generation of a primary embryonic cell line

To recover up to 200 staged embryos, *Drosophila suzukii* females were allowed to oviposit on sterile apple agar plates (Featherstone et al., 2009). Embryos were collected over a 4 h-interval and placed on dH_2O. Next, they were surface-sterilized by immersion in 70% ethanol for 10 min. After being rinsed thoroughly in dH_2O, the eggs were transferred to Petri dishes, where each one of them was cut into halves or thirds, respectively. Subsequently, they were placed on a drop of Schneider's *Drosophila* medium for 24h min at 26ºC. Thereafter, embryos were transferred to 25 cm^2 Falcon flasks without airflow, and the bottle was placed in a 45º angle to allow the cut-embryos reach the bottom of the flask. As the cell monolayer began to grow, the flask was placed horizontally. Schneider's *Drosophila* medium was supplemented with 10% FBS and 1% PS.

5 Results

Goal 1: Isolation of naturally infected *D. suzukii*-specimens and virus identification[5]



Graphical abstract 1. Illustration of the first thesis goal. Berries infested with *D. suzukii* were sampled, and larvae were subsequently used for virus identification.

The main goal of this part of the thesis was to estimate the invasiveness of *D. suzukii* in Hesse (Germany) because no local data have been acquired for this particular pest, so far. Consequently, a sampling was performed to estimate the health status of *D. suzukii* in a non-native range. Collection of larvae and pathogen identification were performed in order to recover naturally-infected insects that could basically contribute to the development of a biocontrol strategy for SWD.

[5] Part of the larvae sampling contained in this chapter has previously been published in Journal of Invertebrate Pathology. Hiebert N, **CARRAU T**, Bartling M, Vilcinskas A, Lee KZ. (2020) Identification of entomopathogenic bacteria associated with the invasive pest *Drosophila suzukii* in infested areas of Germany. J Invertebr Pathol 173: 107389. https://doi.org/10.1016/j.jip.2020.107389

The epidemiological work described in this chapter has been carried out by T. Carrau, K-Z Lee and Vilcinskas A. Viral screening and sequencing was carried out by T. Carrau and project conceptualization was designed by K-Z Lee and A Vilcinskas.

5.1 Sampling of larvae

5.1.1 Location and fruit collection

According to Briem et al. (2018), the best collection sites are in found in a vineyards and orchards, respectively. Sampling was done according to prior agreement with local farmers during the warm summer season, when temperatures reached or exceeded 30°C.

Figure 11. *Drosophila suzukii*-infested cherries. On the left cherry, a SWD female is ovipositing an egg inside of the fruit (white arrow). In the right picture is visible the entrance point (black arrow) of the larva (asterisk).

All orchards and vineyards sampled were found heavily infested with *D. suzukii* larvae. Every single fruit collected was carefully inspected by eye but only berries that displayed entrance holes (Figure 11- black arrow) and/or SWD females ovipositing on the fruit skin were considered for the project (Figure 11- white arrow). As described by Hiebert el at. (2020), cherries were found to be the most heavily infested fruit (mean = 6 larvae per fruit), followed by raspberries (mean = 1.5 larvae per fruit) and grapes (mean = 0.22 larvae per fruit), as illustrated in Figure 12. Fruits displaying distinct signs of fungal or bacterial infection were also collected but stored and analyzed in separate projects (data not shown in this thesis).

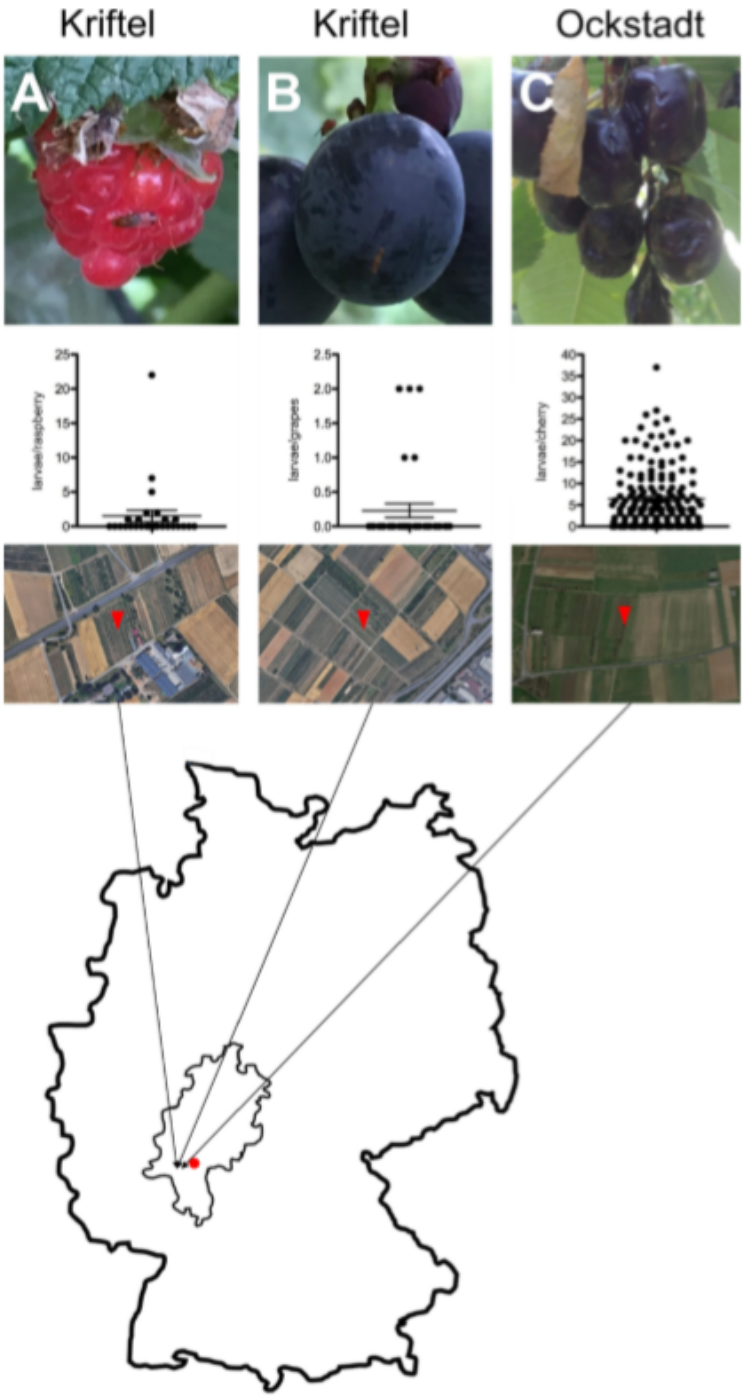


Figure 12. Sampling of moribund *D. suzukii* larvae. Larvae were isolated from (**A**) raspberries, (**B**) grape berries and (**C**) cherries. For each fruit, the top panel shows atypical sample, the middle panel shows the quantification of larvae found per fruit and the bottom panel is a satellite image of the location, with the sampling site indicated with a red arrow. The three satellite images are linked to a line map (outer boundary is the German border, inner boundary is the Hessen border, red dot shows the position of Frankfurt). Figure source, Hiebert et al., (2020)

5.1.2 Screening of infected larvae

By careful inspection with a binocular microscope, a total of 1,093 SWD larvae were obtained. Their perceived health status was recorded, and larvae were accordingly sorted into categories. Out of the total bulk of isolated specimens, the focus was on the dying (44.2%) or dead (21.2%) larvae (Figure 13-A). As an internal control, the remaining healthy larvae were allowed to develop into adults (34.7%). When characterizing the morphological changes of moribund and dead larvae, the presence of intersegmental body extrusions (14.3%) and internal melanization (16.3%) were considered as indicators of poor health status, i.e. bacterial, fungal or viral infection (Figure 13-B). A high percentage of the isolated specimens did not exhibit external anomalies. However, underlying sickness could have triggered a faster decease. A classification scheme was established in which each dead or moribund larva was labeled with one of the following internal codes to monitor the possible presence of natural pathogens: "OS_{NL}", "KR_{NL}" and "Gi_{NL}", for Ockstadt, Kriftel and Giessen, respectively (NL= Number of Larva). Moribund and dead larvae with the same morphology were pooled for each cherry. A total of 57 samples, listed in the Supplementary Table S 7, was obtained by the sampling procedure described above.

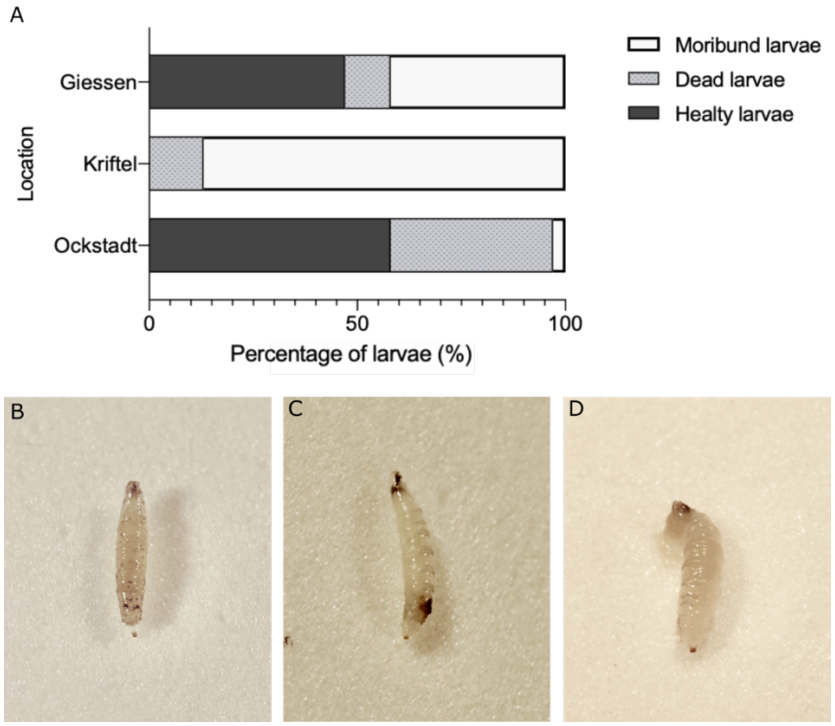


Figure 13. Percentage of the health status of the isolated larvae. For each sampling location, the percentage of healthy, dead, and moribund larvae is shown (**A**). Characteristic external symptoms of dead larvae: Internal (**B**) or localized (**C**) melanization were observed most frequently. Intersegmental body extrusions (**D**) were occasionally recorded.

5.1.1 Rearing of wild-type *D. suzukii*

One wild-type colony was established for each sampling site (Figure 14-A). For this purpose, 80% relative humidity was maintained in the feeding tubes by adding dH_2O on top of the substrate. Adults and larvae were kept separately and monitored daily. Each newly emerged adult was carefully examined in order to ensure it was correctly identified as *D. suzukii*.

For this part of the project, regular supervision of the fly culture was required, and all newly established lines were quarantined until the absence of microbial pathogens was assured. Unrecognized fungal and bacterial infection[6] was screened for by PCR (Supplementary Table S1). No infections were found in any of the wild-type lines. Careful external body examination of adult flies ensured that no surface mites were present in any of the experiments. Up to 6 months of handling time were needed until the three wild-type *D. suzukii* colonies, namely Ockstadt, Kriftel and Giessen (Figure 14-A), reached sufficient population density for experimental use. According to Lee and Vilcinskas (2017), wild strains from Giessen and Kriftel had a single nucleotide polymorphism (SNP) in the last exon of the *pastrel* gene, which might imply an impact on virus susceptibility as described by Magwire et al. (2012). For details, see Figure 14-B.

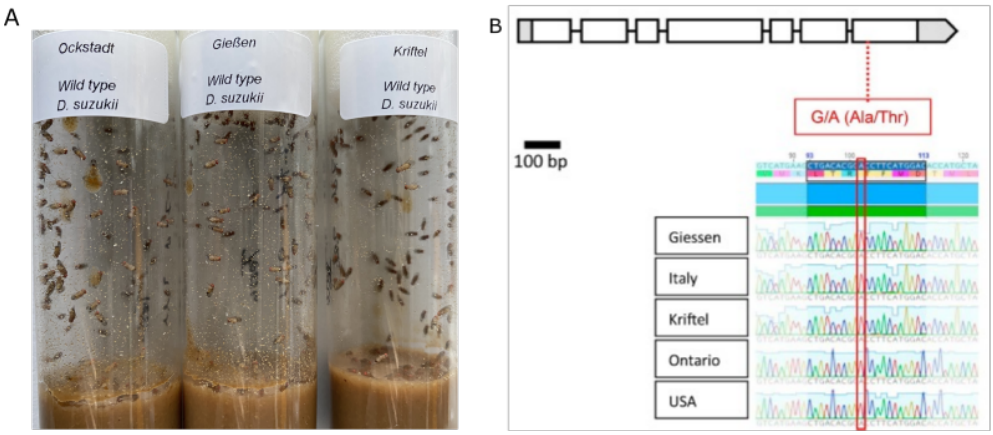


Figure 14. Overview of the wild type *D. suzukii* rearing. (**A**) Three different wild type lines were generated and maintained under laboratory conditions for (**B**) comparison of the virus susceptibility. The structure of the *pastrel* gene and location of the SNP with the strongest effect on virus susceptibility (variable sequences are framed in red). Boxes represent exons, lines indicate introns with scale bar corresponding to 100 base pairs (bp). Figure modified from Lee and Vilcinskas (2017).

[6] The widespread invertebrate bacterial genus *Wolbachia*, harbors endosymbionts, which protect the flies from virus-induced mortality (Hedges et al., 2008).

5.2 Screening for viruses pathogenic to *D. suzukii*

In total, 4.3 Mio events (basecalled bases) and 15,000 reads were generated. Viral sequences of all Illumina® databases obtained showed 45-55% of mapping rate. Finally, hits against four viruses present in the NCBI data base were found: LJV, DAV, MMlV and Teise Virus. All virus identifications were then confirmed by RT-PCR using the previously described data by Medd et al., (2018) that are compiled in the Supplementary Table S2. Viruses were found in 59.6% of all analyzed samples (n = 34/57). Twenty-seven samples were positive to Teise Virus, nine were positive to MMlV, four samples LJV and three to DAV. Six samples appeared to be co-infected with at least two viruses and one (OS26) was positive to three different viruses. All analyzed samples are listed in Table S2.

The most heavily infested fly population was inferred to be the one from Ockstadt, with 51.7% of the samples being infected with Teise Virus, 9.7% with MMlV, 9.7% with LJV and 9.7% with DAV. The Kriftel samples were also heavily infected, showing about 50% prevalence of Teise Virus and 27.2% of MMlV. Finally, one virus was only found in sample from Giessen, LJV, with a 33.3% prevalence (Figure 15).

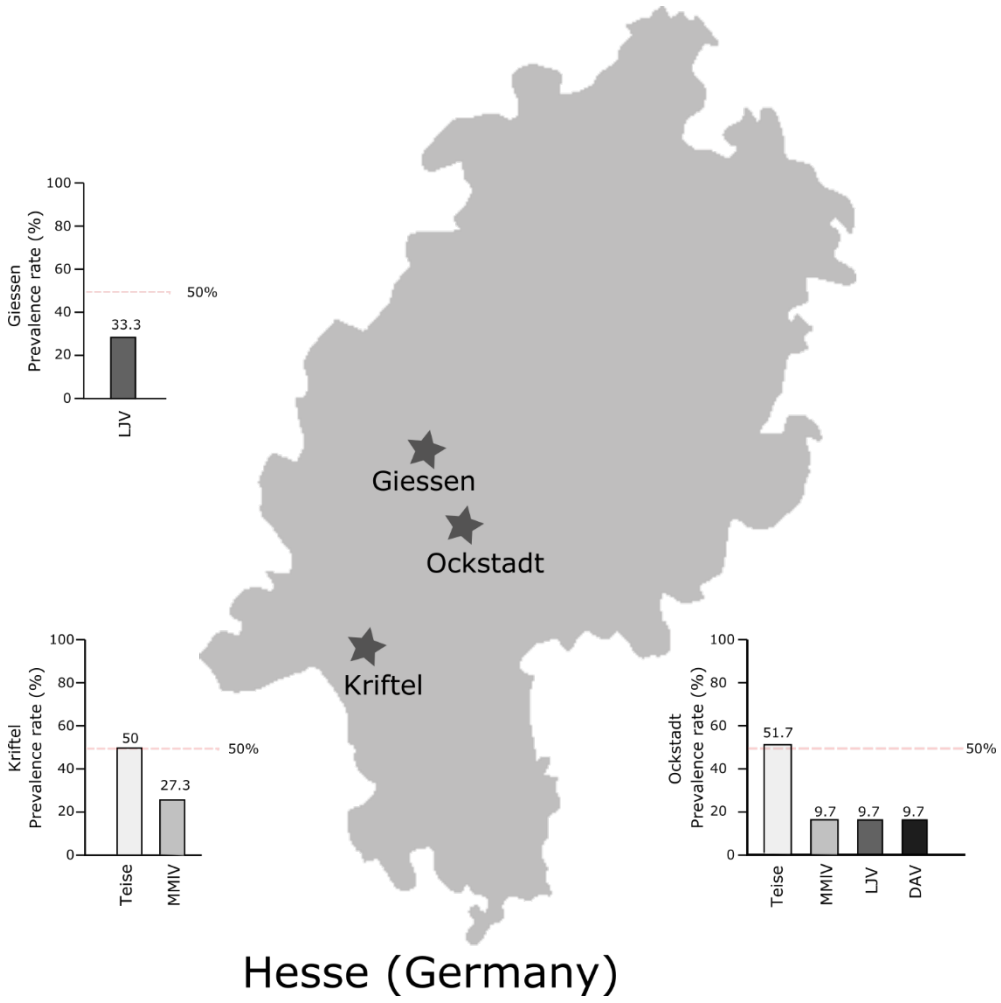
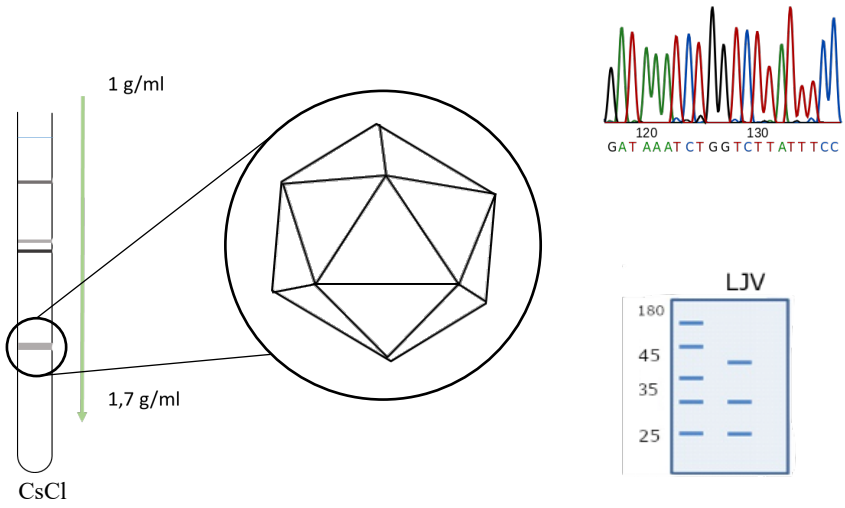


Figure 15. Prevalence distribution of viruses identified from each region. Samples from Ockstadt showed the most diverse spectrum, with up to four different viruses found. In contrast, only one or two viruses were found in samples from Kriftel. The sample originating from Giessen contained only one virus.

Goal 2: Characterization of potential viral candidates for biocontrol of *D. suzukii*[7]



Graphical abstract 2. Illustration of the second thesis goal. Viral candidates were functionally studied, and structural characterization of LJV was performed

The main goal of this part of the thesis was to obtain a suitable single viral candidate for SWD biocontrol. Originally, two different viruses were selected based on the possible biological activity reported in literature. LJV and DAV turned out to be the most attractive candidates to be tested as a possible BCA for SWD.

[7] The preliminary study on the viruses has recently been published: **CARRAU, T.,** Hiebert, N., Vilcinskas, A., & Lee, K. Z. (2018). Identification and characterization of natural viruses associated with the invasive insect pest *Drosophila suzukii*. J Invertebr Pathol, 154, 74-78. https://doi.org/10.1016/j.jip.2018.04.001

The characterization of LJV is in preparation for submission. **CARRAU T.**, Lamp B., Reuscher C., Vilcinskas A. & Lee K-Z (2021). Organization of the structural protein region of la jolla virus isolated from the invasive pest insect *Drosophila suzukii*. Viruses 2021, 13(5), 740. https://doi.org/10.3390/v13050740

5.3 Preliminary characterization of the viral candidates

In vivo-passaging of the viruses was performed in order to increase viral titers. In default of a specific ultra-purification protocol to isolate particles of LJV, a PEG-based concentration approach was developed (Figure 16):

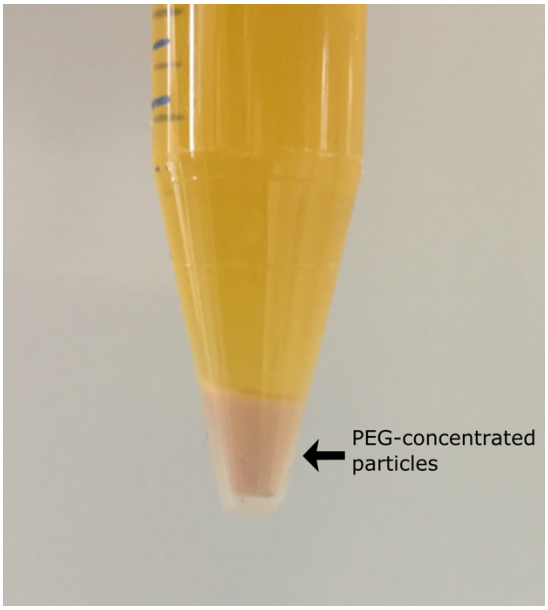


Figure 16. PEG-concentrated particles of LJV. The sample containing infected flies was first homogenized and then centrifuged. The resulting supernatant was mixed with PEG to precipitate the viral particles as indicated by the black arrow. This pellet was subsequently used for the functional experiments presented in this part of the thesis.

First, the presence of a single virus in each of the PEG preparations was confirmed by PCR. This step was necessary because of the intention to analyze each viral candidate separately. In order to analyze the nucleic acid encapsidated in virions, RNA was extracted from the PEG-virus preparations and treated with or without RNase, respectively (Ambrose et al., 2009). Subsequently, both samples were redissolved on a 1% agarose gel and visualized with ethidium bromide (Figure 17-A). In both cases, the non-treated lane showed a smear of nucleic acid ranging from 100 to >3000 nt. Both treated and non-treated samples were then analyzed by PCR. No amplicons were present in the RNase-treated samples. In contrast, positive results were obtained for the non-digested samples (data not shown). These findings suggested that both the DAV and LJV genomes obtained in this experiment comprise single-stranded RNA (ssRNA). Subsequently, SDS-PAGE was used to estimate the size of the protein components. A single protein band of about 42 kDa, equivalent to the size expected for the coat protein, was observed in both

samples (Figure 17-B). Nonetheless, viral proteins were not shown in this preliminary experiment. In order to obtain these data, the purity of this sample had to be improved.

Finally, PEG-concentrated particles were negatively stained and examined by TEM in order to visualize the morphology of the virus. At 100,000 × magnification, non-enveloped, icosahedral particles were observed for both DAV and LJV, with a mean diameter of ~30 nm (Figure 17-C, 17-D). However, impurities are clearly visible in the images below (see Figure 17). Thus, the following steps to improve the quality of the viral sample were carried out.

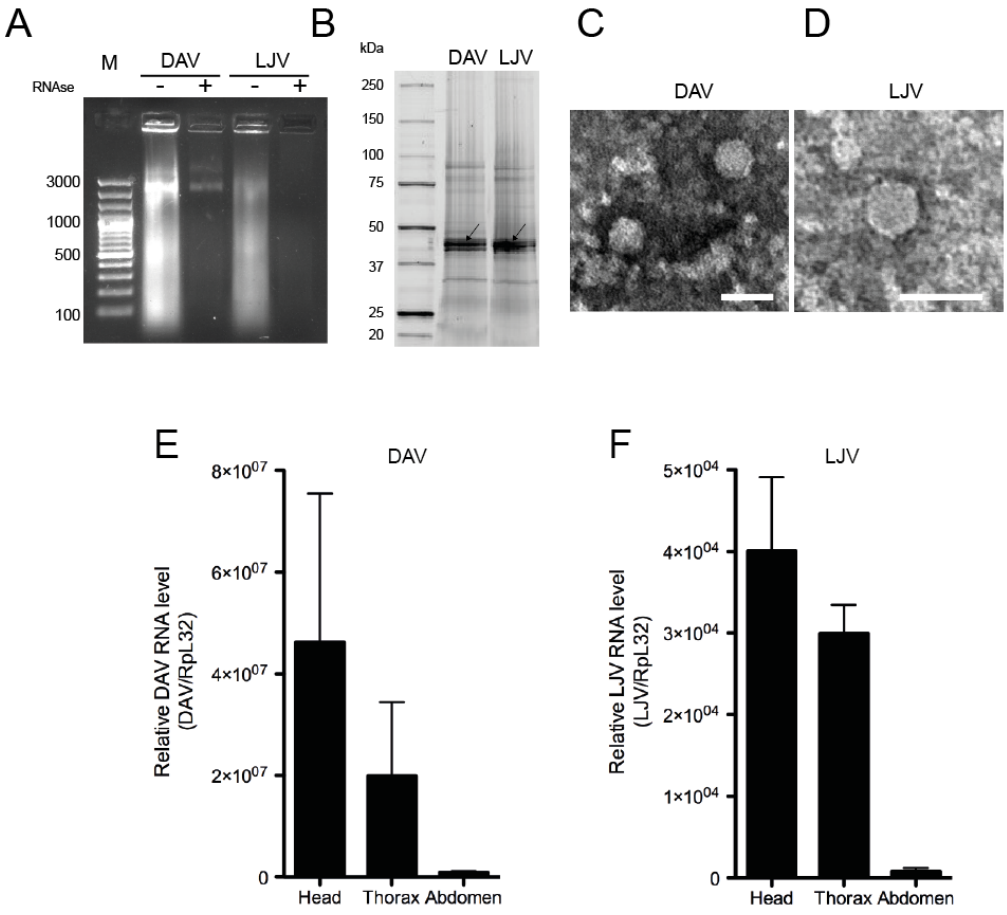


Figure 17. Characterization of DAV and LJV. (**A**) RNA was extracted from precipitated DAV and LJV samples and s non - treated (-) or treated with RNase A (+) samples. Following nuclease treatment, the products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Marker (M) sizes in nucleotides are indicated on the left panel. (**B**) Proteins in precipitated PEG-free samples separated by SDS-PAGE using SDS 4–20% TGX Stain-Free Gels. The capsid proteins are indicated by an arrow. Sizes of the molecular mass markers are shown on the left. (**C**) Precipitated DAV and (**D**) precipitated LJV particles were negatively stained and visualized by TEM at 100 000 × magnification. Scale bar corresponding to 50 nm. (**E**) Relative amounts of DAV RNA or (**F**) LJV RNA in different body parts of *D. suzukii*, normalized to RpL32. The averages and standard deviations (SD) are shown for three biological replicas. Figure adopted from Carrau et al. (2018).

5.3.1 Localization of infection

The subsequent goal was to study the localization of the infection in *D. suzukii*. Consequently, virus-infected female flies were dissected 3 days after injection of DAV and LJV, respectively. Both viruses were detected in all parts of the insects (head, thorax, and abdomen). The relative amount of DAV (Figure 17-E) and LJV (Figure 17-F) genomic RNA in head, thorax and abdomen was determined by quantitative RT-PCR. The highest level of DAV and LJV was found in the head, followed by the thorax, whereas only low levels were found in the abdomen.

5.3.2 Virulence of DAV in *D. suzukii*

In order to study DAV virulence in this new insect model, 3 cohorts of 20 female flies were intra-thoracically injected with either the virus or Tris-HCl (10 mM, pH 7.2) as buffer control. As shown in Figure 18-A, virus-injected flies succumbed more rapidly than then buffer-only injected insects. The median survival time (ST_{50}) of infected flies was at 3 days and most flies succumbed to the infection by day 20, in contrast to the ST_{50} at 16 days in mock-injected flies. Subsequently, the DAV viral load in *D. suzukii* was measured by quantitative RT-PCR. Figure 18-B shows the RNA quantity on day 0, which moderately increased 1 day post infection, followed by a rapid increased of two log values at day 2.

5.3.3 Virulence of LJV in *D. suzukii*

Subsequently, the virulence of LJV in *D. suzukii* was determined as previously described for DAV. Virus-injected flies succumbed to the infection much more rapidly than mock-injected controls, although in this case the ST_{50} was at 8 days in LJV-infected flies compared to 16 days for the control (Figure 18-C). As for DAV, susceptibility to LJV correlated with the viral load as determined by quantitative RT-PCR (Figure 18-D). On day 0, only the quantity of RNA used for injection was detected, but in contrast to DAV a rapid and strong increase in titer (three log values) after 1 day was observed, with this high level of virus RNA persisting for the following 2 days.

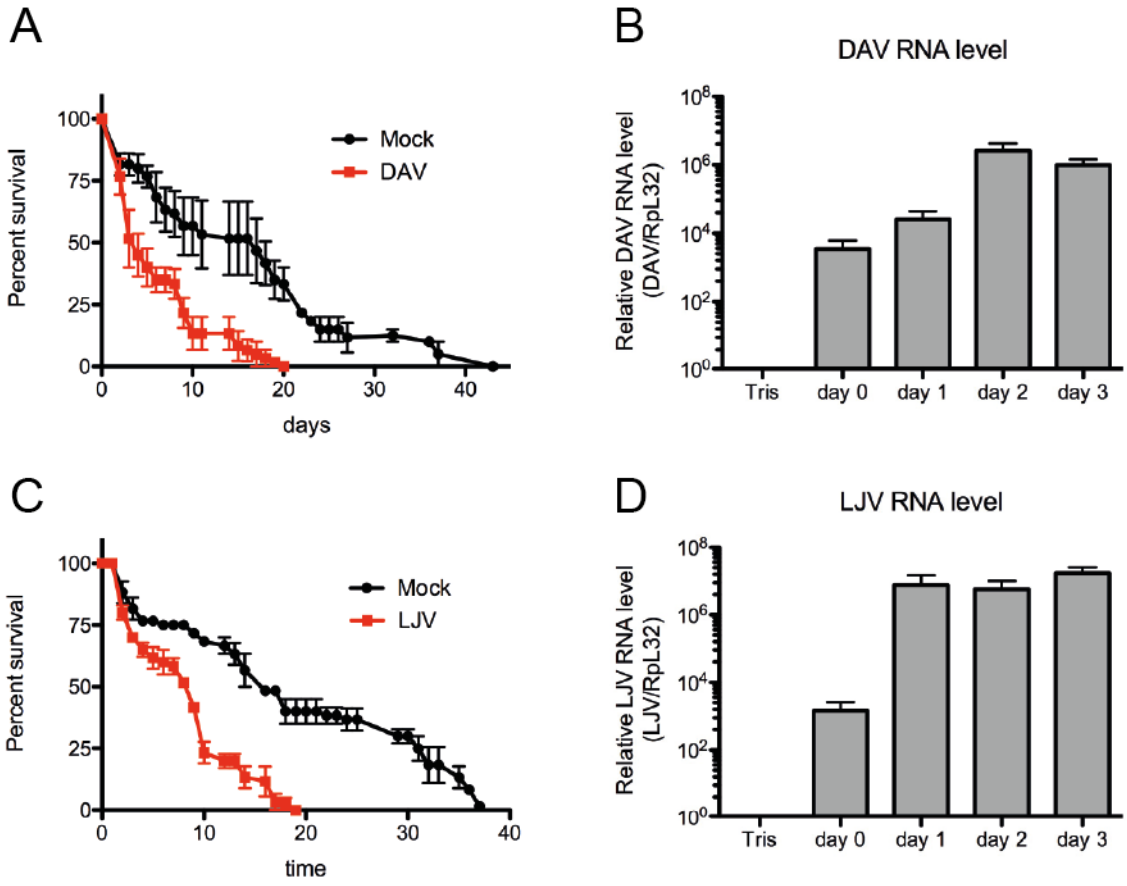


Figure 18. Survival assays and virus titers. Flies (3–7 days old) were infected with (**A**) DAV and (**C**) LJV and the lifespan was monitored daily at 26 °C. Experiments have been performed three times, $P < 0.005$ for (**A**), $P < 0.001$ for (**B**), log-rank test. Quantitative real-time RT-PCR analysis of the accumulation of (**B**) DAV and (**D**) LJV RNA at the indicated time points after infection in *D. suzukii* female flies. Data represent means ± SD of at least three independent experiments, each involving five flies. Figure adopted from Carrau et al. (2018)

5.4 LJV genome organization

The aim of this part of the study was to develop a virus-specific protocol to increase the purity of the viral particles via ultra-centrifugation and, consequently to achieve a close overview of the organization of the LJV protein inventory. After the experiments described above had been completed, survival rate analysis, viral load and infection site, LJV and DAV, were compared in order to determine their potential use as viral BCAs. Finally, LJV was chosen for the above-mentioned characterization because of its relatively high virus-host adaptation, which seemed to significantly impact the ST_{50} of SWD.

5.4.1 Purification of LJV

Initially, a 20% sucrose cushion was applied to the viral pellet (Figure 19-A). This pellet was subsequently isolated and transferred into a CsCl gradient, which resolved a turbid band of cellular material with a buoyant density of 1.15-1.20 g/ml (Figure 19-B, white arrow) and an additional, strong band with a buoyant density of 1.36 g/ml, which turned out to contain the LJV virions (Figure 19-B, black arrow). High resolution LJV-TEM microscopic pictures were taken in order to visualize the LJV virions in this band. Notably, the quality of the microphotographs taken is superior to any previously published photographic material. Results were, however, in complete agreement with previous findings (Carrau et al. 2018), and the virus particles found exhibited the typical structures of LJV (Figure 19-C). Additionally, co-purified material, consistent with disintegrated virions, was also found in the analyzed sample (Figure 20-D, black arrow).

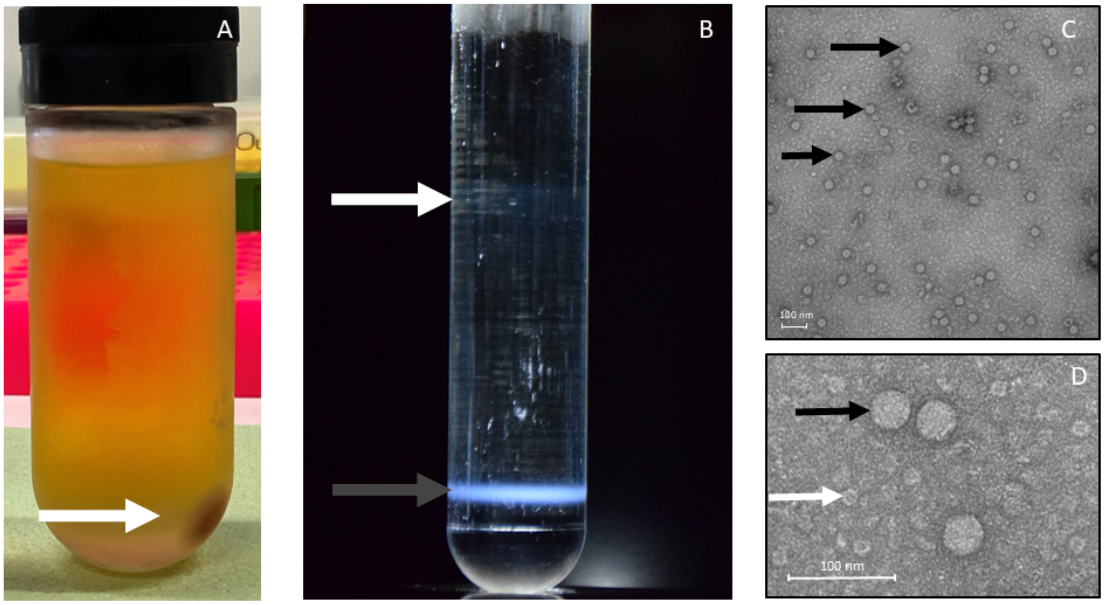


Figure 19. LJV purification. A 25% sucrose cushion was first performed (**A**) to pellet (white arrow) the virus particles. (**B**) CsCl gradient purification of LJV. (C) TEM image of purified LJV particles in negative contrast at 85,000 × magnification. (**D**) TEM image of purified LJV particles in negative contrast at 140,000 × magnification. Scale bar corresponding to 100 nm. Figure modified from Carrau et al. (2021).

5.4.2 *N*-terminal sequencing and capsid processing

In order to unravel the unknown sequence of the structural protein-coding region of LJV, *N*-terminal sequencing of the structural polyprotein was carried out. A dual approach was used for this part of the project:

At first, an initial attempt was made to resolve the proteins in 10% polyacrylamide gels stained with Coomassie brilliant blue. To this end, up to 1,500 flies were injected. After three days of incubation (see Material an Methods 4.3.1, page 22), viral particles were obtained by ultra-centrifugation in a CsCl gradient, as previously described. Subsequently, these particles were used to separate and visualize representative protein bands (Figure 20-A), two of which were resolved from the purified virions (B1 and B2), as illustrated in Figure 20-A. Finally, the two bands were excised from the gel and *N*-terminally sequenced by ED. Virion-band B1 (33 kDa) exhibited the characteristic cleavage motif DKPYDDQRVQ. In contrast, the results for B2 (29 kDa) were unsatisfactory (Figure 20-A). Consequently, an alternative method had to be employed.

The second approach was based on separation in a 12.5% SDS-PAGE gel after IEF, using an immobilized pH gradient from pH 3.0 to pH 10.0. After IEF, the gel was blotted onto a PVDV membrane and stained with Coomassie brilliant blue.

For this method, an excess of 6,000 flies was required. Virions were again purified in a CsCl gradient, as described above. After IEF, the Coomassie-stained protein spots were cut out of the membrane and subjected to ED and LC-MS/MS analysis. As shown in Figure 20-B, five spots were clearly visible in the 2D-SDS gel. Minor contamination of virus particles by SWD host proteins was concluded from the form of spots C1 and C2. Additionally, five spots attributted as so-called viral proteins (VP) were also found.

These VPs belonged to LJV structural components that organize the viral capsid. For the purpose of this thesis, VPs were arranged following previous conventions reported data for picornaviruses. The order of the major capsid proteins was as follows: VP2, VP4, VP1, and VP3 (Kalynych et al., 2016, 2017; Škubník et al., 2017). VPs were then attributed as follows: V1, V2 and V3 were assigned to VP1; V4 and V5 spots were assigned to VP2; and was VP3 also detected in V1. The apparent molecular weight

of the designated VP1 was 32 kDa, VP2 had an apparent molecular weight of 28 kDa and, finally, 24 kDa was the apparent molecular weight of VP3.

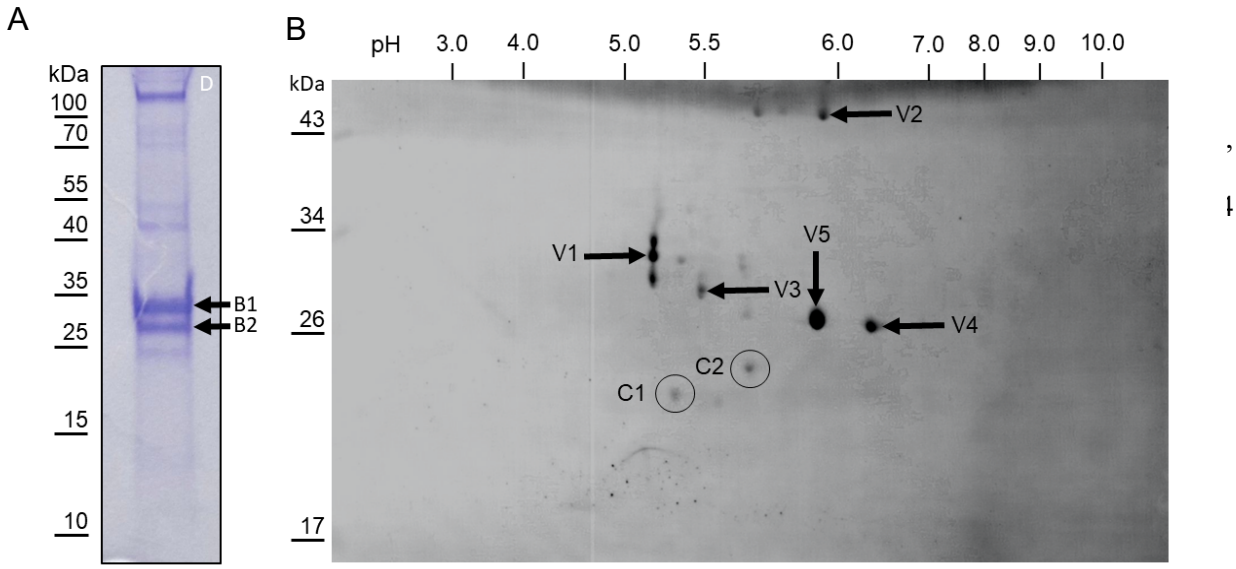


Figure 20. Visualization of the protein bands. (**A**) Dominant protein bands (B1 and B2) and (**B**) *N*-terminally sequencing by ED after IEF using an immobilized pH gradient from pH 3.0 to pH 10.0. The apparent molecular weight of marker proteins that were separated in parallel are shown on the left, the pH range of the gradient matrix is indicated above the blot. Modified figure from Carrau et al. (2021).

Analytical efforts could then be focused on devising the layout of the structural protein region of LJV based on the obtained *N*-terminal sequencing data. The identity of the leader protein (L-protein) was deduced from the N-terminus of VP2, where the C-terminal cleavage site was found (Figure 21, detected cleavage site in red). Then, the full sequence of VP2 was determined with both of approaches: using the *N*-terminal sequencing by ED and by LC-MS/MS after tryptic digestion (Figure 21, detected amino acids in bold). Nonetheless, the structure of the C-terminus of VP2 could be not resolved by MS/MS analyses (Figure 21).

The peptides originating from the smallest protein, VP4, were detectable due to a conserved Q/M/E cleavage site (Figure 21, detected cleavage site in red). They were found right after the peptide sequence of VP2, downstream of the supposed N-terminus of VP4. However, they were not found in the LJV particle analyses. The N-terminus of VP4 reported in this work is reasonably compatible with previous assumptions (Figure 21). Additionally, the unusual C-terminal cleavage site of VP4 was deduced from

the N-terminus of VP1, found by ED (Figure 21, relevant amino acids in bold). The structure C-terminus of VP1 and of the N-terminus of VP3 were detected as neighboring peptides in MS by their typical Q/M/D cleavage sites (Figure 21, detected cleavage site in red). However, these proteins could not be experimentally mapped. Nonetheless, the C-terminal peptide of VP3 could be detected because it is devoid of characteristic basic amino acid residues and their typical cleavage sites.

Leader protein (20.3 kDa, IP 7.80)	MQSFVEGIHNMEQLDNGRIKRSKHIPKGFKPICNRQYETCKDESGRGYINISFKPLDNTDSR ETLACALERFARLRLKRKSNRVNRPNLTALFNDWVCGVTILEKQTSRAQERHNQQGDRTRYV ERNTYVLQFMDGMLRVNCSFKSFKFRNLQEIIYEAIIMELLSIPQPQ
VP2 (26.3 kDa, IP 6.8)	**ME**DDGGQGDTTQDSDKAGNTIITRDADQSVAKEEGIAIPQLISICSTEPMHQFESITNRWMA LPSIEVKTSDAFNTILQTYNLPSFLYSENSAPNMMPFENFMFGKYDIWFKFVVNANKFHVGK VLASVKYDSYQIDTLRNSLPALVSRPHVMMDLAANNEGMLCVPFKYHRTFVRNASLANSQYG SKAAQYASVVLSIMSPLAAAVGAPSNMYIRPFYAIKKASFTGMSYKIPVVQ
VP4 (2.3 kDa, IP 7.80)	METAGAVVSMLDKGLKICGAIKNM
VP1 (47.8 kDa, IP 6.27)	**DK**PYDDQRVQQVILRPRMNFQTGVGASDSVPLRLDPLAQTTYLPDHEYPDDPTTVLDIAKIW GFAGAFTWKASSTEGSELFNIPIEPTWRNRDSWAGVPTPLEYISSMYQFWSGPLEVRLDFVS NAFHTGSIMLSAEFGRASTNVEQSGSTYTKVFHLGNQKTVSFVIPYIYDTVWRRTSTVPFSV LRTNLDASARSGQISTMRANSSAFKVRVINKLVPVQSVVQDIQVLVFIRAADTFTLHSPIMS NMLNSEQVYALQDFPGNYPLENVSELSKISLTDSVREPGAVQVS/HKWMKGQTPDQIQNAIL VTNEGTILSSNPDHPLIDPKNVSTAPDSKIESGIIAAPNPASKYFWVNSQGSRITYNPSILP PDDYQHVVLPDFEAKKDAYYWNTARTRRYRYNRTYRTFDIGGGVWMDQPAFAQ
VP3 (28.9 kDa, IP 5.44)	MDDGVKEDTDPTETFQIGTNRNNIQTVESHIRIKDILRRPVCIVSHFNIPAYRNVAAGATLS VNPFFVPCLPPSHMITYGTSANRIFTPLIGRSAHTHLLDLFRFWRGSQRYSIISHRITGAPI YAVYVPHSGAMNCGTVTFLTVDLLDTNCPPSSFGLATEVMIPSVNPTMSIEVPYETENNWTL MQCENWERNFSWRDHGDYNVGHIVLWSEEAFTCDIFWSAGDDFEIKNFLGPPPVLAPFSKFA LSDNHPSSQ
NSP region	MEDFSPRERPSLARSIYDRISTPLACAGAA…

Figure 21. Scheme of polyprotein processing in LJV structural protein region. The underlined sequences represent peptides that were detected in MS analyses of the individual protein spots after 2-D SDS-PAGE. The amino acids in bold at the N terminus of VP2 and VP1 were determined by ED of the mature proteins from virions. Cleavage sites highlighted in red. **NSP**: Non Structural Proteins; **VP**: Viral Proteins. Figure modified from Carrau et al. (2021).

Finally, the structural protein region (P1) was compared with those from other well-characterized Iflavriuses: Slow Bee Paralysis Virus (SBPV), DWV, and Sackbrood Virus (SBV) (Figure 22). The presence of one of the so-called 'viral genome-linked proteins' (VPg), 5'- and 3'-untranslated regions (UTRs) as well as an internal ribosome entry site (IRES) was also expected but could not be confirmed[8]. The structural polyprotein organization of LJV following the L-protein is schematically illustrated in

[8] VPg is a highly heterogeneous class of small proteins, covalently bound to the 5'-end of most RNA viruses (including iflaviruses). It is crucial for RNA stability, genome replication, translation, and movement (Hébrard et al., 2009; Ng et al., 2008; Steil & Barton, 2009). These processes also involve the 5'- and 3'- UTR as well as numerous host factors (Belsham, 2009). Translation is probably initiated by an IRES in the 5'-UTR (Ongus et al., 2006; Roberts & Groppelli, 2009), thus avoiding the host's *cap*-dependent translation (Belsham, 2009).

Figure 22. Its structural region is followed by non-structural proteins (NSP), which were not studied in this project.

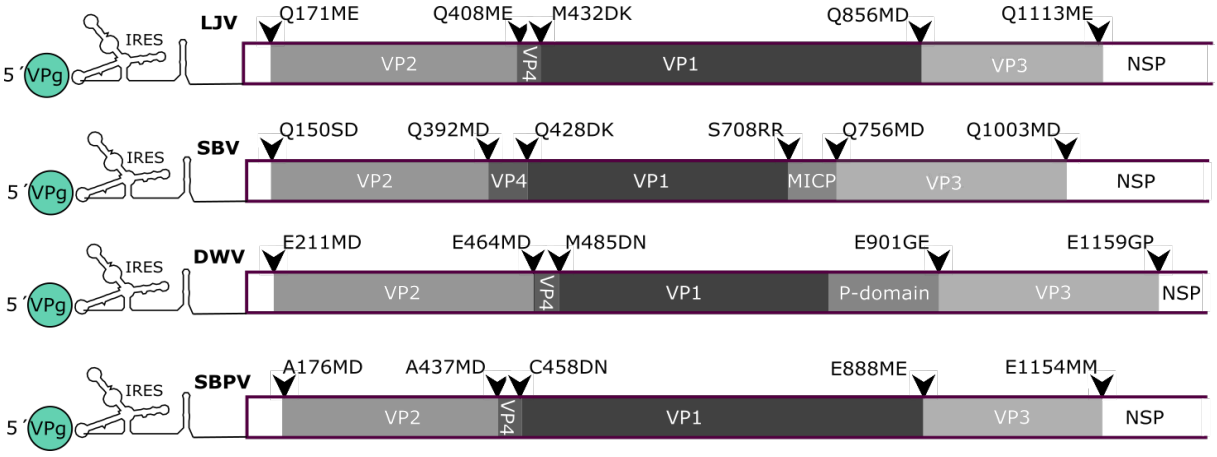


Figure 22. Organization of P1 polyproteins of different Iflaviruses. A scheme of the 5‘-UTR including the IRES element and the VPg (green circle) is shown. The leader protein is indicated as white bar, while capsid proteins (VP2, VP4, VP1 and VP3) within the P1 are in greyscale. The unusual minor capsid protein (MICP) from SBV, represents a processed protusion (P) domain present in DWV. The start of the non-structural protein region (NSP) is indicated in white. Arrowheads indicate cleavage sites as annotated. Note the similarities of the P1 cleavage sites of SBV and LJV. **IRES**: internal ribosome entry site; **VPg**: viral genome-linked protein; **NSP**: non-structural proteins. Figure from Carrau et al. (2021).

5.4.1 Sequence determination and phylogenetic analysis

As a follow up, the full sequence of LJV was determined via RT-PCR using a traditional Sanger sequencing approach (Primers listed in Supplementary Table S3). The complete genome of LJV is 10,266 nt long, excluding the 3’-poly-A tract of variable length. The genomic sequence was searched for Open Reading Frames (ORFs). One major ORF encoding a hypothetical polyprotein of 3,047 amino acids (aa) was found, flanked by a long 5’-UTR of 918 nt and a short 3’-UTR of 204 nt, again excluding the 3’-poly-A tract.

Terminal sequences were determined by 5′- and 3′-RACE analysis. When compared to other LJV isolated from *D. melanogaster,* a low diversity in the 5’-terminal end was observed. Nonetheless, when this particular 5’-sequence is compared to the one of other Iflaviridae, it is of high diversity (Grabensteiner et al., 2001; Murakami et al., 2014; Seitz et al., 2019) as further illustrated in the Discussion section 6.3.3, page 67 (Figure 39).

Next, the high identity of the results presented here with the published genome of LJV was confirmed by a BLAST search. The LJV isolate studied in this thesis was most closely related to an isolate obtained

via Next Generation Sequencing (NGS) by Shi et al. (2018) in *D. melanogaster* (GenBank accession number MH384278.1). These findings were confirmed by a phylogenetic analysis performed on the previous ORF region, which encodes the vRdRp. The resulting output resolved the present strain of LJV as part of a monophyletic group. As indicated in Figure 23, it includes all Australian strains obtained by Shi et al. (2018):

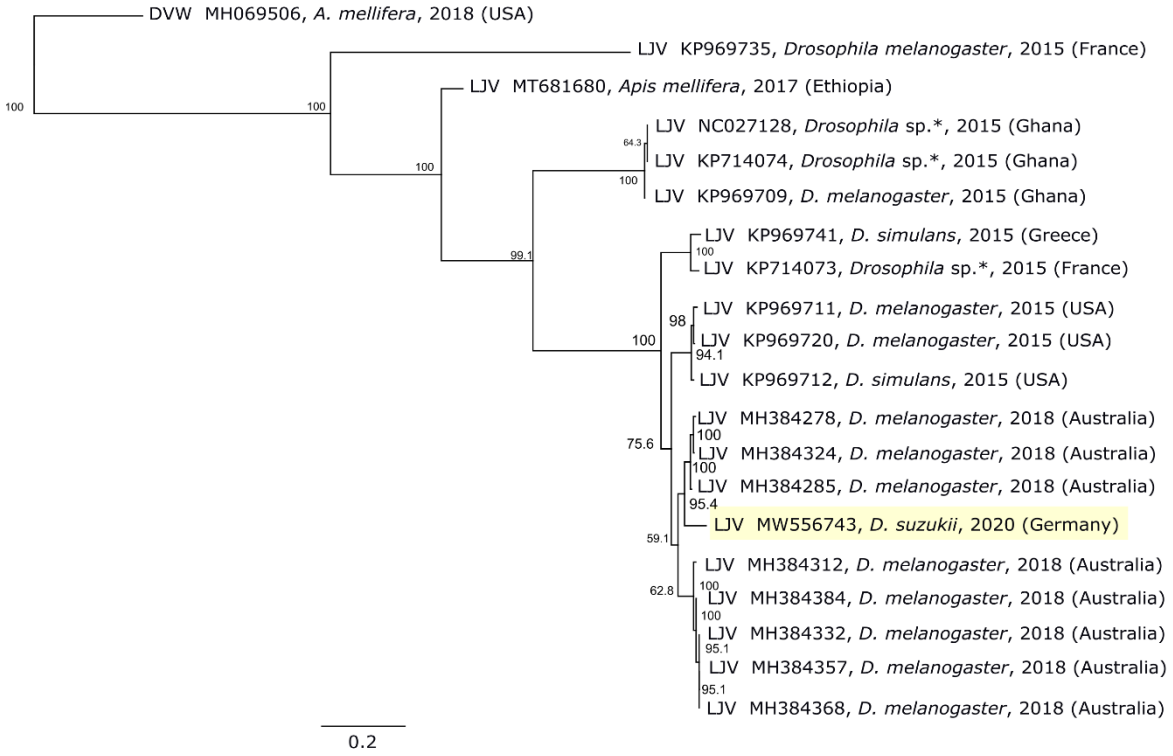
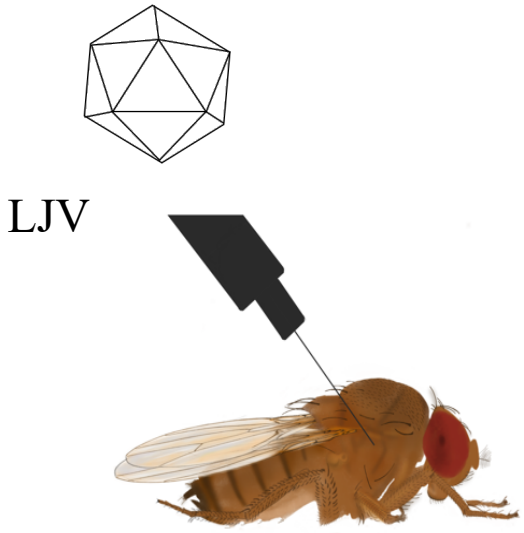


Figure 23. Phylogenetic analysis of LJV strains. The phylogenetic tree was constructed using the neighbor-joining method with a ClustalW alignment of the vRdRp (483 bp) gene of different published full-length LJV sequences from Drosophilids (n = 18) and from *Apis mellifera* (n = 2). A star (*) indicates sequences obtained from pooled *Drosophila*. Bar indicates substitutions per sites. Bootstrap values are indicated on each node as a result of 1000 replicates calculated.

To the best of the author's knowledge, the structural protein region and the subsequent molecular characterization of the new Iflavirus described in this thesis are novel contributions to science.

Goal 3: Biological characterization of LJV and cell culture[9]



Graphical abstract 3. Illustration of the third thesis goal. Biological features of the new LJV were studied to gain insight into the nature of this particular Iflavirus.

Once the prevalence, phylogenetic analysis, and genomic features of LJV had been described (Carrau et al., 2018; Carrau et al., 2021), the next set of experiments aimed at understanding the fundamental biological features of this Iflavirus. First, the presence of LJV across different developmental stages and organs of *D. suzukii*, as well as the transmission route to other flies were investigated. Subsequently, *in vivo* serial passage experiments were conducted under controlled conditions to gain insight into the host-virus adaptation of LJV.

[9] The biological characterization of LJV contained in this chapter has been carried out by Carrau T, Lee KZ and Vilcinskas A. Experiments were performed by Carrau T. Project conceptualization was designed by Lee KZ and Vilcinskas A.

The work on the hemocyte characterization and cell culture contained in this chapter will be submitted for publication. **CARRAU T**, Thümecke S, Silva L, Hermosilla C, Taubert A, Vilcinskas A. & Lee K-Z. Experiments were performed by Carrau T, Thümecke S, Silva L. Project conceptualization was designed by Hermosilla C, Taubert A, Lee KZ and Vilcinskas A. Cellular innate immune response of invasive pest insect *Drosophila suzukii* involves the release of extracellular traps against *Pseudomonas entomophila.*

5.5 Biological characterization of the new LJV

5.5.1 LJV production system

For the subsequent biological characterization of LJV, adults, pupae and larvae were evaluated as potential hosts. This was carried out using qRT-PCR and single animal extracts. The injection of adult female flies with 4.6 x 10^5 G was performed with 46 nl of CsCl-purified virus particles per insect. This resulted in substantial virus growth after three days of infection showing an average of 1.2 x 10^9 GE per fly measured by RT-PCR in the fly homogenates. In contrast, injection of larvae and pupae with LJV resulted in low virus yields with average RNA levels of 2.4 x 10^6 GE per larva and 2.0 x 10^6 GE per pupa. Hence, *D. suzukii* adults were confirmed to be the most specific host for LJV infection.

5.5.2 Replication organ

Previously, Carrau et al. (2018) showed that LJV infection is mostly located in the head of the affected fly. Nonetheless, the transmission route of this iflavirus is still unknown. Related viruses have shown to be horizontally transmitted via regurgitaion or oro-fecal contamination of the larval diet, especially in laboratory-reared insects (Murakami et al., 2014). Therefore, insects were injected with the virus and dissected 3 days post-injection. Subsequently, the relative amount of LJV genomic RNA in the fat body, gut and ovaries was determined by quantitative RT-PCR. The highest viral level was found in the fat body, followed by the gut with lower relative expression. Last but not least, almost imperceptible viral levels were detected in ovaries (Figure 24). The latter two results might suggest a possible horizontal transmission route.

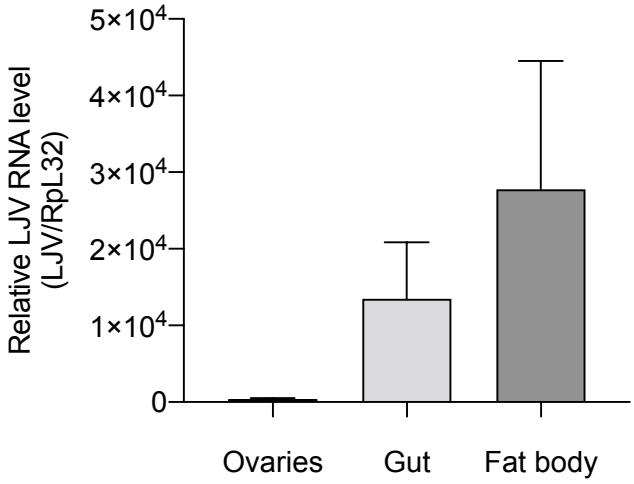


Figure 24. Relative amounts of LJV RNA in different organs of *D. suzukii,* normalized to the ribosomal protein mRNA RpL32. Data represent means ± SD of at least three independent experiments.

5.5.3 Feeding experiments with LJV

In order to verify the horizontal transmission route, specimens were fed with CsCl-purified virus particles. Adults were infected following the protocol of Ferreira et al. (2014) for the oral infection of DCV in *Drosophila melanogaster*. Larvae fed in LJV-infected food vials were allowed to develop into adults to observe any upcoming infection. However, no LJV infection was detected in artificially-fed flies and larvae, respectively.

Although the presence of the virus in the diet was as well confirmed by qPCR, the possibility of virus transmission via oro-fecal route was not confirmed under the experimental conditions applied in this thesis.

5.5.4 Fitness assays

As mentioned aboved, LJV infection is mostly located in the head. For this reason, a fitness evaluation of the infected insects was carried out. Asymptomatic infection with rhabdovirus, which targets the nervous system, has shown to be lethal for *Drosophila* under environmental stress, i.e. in the presence of CO_2 (Chow et al., 2017). The virulence of LJV in *D. suzukii* was in agreement with previous findings described by Carrau et al. (2018) where statistically significant differences were found between LJV-infected flies and sham-injected controls (Figure 25). Nonetheless, no influence of CO_2 on the virus-infected flies could be observed. Consequently, LJV did not increase its lethality after exposure to CO_2.

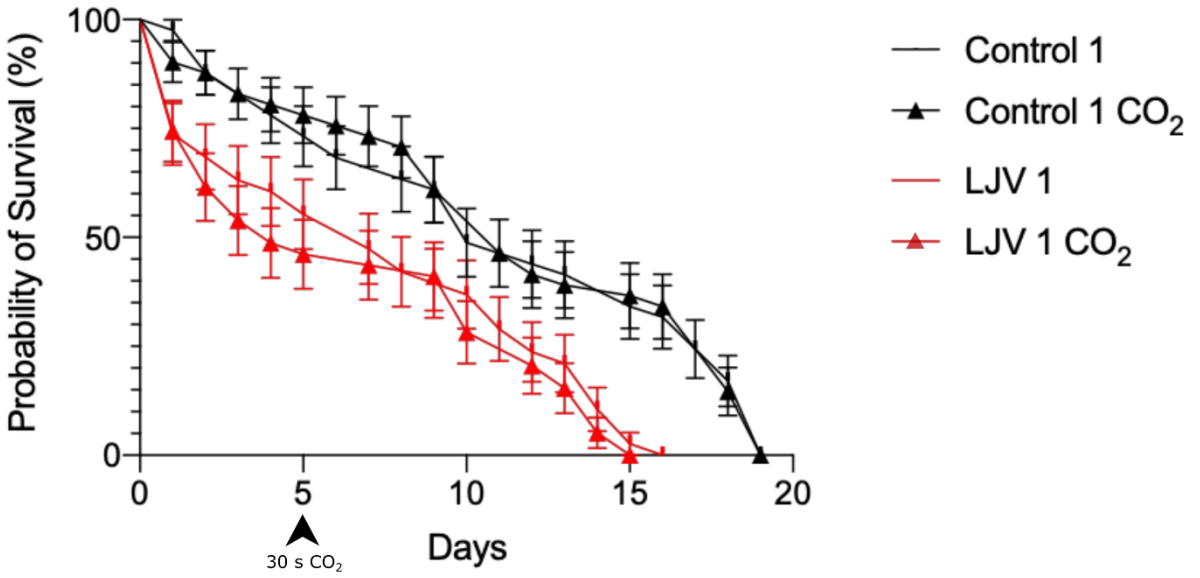


Figure 25. Survival assays after CO_2 exposure. Flies (3–7 days old) were infected with LJV and the lifespan was monitored daily at 26 °C. The arrow underneath the abscissa points towards the day of the CO_2 exposure for 30 seconds. Experiments have been performed three times. $P < 0.005$ for LJV- and mock-injected flies; $P > 0.005$ for LJV CO_2- and LJV-injected flies.

In a first experiment, the recovery time required after 30 s of CO_2 exposure was tested (Figure 26-A). No statistically significant differences were observed between both groups to recover from the CO_2 exposition. In the next experiment, the locomotor ability of infected flies was investigated, using the climbing assay. This experiment was design in order to detect any possible deficiency in locomotor abilities inflicted by LJV on *D. suzukii*-infected flies. The climbing assay is widely used method on *Drosophila* to measure the motor activity that requires a brain circuit (Riemensperger et al., 2013). Flies of both groups were tested the fifth day post-injection. Notably, SWD does not seem to show the tendency to climb upwards against gravity, as in the case of *D. melanogaster*. In contrast, *D. suzukii* seems to remain closer to the surface of the substrate. In both groups, only 30% of the fly population climbed higher than 5 cm (Figure 26-B).

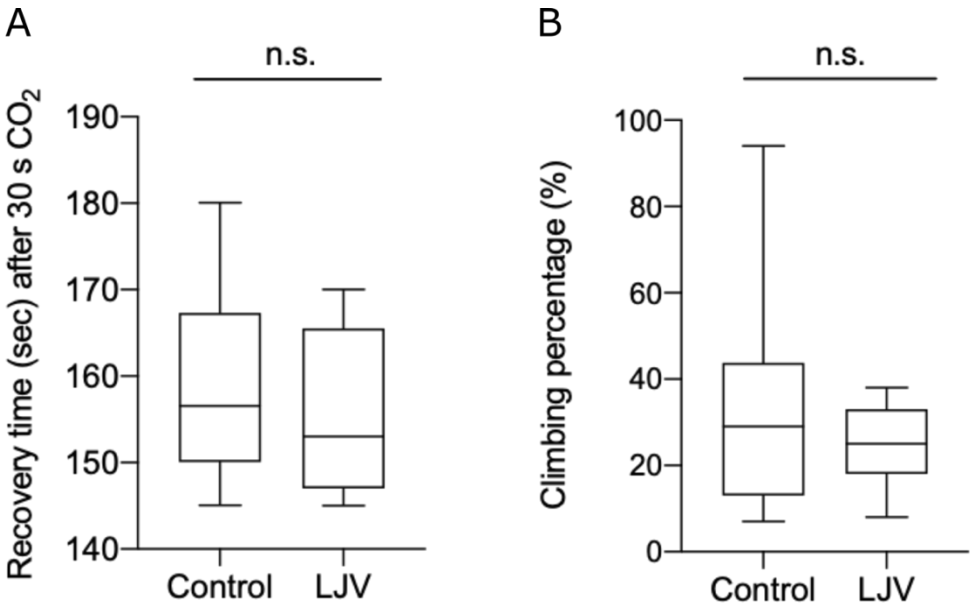


Figure 26. Graphical representation of the fitness assay performed in *D. suzukii*. Box plots representing the recovery time (in seconds) (**A**) or the climbing percentage (**B**) that *D. suzukii* flies required after 30 s of CO_2 exposure. Data represent means ± SD of at least three independent experiments.

5.6 *D. suzukii* immunity against LJV

In order to understand the immune response of *D. suzukii* to LJV, the relative expression of different genes involved was determined by quantitative RT-PCR.

Therefore, the expression of genes that encode putative AMPs was analyzed: two drosomycin-like-6 genes (*drsl6_6667* and *drsl6_7846*). Subsequently, two genes that activate the JAK/STAT signaling pathway were also profiled: the ligand Unpaired 3 (*upd3_b*) and virus-induced RNA 1 (*vir-1_9844*). The latter one is a promoter and general marker of the induction of antiviral response. Finally, a gene encoding negative regulation of the JAK/STAT pathway was profiled: suppressor of cytokine signaling 36E (*socs36E*).

All of these expression profiles are summarized for every gene (Figure 27). Notably, there is a clear pattern of response induction to the infection. The *drsl6* genes are upregulated in this experiment and *vir-1_9844* was moderately expressed. This pattern was even more pronounced for *upd3* and *socs36E.* In this last scenario, a strong expression of *upd3* and *socs36E* was observed after LJV infection.

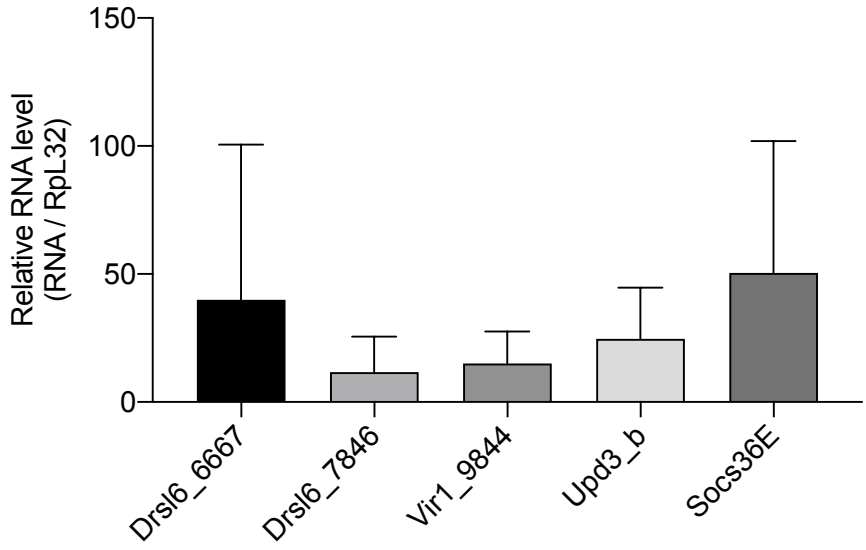


Figure 27. Analysis of immunity-related and stress-response gene expression for each gene. The fold-change in gene expression compared to uninfected controls and normalized to the ribosomal protein mRNA RpL32. Data represent means ± SD of at least three independent experiments.

5.7 LJV serial passage experiment

For a better understanding of viral selection and LJV-host adaptation, a serial passage experiment was conducted. This work was designed to systemically investigate potential changes in the infection development of LJV in SWD as the target host. Flies were inoculated with four different LJV isolates (Figures S1, S2 and S3). Each isolate was used as an inoculum to generate one *D. suzukii*-infected line. After the injection of each passage, insects were allowed to develop the infection for 3 days, then the following passage was carried out. A constant amount of each isolate was used to carry out the following passage. For the purpose of this thesis, only one isolate was examined: $LJV_{OS20.}$

Figure 28 represents the relative fold expression for each passage with LJV. It increased after passage (P) 8 and remained constant for the rest of this experiment.

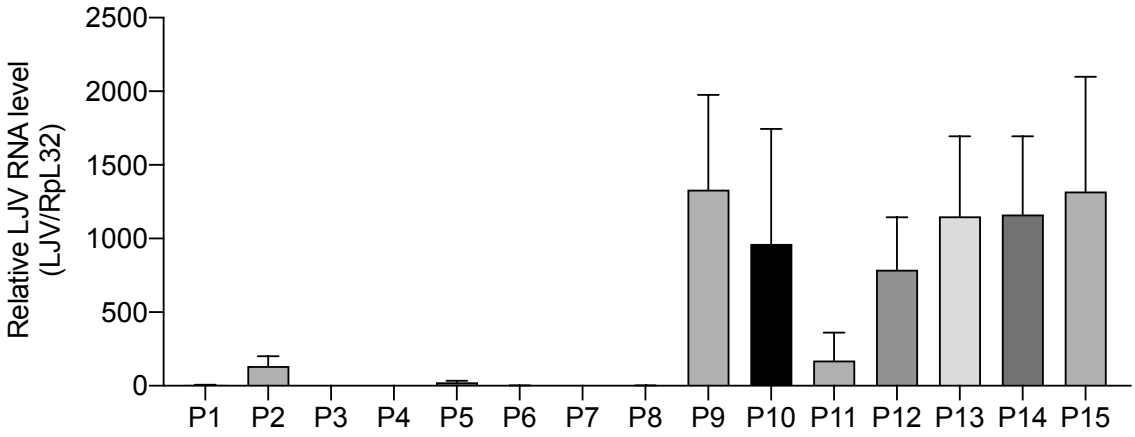


Figure 28. Overview of the passage experiment performed with the isolate LJV_{OS20}. The amount of RNA for every passage was calculated, normalized to the ribosomal protein mRNA RpL32. Experiments were performed with cohorts of 20 *D. suzukii* flies. **P**: Passages

Subsequently, a complete short-read sequencing of both isolates (LJV_{OS20P8} and LJV_{OS20P9}) was performed. Virus coverage showed a difference when mapping both sequences (Figure 29). Complete LJV_{OS20P8} and LJV_{OS20P9} sequences were generated by *in silico* assembly of high-throughput sequencing data. However, such Illumina datasets often come up with problems in the assembly of homopolymeric sequence stretches, as shown at the 5'- and 3'-ends (Figure 29). Additionally, coverage showed two regions without meaningful consensus as also illustrated in Figure 29. The second of these non-meaningful consensus was present in both mapped sequences. Sequence analysis of the mapped isolates revealed eleven single-nucleotide polymorphisms (SNPs). Five of them were present in the L-protein and six in the VP2 of the structural protein region. In agreement with the revision of Robert and Pelletier (2018), the most commonly found SNPs in this part of the study were transitions (A ↔ G or C ↔ T), followed by transversions (A ↔ C or T; and G ↔ C or T).

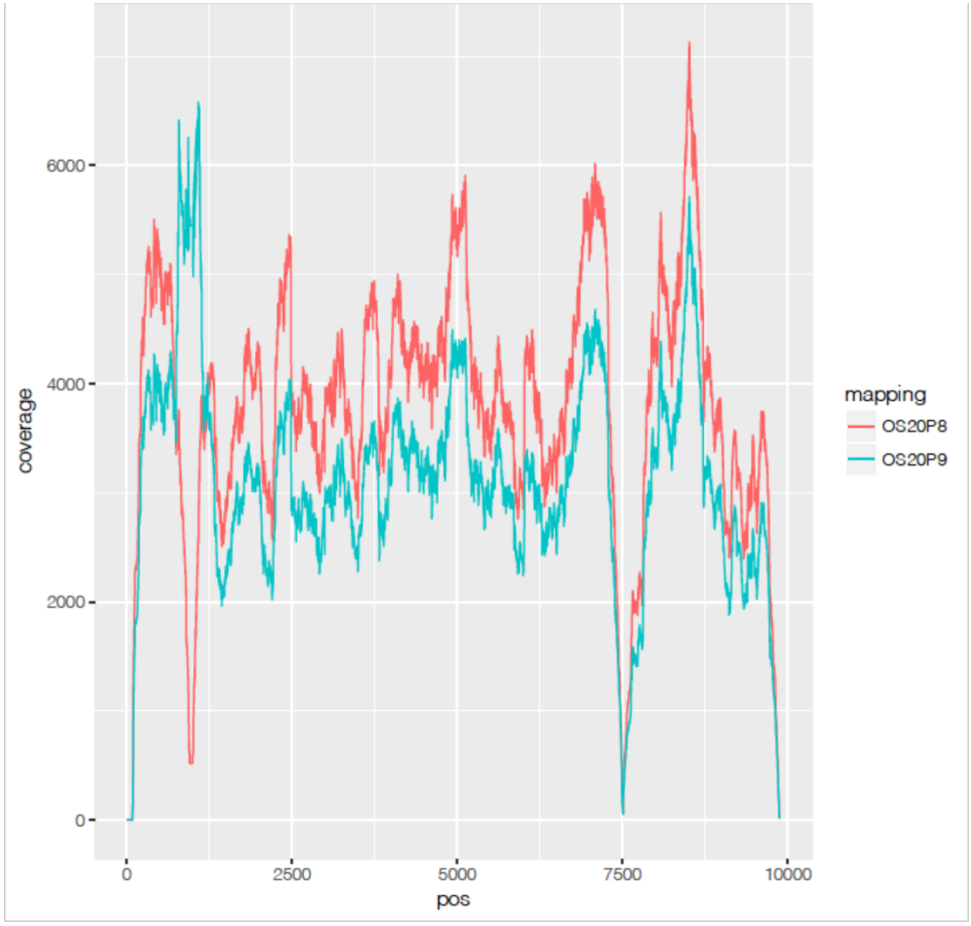


Figure 29. Short-read sequencing of both passages. Mapping of both isolates indicate a difference in coverage.

Sequences were manually translated, and an amino acid exchange from threonine to serine was observed in the polyprotein region of LJV, at the VP2. This phenomenon is illustrated in Figure 30.

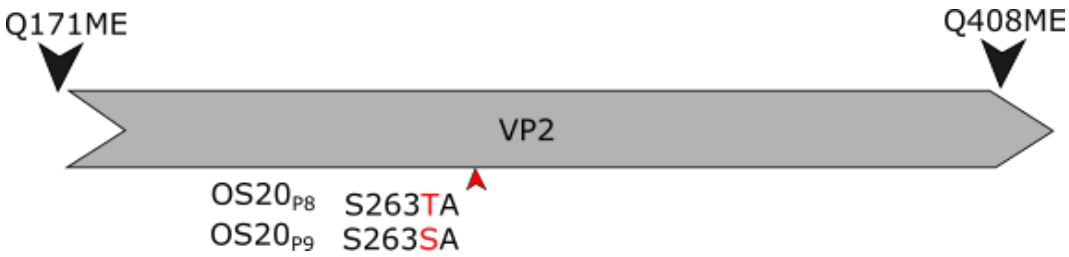


Figure 30. Translation of the affected codon in the one SNP present in the VP2 of LJV. Black arrowheads indicate cleavage sites and red arrowheads indicate the position at the amino acid exchange.

In order to measure a possible increase of virulence, isolates LJV_{OS20P8} and LJV_{OS20P9} were used to perform a survival assay. Isolate LJV_{OS20P8} showed a ST_{50} of 15 days, and most flies succumbed to the

infection by day 20 (Figure 31). In contrast, the ST_{50} of LJV_{OS20P9} was at 8 days in infected flies. All infected cohorts succumbed by day 14 (Figure 31).

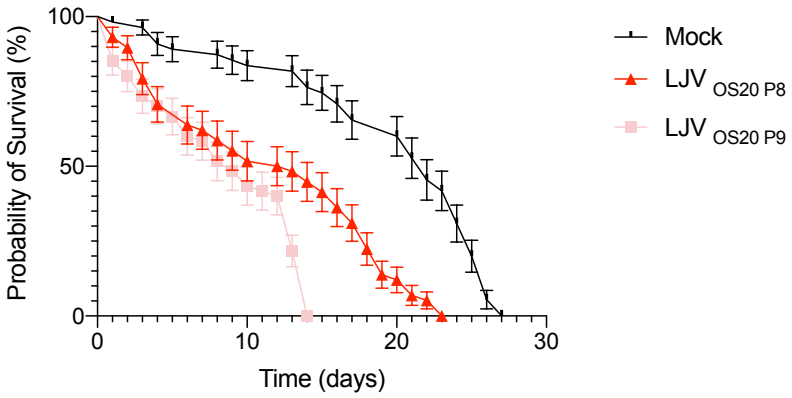


Figure 31. Survival assay carried out with the isolates obtained after P8 and P9. Flies were infected with LJV LJV_{OS20P8} and LJV_{OS20P9}, respectively. Their lifespan was monitored daily at 26°C. Data represent the mean ± SE of at least three independent experiments. Log rank test: ***$P < 0.0001$.

5.8 *Drosophila suzukii* cell culture

Finally, a study of two different cell lines of *D. suzukii* was carried out. This was done in order to illuminate unknown aspects of the immune cellular response of this insect. Subsequently, preliminary steps to establish a primary embryonic cell line cell were made.

5.8.1 Hemocyte characterization

The hemocytes collected from *Drosophila suzukii* larvae adhered to the glass bottom of the cell chamber after 30 min (Figure 32). In contrast to previous studies that used Schneider's *Drosophila* medium, melanization was constantly found to occur when using this particular medium, even if PTU was added (Hiroyasu et al., 2018). Therefore, a protocol avoiding these shortcomings had to be designed. Notably, successful isolation of *D. suzukii* hemocytes could be achieved by using Grace's insect medium, supplemented as described in Material and Methods 4.5.1 (see page 28). This medium was found to avoid melanization, even without the commonly used PTU supplement, as observed in Figure 32.

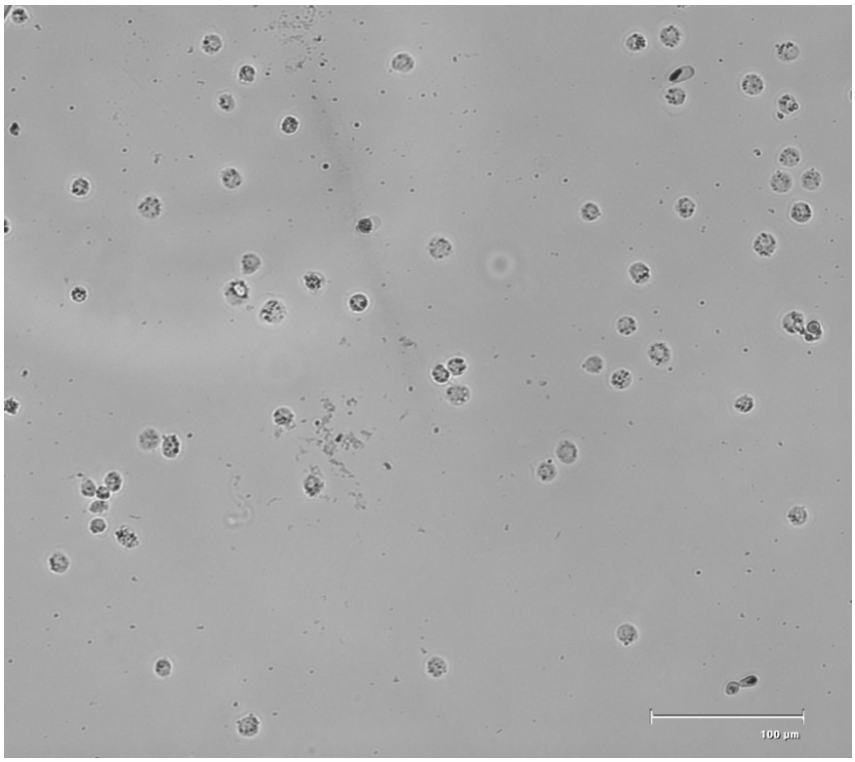


Figure 32. Freshly recovered *Drosophila suzukii* hemocytes. Scale bar corresponding to 100 µm.

The size of freshly recovered cells was in the range of 10 to 30 µm (Figure 32). Plasmatocytes were highly predominant (89.9%), followed by crystal cells (7.5%), while lamellocytes were rarely present (2.6%) as illustrated in Figure 33-A. Plasmatocytes were larger, reaching an average diameter of 35 µm with irregular margins (Figure 33-B). Crystal cells were smaller and darker, mainly because of the presence of crystal inclusions. These cells appeared almost completely round, reaching an average diameter of 10-15 µm (Figure 33-B). Lamellocytes were by far the largest cells isolated, displaying correspondingly large nuclei. As can be deduced from Figure 33-B, they approximately reach 40 µm in diameter.

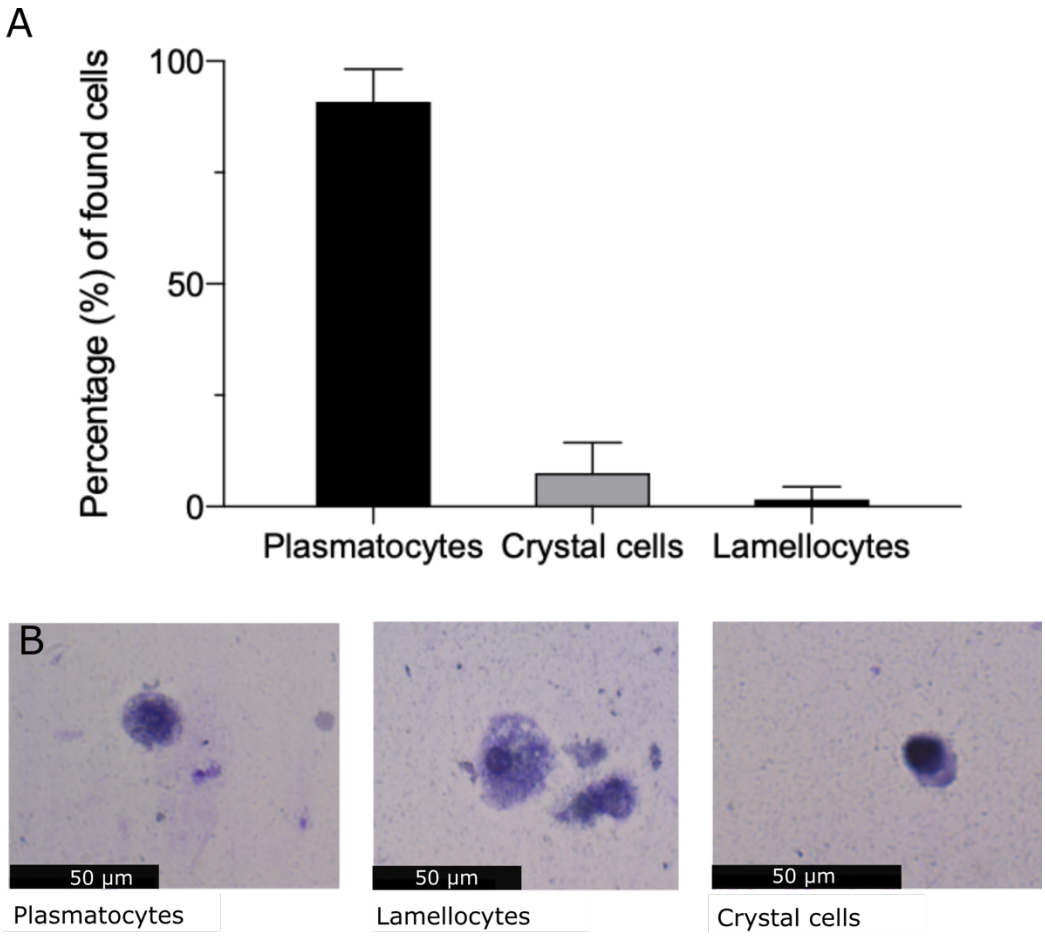


Figure 33. (**A**) Distribution of the different cell types isolated from *D. suzukii.* (**B**) Morphological description of the different types of hemocytes as described in the text. Scale bars correspond to 20 µm.

The interesting cell type for the project were the plasmatocytes because they play a decisive role in the immune response of *D. suzukii*. The presence of these cells was unambiguously confirmed by using specific antibodies (Figure 34). To the best of the author's knowledge, these antibodies have been used to detect plasmatocytes of SWD for the first time. Phalloidin staining revealed that roughly ⅓ of them developed pseudopodia as illustrated in Figure 34.

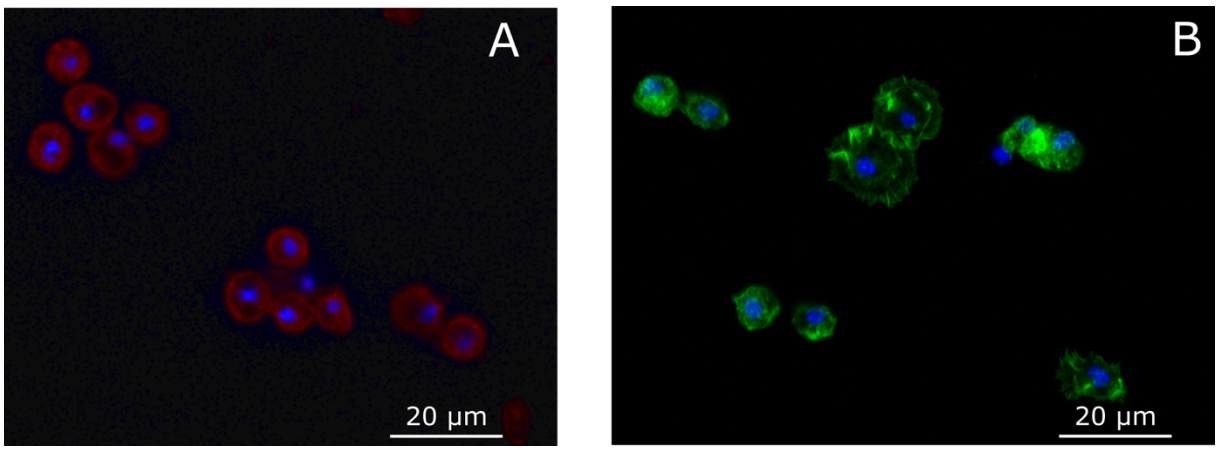


Figure 34. (**A**) Immunostaining of *D. suzukii* plasmatocytes, nuclei stained with DAPI (blue) and anti-Nimrod C1 (red) and (**B**) Confocal microscopy of *D. suzukii* plasmatocytes: nuclei stained with DAPI (blue) actin stained with Alexa 488 phalloidin (green). Scale bar corresponding to 20 µm.

5.8.1.1 Cell viability

Viability of the plasmatocytes obtained was measured with a comparative staining procedure involving the staining agents Hoechst 33342 and SYTOX Green (see Materials and Methods 4.5.2, page 29 and Figure 35-A). Hoechst was used here to mark all cells present in the slide, regardless of their viability state. Combined with a superimposed visualization by SYTOX Green, dead and apoptotic plasmatocyes can be differentiated from viable ones (Figure 35-A). This method was used to quantify relative plasmatocyte viability in each experiment (Figure 35-B). Half of the isolated hemocytes died within two hours post isolation. However, approximately 10% of the population survived up to 3 hours, which is a period to perform experiments to study extracellular traps as a decisive part of the immune response (data not shown).

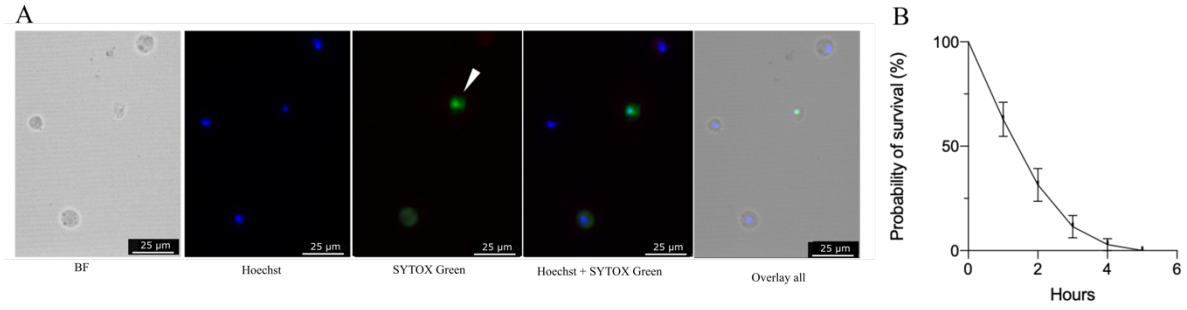


Figure 35. Cell viability assay using SYTOX Green and Hoechst staining. (**A**) Culture with of live (blue) and dead (green) cells. Scale bar corresponding to 25 µm. (**B**) Graphical representation of the survival probability of the isolated *Drosophila suzukii* hemocytes.

5.8.2 Embryonic cell line

The *Drosophila melanogaster* S2 permanent cell line is one of the most widely known and used insect lines in history. This cell line was obtained by Schneider (1972) from late embryonic stages of the fruit fly, *D. melanogaster*, and has also been used for the development of virus-based IPM strategies (Gu & Knipple, 2013). Primary cultures from SWD embryos were established, following the above methodology (see Materials and Methods 4.5.3, page 30). The morphology of the cells was round to ovoid (Figure 36), as previously described by Schneider (1972) for *D. melanogater*. These cells did not form a monolayer but instead coalesced into slow-growing, colony-like structures. In order to increase their growth rate, different concentrations of FBS (10%, 20%, and 50%) were tested in a modified Schneider's *Drosophila* medium but no significant changes could be recorded and the lifespan of the cells averaged 7 days.

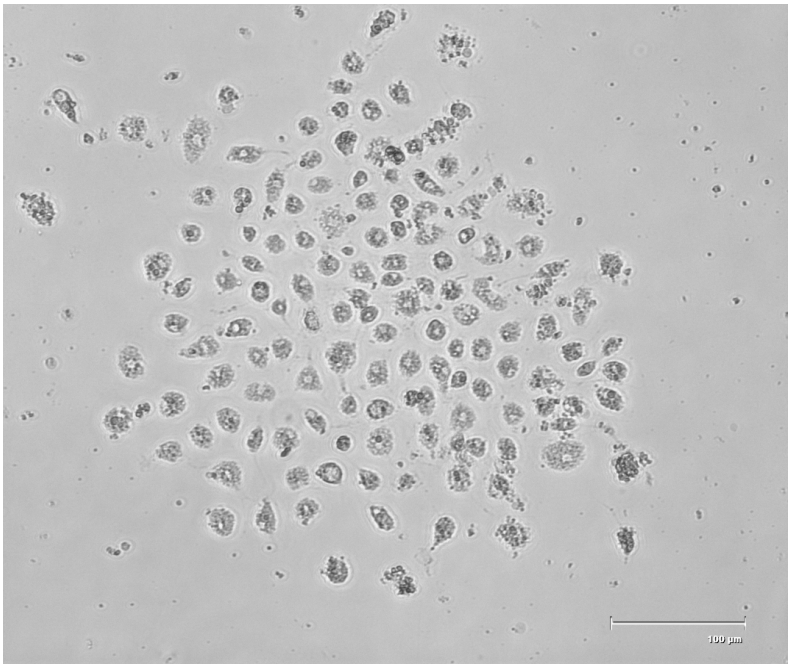


Figure 36. *Drosophila suzukii* primary embryonic cell forming patches. The scale bar corresponds to 100 µm.

In this chapter, the first results on the way to a biological characterization of LJV were described, followed by a study of the immune-related cells, the hemocytes, of *D. suzukii*. Both parts are notable achievements because no data in those fields have been published so far. Last but not least, a host-specific primary cell line was successfully established that will be used for the virus-based IPM strategies.

6 General discussion

In the past decade, *Drosophila suzukii* has become a major pest, causing millions of euros in yield losses to the global agricultural industry (De Ros et al., 2015; Farnsworth et al., 2017; Goodhue et al., 2011). Originally restricted to South-East Asia, it turned into a highly invasive species, which is now considered as a serious threat to modern horticulture in the Middle East, Europe, North and South America (Bolda, 2008, 2010; Deprá et al., 2014; Maier, 2012; Lue et al., 2017). Currently, three reasons are discussed for its invasiveness: the short generation cycle, combined with a high reproduction rate, and its characteristic serrate ovipositor (Lee et al., 2011; Atallah et al., 2014), which facilitates the females to lay their eggs directly in ripening fruit (Tochen et al., 2014; Wiman et al., 2014). To date, no effective measures have been reported to control this invasive species chemically, biotechnologically or biologically. Consequently, *D. suzukii* attracts considerable interest of the scientific community because specific, highly effective and environmentally friendly control measures are urgently required. Viruses have been known since 1951 (Krieg & Franz, 1989) to offer the potential for host-specific and environmentally friendly biocontrol of pest insects (Hunter-Fujita et al., 1998). The close association between insects and insect-pathogenic viruses, which results from more than 200 million years of mutual interaction, has evolved a rich diversity of entomopathogenic viruses that may act as important antagonists (Miller, 1997). So far, more than 1,100 viruses, roughly half of which belonging to the Baculoviridae, have been reported to infect more than 20 different insect orders. However, there is reason to predict that a multitude of them remains to be discovered (Eberle et al., 2012; Miller, 1997).

6.1 Goal 1: Isolation of naturally infected *D. suzukii*-specimens and virus identification

Drosophila suzukii has been recorded in Germany since 2011 (Asplen et al., 2015; Calabria et al., 2012; Deprá et al., 2014; Walsh et al., 2011). In order to evaluate the implications of these findings towards potential invasiveness, an exhaustive sampling of SWD larvae was conducted in the federal state of Hesse, followed by an extensive screening for viruses. A simultaneous screening for bacterial antagonists was recently reported by Hiebert et al. (2020).

6.1.1 Establishment of wild-type rearing

Wild-type populations of *D. suzukii* reared under controlled laboratory conditions offer the possibility to study genetic polymorphisms that can influence their susceptibility to natural pathogens, such as viruses (Magwire et al., 2012). In their work, performed on those SWD lines established during the thesis reported here, Lee and Vilcinskas (2017) studied the *pastrel* gene associated with virus resistance. They found *Giessen* and *Kriftel*, i.e. two wild-type strains of *D. suzukii*, to contain the sensitive allele. When flies were injected with DCV, CrPV, or FHV, respectively, they succumbed more rapidly than flies exposed to injected mock controls. These experiments with model viruses known from *D. melanogaster* were the first, preliminary findings towards biocontrol of SWD. Based on the above study, *D. suzukii* was started to be established as a new model organism for contemporary research on entomopathogenic viruses.

6.1.2 Epidemiology of *Drosophila suzukii* viruses

The search for natural viruses of SWD was the next goal. Upon careful inspection of *D. suzukii* larvae, dead or moribund specimens were separated and characterized according to their main external features. Some of the characteristics found in the larvae studied, such as internal melanization, intersegmental body extrusions, and dorso-ventral flattening, were used as indicators for a potential infection by entomopathogens. A widely known example is the *Infectious flacherie virus,* described in silkworms as highly prevalent in a subset of weak-looking larvae with internal dark brown coloration (Paillot, 1929). Although external pathologies could be related to a wide range of causative agents, the possibility that these features were, indeed, caused by entomopathogens in the sampled larvae remains open.

Subsequently, a screening for all viruses pathogenic to drosophilid species was performed. It should be mentioned here that the virology of SWD has not been extensively studied, so far.

The best approach for this goal was a strategy based on those RT-PCR virus-specific primers described by Meed et al. (2018) and Webster et al. (2015). Their work was based on a global drosophilid sampling, which resulted in the description of approximately 50 new viruses affecting all the *Drosophila* species in their study. Four of those viruses were present in the samples analyzed in this thesis. Teise Virus, LJV and MMlV were found in two out of the three locations sampled, suggesting a high prevalence among the *D. suzukii* populations in the federal state of Hesse. As listed in Supplementary Table S2, LJV and DAV were present in 7.1% and 5.3% of the analyzed samples, respectively. These findings are in agreement data of Medds et al. (2018), who showed Teise Virus as the most prevalent species – both in invasive and native populations of *D. suzukii*. Moreover, flies highly infected with LJV, DAV or MMlV were found in this thesis, i.e., in the non-native range of *D. suzukii*. This result confirms data of Medds et al. (2018). Taken together, the findings presented here imply a great adaptability and variability of this invasive pest to viruses that could be a threat for the biodiversity of native drosophilids (Power et al. 2004; Daszak et al., 2000). The interesting and novel pathogen spillover resulting from the recent range expansion of this fruit fly is a starting point to elucidate the complex interactions of multi-host/multi-parasite systems. Consequently, more detailed studies should be performed in order to fully understand the relationships in such multi-trophic networks.

Recently, Hiebert et al. (2020) demonstrated that *D. suzukii* larvae carry a plethora of bacteria, exhibiting detrimental effects on the survival rate of the flies. They also described notable differences between the cultivable bacteria isolated in the three different locations. However, the viruses described in this thesis appear to be more homogeneously distributed amongst the sampled fly populations. Future work with a wider perspective on the microbiome of SWD is urgently required in order to draw more solid conclusions.

6.3 Goal 2: Characterization of potential viral candidates for biocontrol of *D. suzukii*

Up to date, not a single natural virus from SWD has been isolated. The novelty of this part of the thesis was the pioneering identification and description of two naturally occurring viruses (Carrau et al., 2018). This was done by passaging and maintaining the viruses in injected flies in order to obtain the highest viral yield possible in the final samples. The ultra-centrifugation steps involved in this procedure can be a technically challenging process. Viral particle ultra-purification is standard practice to purify, analyze, and identify the genome of viruses. This technique includes a particle-refining process to achieve a high level of viral purity. However, steps aiming towards an upscaling strategy were taken by replacing the ultra-centrifugation by precipitation as described. The process, described by Carrau et al. (2018), is based on the use of PEG and the homogenate of virus-injected flies to produce a concentrated viral sample. The latter was then used to conduct a functional characterization and some preliminary morphological studies on both viruses.

6.3.1 *Drosophila* A Virus

Drosophila A virus was the first potential candidate to be investigated as a BCA. It is a positive-sense RNA virus, which has been studied in *D. melanogaster* since the 1970s (Plus et al., 1975). This particular virus, related to the *Permutotetraviridae*, has an unusual T=3 icosahedral core and a permuted vRdRp (Ambrose et al., 2009). This particular set of features is only found in a subset of the double-stranded RNA viruses in the families *Birnaviridae* and *Tetraviridae* (Ambrose et al., 2009). Medd et al. (2018) have reported the presence of this particular virus in *D. suzukii* at new areas outside its original range, including sites in Japan, France, and the UK. This virus is particularly known as an important pathogen of *D. melanogaster*, and, along with DCV and DPV, is responsible for ~40% of viral infections in wild fly populations (Jousset et al., 1972; N. Plus & Duthoit, 1969; Nadine Plus et al., 1976).

Notably, this thesis presents the first functional studies and preliminary characterization of the DAV isolated from *D. suzukii*-infected flies (Carrau et al., 2018). The identity of the ssRNA virus was confirmed by performing an analysis of the nucleic acids encapsidated in virions, as previously published by Ambrose et al. (2009). Expected similarities were achieved when the DAV-precipitated particles

were treated with RNase, thus confirming the presence of DAV in our isolate. Additionally, the structural proteins of the virus were analyzed by SDS-PAGE, which yielded a unique and intense protein band of 42 kDa in size. This is in perfect agreement with data reported by Ambrose et al. (2009).

When bioassays were carried in *D. suzukii*, DAV replicated in these flies, thus confirming host-virus compatibility. Further genome analyses might lead to a better understanding of the possible adaptation of this isolate to *D. suzukii*. When flies were dissected three days after viral inoculation, DAV was preferentially localized in the insects' heads rather than in the thoraci or abdomina, suggesting it may preferentially infect the brain, ganglia, and nerves.

6.3.2 La Jolla Virus

La Jolla Virus was the next biocontrol candidate studied. It is a recently described species of the Iflaviridae (Webster et al., 2015). According to Shi et al. (2018), the size of LJV genome is 10,250 nt, and it presumably carries a VPg and a 3' end poly-A tail. Mediated by an IRES, a hypothetical polyprotein of 3,057 aa is translated from an ORF.

For comparability purposes, the same experiments were carried out as reported above for DAV. The ssRNA identity of this Iflavirus was confirmed and SDS-PAGE was also performed for the analysis of the structural proteins of the virus. Similar results as for DAV were obtained.

Host specificity assays were carried out as described for DAV. These experiments, which have not been reported in literature before, showed significant replication of this particular virus, thus indicating host-virus specificity. Remarkably, LJV appears to infect a variety of tissues in *D. suzukii*; however, a clear preference to the central nervous system, has been observed.

An extensive literature search revealed that – in contrast to DAV – no genomic studies of LJV and its deadly effect on *D. suzukii*-infected flies have been published yet. Therefore, this Iflavirus was a suitable candidate to be extensively studied in this thesis.

6.3.3 LJV as candidate for biocontrol of *D. suzukii*

Currently, insect viruses are increasingly scrutinized as biopesticides. The main focus of research is still on Baculoviruses, which have been commercialized because their entomopathogenic potential is well-known for decades (Prasad & Srivastava, 2016). However, short-term effects on the host and long-term

biological control have only been achieved in a few cases, such as in the well-known *Oryctes* virus for biocontrol of the Rhinoceros beetle (*Oryctes rhinoceros*) in South-East Asia and the Pacific Islands (Huger, 2005; Wang et al., 2007). Notably, more sustainable success of baculoviruses in IPM has been reported when they are combined with other BCAs (Moreau & Lucarotti, 2007). Bird & Elgee (1957) reported on a sawfly virus transmitted by the two highly specialized parasitic wasps, *Dahlbominus fuscipennis* and *Exenterus claripennis*. This was one of the first examples of successful virus-mediated biocontrol, regulating the population of *Gilpinia hercyniae*, the European spruce sawfly, in North America. The broad and widely accepted concept of IPM includes the use of one or more pest-specific natural predators, entomopathogens, as well as preventive cultural measures, alongside with the responsible use of chemical pesticides. A sophisticated combination of several approaches is the core concept of IPM. Consequently, the use of a mixture of different viruses is a valid alternative within the IPM framework. In nature, the interactions between different virus species are often regarded as an inevitable consequence of multiple, simultaneous infections of a given host (Berényi et al., 2006). Recently, the combination of a baculovirus and an iflavirus has been shown to increase both the infectivity and physical stability of the viruses towards *Spodoptera exigua* (Jakubowska et al., 2016).

In most cases, iflaviruses seem to cause inapparent sublethal infections in insect hosts, although previous findings in honeybees and silkworms argue for lethal infections of those particular host species. Notably, Carrau et al. (2018) were able to demonstrate a deleterious effect of LJV on the lifespan of *D. suzukii*. This result is in perfect accordance with the previously published reports (Aizawa & Kurata, 1964; Ribière et al., 2010). Not only could the lethal effect on flies be shown, but also a high host-specificity with significant viral replication within the fly.

6.3.3.1 LJV purification and genome characterization

For this purpose, a protocol to concentrate the viral particles by ultra-centrifugation was developed, consisting of a glucose cushion that is subsequently purified in a CsCl gradient. The small amount of virus found in a single fly is challenge in itself, with roughly 6 g of female flies (~6,000 flies) being needed to obtain enough viral particles. Flies were allowed to develop the infection for 3 days. This part of the project is more extensively discussed in the published work of Carrau et al. (2021).

Recovered particles were then examined morphologically via EM, which conclusively proved a correct ascription to the family Iflaviridae. Virion size was approximately 30 nm, which falls within the scale reported for other Iflaviruses such as the DWV (Kalynych et al., 2017). The overall appearance was consistent with that reported for other insect RNA viruses such as nodaviruses, tetraviruses and picornaviruses (Johnson & Reddy, 1998; Schneemann et al., 1998; Tate et al., 1999). Additional work to fully characterize LJV would imply the use of cryogenic electron microscopy (CryoEM) and a reconstruction of the genome in order to determine the structures of LJV particles.

In Iflaviruses, structural polyproteins are preceded by the L-protein. In picornaviruses, this protein seems to be an indicator of pathogenicity because it is the effector of RNA translation and protease activity of virus and host (Glaser et al., 2001; Guarné et al., 1998; Hinton et al., 2002) as illustrated in Figure 37. The L-protein of LJV, which is encoded by 171 aa in this particular strain, was successfully sequenced in this thesis.

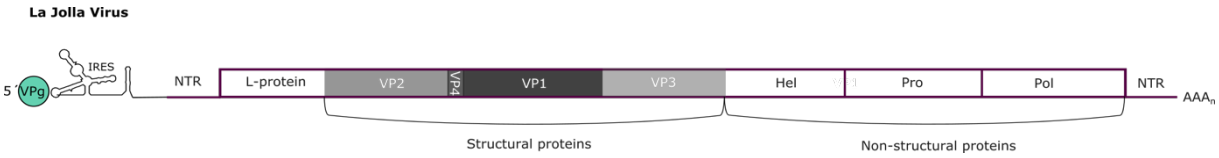


Figure 37. Schematic diagram of the genome organization of LJV. The genome-linked protein (VPg) at the 5'-end and the structural proteins (VP1–VP4) are shaded. Additionally, the L-protein is located at the beginning of the ORF, and the non-structural proteins are found in the third part of the genome. LJV displays a poly(A) tail on the 3'-end.

Adjacent to the L-protein, structural proteins are located behind, i.e., in the N-terminal third downstream. The structural proteins of LJV were found to have the same organization as those of SBPV and DWV, respectively (Procházková et al., 2018; Kalynych et al., 2016; Škubník et al., 2017; Kalynych et al., 2017). Additionally, it was found that the cleavage sites of these polyproteins are highly conserved amongst all published strains of LJV, as illustrated in Figure 38. Adjacent to the structural polyproteins, a sequence of non-structural proteins is found at the C-terminus. In Iflaviridae, non-structural polyproteins contain a conserved helicase–protease–polymerase (Hel–Pro–Pol) module of core replication domains (Le Gall et al., 2008), as illustrated in Figure 37. These enzymes are responsible for proteolytic processing of viral polyproteins, a crucial step in genome replication and capsid assembly (Dougherty & Semler, 1993). Ye et al. (2012) reported that the cleavage sites are highly conserved in all Iflaviruses.

The non-structural proteins are found at the C-terminus. Remarkably, two thirds of this polyprotein consists of multiply conserved domains (de Miranda et al., 2010; Procházková et al., 2018), as illustrated in Figure 37. Although the N-terminal sequencing of the non-structural part is not considered here, it seems likely that LJV follows this same pattern. Further work must be done to complete its genome sequence.

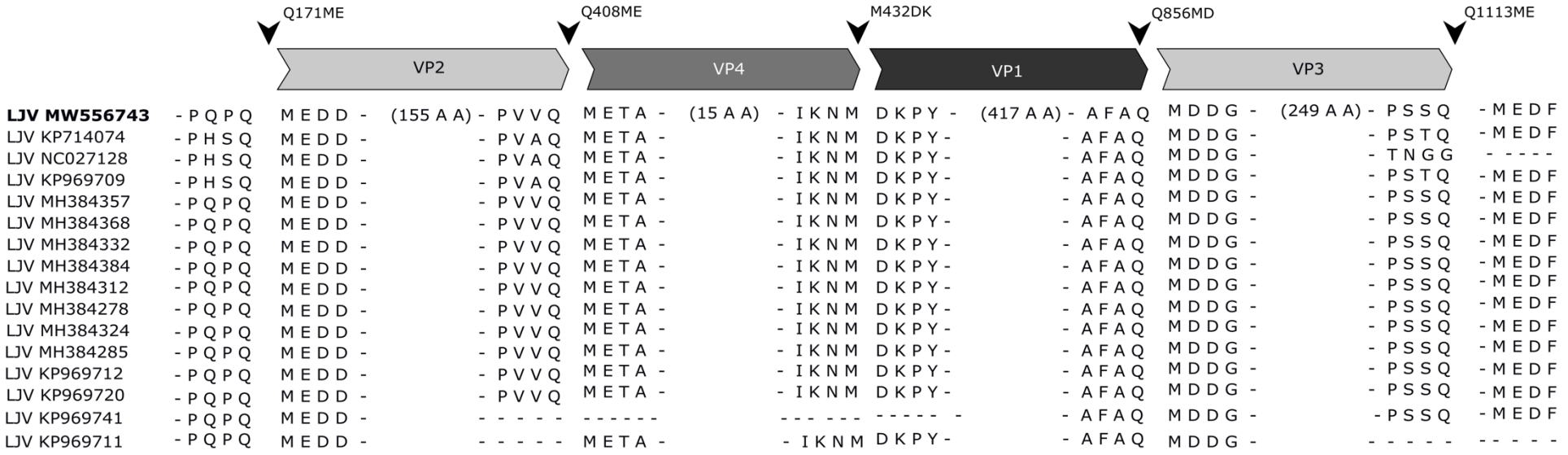


Figure 38. Multi-sequence alignment of the P1 cleavage sites in LJV. Comparison of the P1 cleavage sites in LJV strains. Capsid proteins (VP2, VP4, VP1, and VP3) within the P1 are drawn in greyscale. Arrowheads indicate cleavage sites as annotated. Note that all assumed cleavage sites within the structural protein region are highly conserved.

When comparing the outcome of the RACE-PCR with previously published data, the 5'-UTR elements appear complete and include a hitherto unknown stretch of 10 nt (GAAAAGTAGT) at the 5'-end of the genome. As described by Carrau et al. (2021), this might be an indication of specificity for this particular strain. Similarly, the six nucleotides in front of the 3'-poly-A tract (ATATAT) are a distinctive motif of the strain described in this thesis. However, its apparent uniqueness may be owed more to oversight because the other (nearly) complete LJV sequences in the database were generated by *in silico* assembly of high-throughput sequencing data (e.g., Illumina datasets), which are often problematic for the resolution of homopolymeric sequence stretches. This sequence was used as a basis for a detailed analysis of the LJV virions (Figure 39).

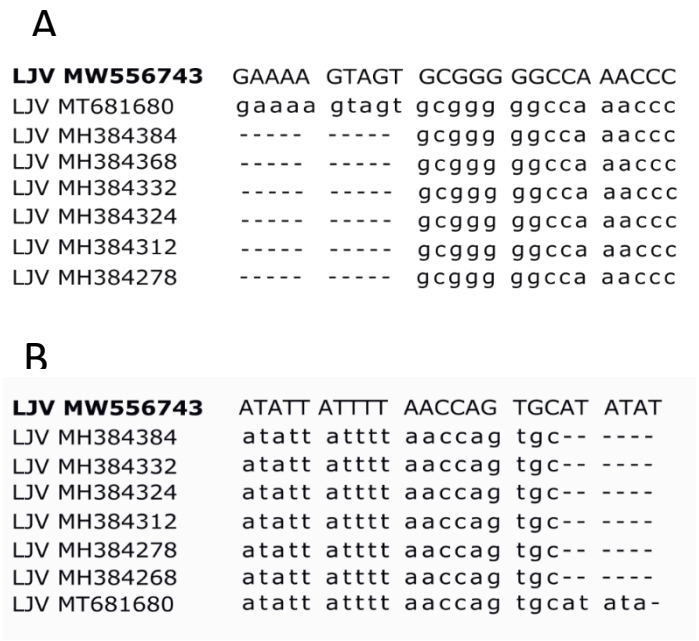


Figure 39. Comparison of 5'-UTR and 3'-UTR sequences described from known LJV strains. (**A**) 5'end-UTR comparison of LJV studied here (MW556743) with seven other strains. (**B**) 3'end-UTR comparison of LJV (studied here (MW556743) with seven other strains.

6.4 Goal 3: Biological characterization of LJV and cell culture

The third goal of the thesis is the detailed study of the main infection features of LJV. In other words, a biological characterization of the virus was performed.

6.4.1 Infection route of LJV and fitness study

When comparing the infection between larvae, pupae, and adults, the latter appeared to be the preferential target. However, it still remains unknown how infection is transmitted. Injection and dissection of different organs of adult flies was performed in order to identify a possible transmission route. A considerable amount of LJV was found in the fat body and in the gut. Horizontal transmission has been reported for some Iflaviruses (Murakami et al., 2014; Ottati et al., 2020). Nonetheless, in contrast to the situation for other picorna-like viruses such as Nora virus, no systemic infection developed upon oral administration (Habayeb et al., 2009). This suggests that LJV might not able to cross the gut barrier. The absence of the virus in the ovaries seems to indicate that LJV is not transmitted vertically as previously reported for other Iflaviruses, such as the honeybee DWV (Aizawa et al., 1964; Yue et al., 2007). Altogether, the results presented here suggest that there may be a potentially complex interaction:

One possibility would be spillover through parasitoid wasps, as discovered for the Moku virus, another recently described Iflavirus (Mordecai et al., 2016). The second possibility, a complex vector-host relationship between an ectoparasite and the virus, cannot be excluded either. Such scenario was reported for the DWV of the honeybee and the notorious mite *Varroa destructor* (Posada-Florez et al., 2020).

As outlined in the Results section 5.3.3 (see page 41), the virus was mainly present in the insects' head. In order to verify the possible neurological symptomatology in the infected *Drosophila*, flies were experimentally exposed to stress. This effect has already been described to be caused by asymptomatic viral infections of *Drosophila*. One of the most widely known examples is the asymptomatic Rhabdovirus infection that has shown to be lethal for *Drosophila* under environmental stress conditions such as excess of CO_2 (Chow et al., 2017). LJV-infected flies exposed to CO_2 did not display pronounced loss in viability after CO_2 anesthesia. In contrast to rhabdoviruses, this particular Iflavirus does not increase sensitivity to environmental stress. In order to detect possible deficiencies in locomotor abilities

of LJV-infected *D. suzukii*, a "climbing assay" was used. It measures a motor activity that requires an unimpaired brain circuit (Riemensperger et al., 2013). In contrast to *D. melanogaster*, SWD does not seem to have the tendency to climb upwards against gravity. Nonetheless, LJV did not seem to affect the locomotor system of *D. suzukii* – even after systemic infection. For these reasons, a detailed study of the underlying pathologies inflicted by LJV onto *D. suzukii* requires further and more case-specific experiments for any substantial conclusion to be drawn.

In a next step, expression profiles for four *D. suzukii* genes, coding for AMPs, were analyzed. The expression of three drosomycin-like genes was found to be upregulated. This result is in accordance with Zhu et al. (2013), who suggested that drosomycin might play a relevant role in viral immunity – different from that of other AMPs. Similar responses were observed for the genes encoding the JAK/STAT pathway (*socs36E*, *vir-1* and *upd2*), which were upregulated after infection of the flies with LJV. For instance, Dostert et al. (2005) suggested that the function of the JAK/STAT pathway in the control of viral infections has been conserved throughout evolution, and fruit flies seem to adhere to this theme in their innate immune system.

6.4.1 LJV serial passage experiment

Serial passage experiments were carried out, which are a well-established tool for the experimental study of evolution and host-virus adaptation (Ebert, 1998). Passaging was performed *in vivo* and designed to modify lethality and multiplication of the LJV isolates. From the overall biological replicates (n = 57; Supplementary Figure S1 and S2), LJV_{OS20} increased its replicability after the 8^{th} passage. In addition, several SNPs were found in between both sequences. One of the previously mentioned changes had a translational effect on VP2. This change might have increased the virulence of the virus and its ability to adapt to the host, ultimately by increasing its replication rate (Figures 29 and 31). Nonetheless, S ↔ T is a homologous exchange, so that a drastic influence on the phenotype is unlikely, but cannot be excluded. Nucleotide exchanges could alter RNA structures that are important for replication or genome stability. A precise clarification of the significance of the individual mutations could only be analyzed with a reverse genetic system. RNA viruses are notorious for their high mutation rates, and positive-strand viruses may have higher mutation rates than negative-strand RNA viruses (Domingo and Holland

1997; Drake and Holland 1999). However, the assessment of viral mutation rates and potential implications in comparative evolution studies are outside the aims and scope of this thesis. Further studies, combining gene technology with additional survival assays for each passage, will be inevitable to draw robust conclusions.

In accordance with Ebert (1998), the findings presented here might indicate an adaptation of the pathogen to a new environment. This phenomenon is characterized by an increase in virulence caused by the pathogen's enhanced ability to grow in the new host. The specific scenario presented here, could possibly indicate that LJV has recently switched hosts, most probably by jumping from another drosophilid to *D. suzukii*. This theory might be supported by previous phylogenetic studies (Medd et al, 2018; Carrau et al., 2021). Nonetheless, parallel experiments in other drosophilds would be useful to reinforce this hypothetical conclusion. On the other hand, there are also records of increased virulence after serial passages in its original host. For instance, Chapuis et al. (2011) concluded that the lack of selective pressure, otherwise present in the natural environment of the virus, might triggered this evolution towards higher virulence. Nevertheless, more detailed experiments should be performed in order to contribute to the understanding of this complex host-virus relationship.

6.4.2 *Drosophila suzukii* cell culture

6.4.2.1 Hemocyte characterization

The establishment of specific protocols adapting current hemocyte isolation procedures to *D. suzukii* is crucial for the investigation of its cellular immune system (Harrison et al., 1995; Luo et al., 1995). High abundance of plasmatocytes and fewer circulating lamellocytes was observed, a situation common to other drosophilids whose hemolymph has been studied in more detail (Rajak et al., 2014). As previously described by De Lavine et al. (2002), hemocytes can be used to investigate specific cellular defense reactions such as phagocytosis, microaggregation, nodulation, and encapsulation. All of these immune responses are of high medical relevance. Consequently, they may influence similar interactions between insects and their pathogens or parasites.

Previous work suggests that the evolutionary success of pest insects, among other reasons, can be attributed to their sophisticated immune system, which allows them to effectively defeat pathogens and

parasites in their environments (Gegner et al., 2018; Vilcinskas et al., 2013). The role of insect innate immunity in the successful colonization of novel ecological niches is clearly demonstrated by invasive insects. A robust immune system is proposed to assist invasive species in colonizing habitats that have previously been occupied by unfamiliar pathogens (Lee & Klasing, 2004; Poyet et al., 2013). This phenomenon has recently been confirmed for the invasive harlequin ladybird, *Harmonia axyridis* (Gegner et al., 2018). In the case of *D. suzukii*, several established biocontrol strategies such as the use of natural enemies as BCAs have been studied in laboratory scale. When *D. suzukii* larvae are parasitized by wasps, higher numbers of plasmatocytes, crystal cells, and lamellocytes are produced compared to larvae of *D. melanogaster* (Kacsoh et al., 2012). This suggests that *D. suzukii* has evolved a strong immune response to parasitism. Recently, Schetelig et al. (2018) reviewed environmentally sustainable pest control options for *D. suzukii* and concluded that no effective BCA has been reported for this insect pest.

Consequently, targeting of its cellular immune response has to be exploited in more detail to increase the sensitivity of this pest towards its natural enemies. In this context, the availability of a protocol for primary hemocyte isolation and characterization of plasmatocytes, as established here, enables the *in vitro* testing of its immune response. Notably, the first description of specific antibodies for plasmatocyte characterization was performed in this thesis. These antibodies have been described for expression of the P1 molecule, NimC1, confined to cells with plasmatocyte morphology at all stages of the larval development and the adult life (Kurucz et al., 2007). These findings are in agreement with previous reports on larval hemocytes (Kurucz et al., 2007). Because plasmatocytes are able to detect a foreign body, they activate the cellular immune response of the host by forming a capsule. This cascade eventually kills the parasite – either by asphyxiation or by local production of cytotoxic free radicals, quinones or semiquinones (Nappi et al., 1995; Russo et al., 1996). Hence, the isolation protocol described here can be used to study the immunology described above, which is involved in the SWD host-pathogen response.

6.4.2.2 Embryonic cell culture

The importance of any primary cell culture is that it closely resembles *in* vivo-conditions (Carter & Shieh, 2010). Nonetheless, it is also the decisive first step for the establishment of permanent lines. Once

this aim has been achieved, mass production of either viruses or bioactive secondary metabolites for IPM is possible (Smagghe et al., 2009).

The successful establishment of dipteran cell lines has usually requires embryonic stages, such as the S2 cells described by Schneider et al. (1972). This is mainly due to the mitotic activity patterns in the first 6 to 8 hours of the embryo's life, which are different from those associated to the latter organ differentiation that occurs in the following stages. It is assumed that this mitotic activity can be maintained *in vitro* when using the optimal age range of donors.

In this part of the thesis, replication was observed, but the lifespan of the cells was relatively short, averaging 7 days. According to Schneider et al. (1972), the culture was supplemented, but this did result in any observable differences.

In a nutshell, these findings indicate the need for future work aimed at optimizing certain biotic and abiotic conditions of the culture media before substantial progress can be achieved in this field.

6.5 Conclusions and perspective of the project

Currently, the economic impact of *D. suzukii* to the agricultural industry accounts for millions of € in crop losses (Farnsworth et al., 2017; Goodhue et al., 2011; De Ros et al., 2013). In 2009, losses of more than 500 million US $ were recorded for different cultivated fruit crops i.e., strawberries, blueberries, raspberries and blackberries, (Walsh et al. 2011). In 2010, Europe lost 80% of the strawberry yield because of the *D. suzukii* infestation (Lee et al. 2011). In the Trentino region (Italy) alone, the losses in fruit cultivation (cherries, raspberries, and blueberries) were estimated at 3 million € due to SWD infestation (Wenneker et al., 2015). This, combined with the rapid global spread of this insect, makes *D. suzukii* a serious threat for modern horticulture. Hence, it is increasingly more imperative to find effective and ecologically safe IPM strategies for its control. Notably, viruses offer the possibility to develop host-specific and environment-friendly control agents (Hunter-Fujita, 1998).

As outlined in this thesis, the approach to contribute to a fundamental understanding of the epidemiology of SWD viruses resulted in highly valuable findings: four viral candidates were obtained that can potentially be used as BCAs; however, some of them remain to be characterized. During this first viral screening, LJV was studied in greater depth. Besides DAV and LJV, MMlV turned out to be another interesting virus, and, to the best of the author's knowledge, unstudied species. As a new pathogen of *D. suzukii*, this latter virus represents another potential candidate to be considered as a BCA.

During this thesis, unknown biological and genetic viral features were studied, and a clear, first picture of LJV was drawn. Nevertheless, further work will have to be performed in order to understand the transmission of this particular virus. Horizontal transmission appears most likely, but additional experiments have to be conducted in support of this hypothesis. A formulation to implement LJV oral infection could be an elegant solution to address this question.

The genome characterization discussed here, together with the implementation of reverse genetics for RNA viruses, will allow to create and study a desired phenotype under laboratory conditions. Most probably, these tools might unravel the unknown biological characteristics of these viruses.

Nonetheless, additional experiments and investigations are necessary to accomplish the mass production of any desired viral candidate. The establishment of SWD-specific cell lines could result in a permanent cell line, given that the physiological parameters of the medium can be optimized. The steps summarized

in this outlook are recommended to be performed to successfully isolate and eventually produce natural SWD viruses in large scale in order to develop an ecologically friendly and sustainable BCA for this insect pest of global economic importance.

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Appendix

Table S 1. Primers used to detect *Wolbachia* infection in the flies. **F**: Forward primer; **R**: Reverse primer.

Oligo name	Sequence (5'-3')	Product size (bp)
*Wolbachia*_F	TGGTCCAATAAGTGATGAAGAAAC	610
*Wolbachia*_R	AAAAATTAAACGCTACTCCA	

Temp	Time	Description
95°C	5 min	Initial denaturing
35 Cycles of		
94°C	30 sec	
55°C	30 sec	
72°C	90 sec	
72°C	7 min	Final extension

Table S 2. List of primers of the known drosophilid viruses (Medd et al., 2018; Webster et al., 2015). **F**: Forward primer; **R**: Reverse primer.

Oligo name	Sequence (5'-3')	Product size (bp)
DCV_F	AAAATTTCGTTTTAGCCCAGAA	250
DCV_R	TTGGTTGTACGTCAAAATCTGAG	
DAV_F	AGGAGTTGGTGAGGACAGCCCA	146
DAV_R	AGACCTCAGTTGGCAGTTCGCC	
Nora_F	ATGGCGCCAGTTAGTGCAGACCT	410
Nora_R	CCTGTTGTTCCAGTTGGGTTCGA	
Bloomfield virus_PCR_a_F	ATTTTTGGACTCAGATTGG	900
Bloomfield virus_PCR_a_R	GCCAAAATACTTGTTCCAG	
Bloomfield virus_PCR_b_F	CTATGGTTATCGATTGCATGGTCC	1000
Bloomfield virus_PCR_b_R	GTAAACAAATCAAAACCATC	
Chaq virus_PCR_F	AACAGAACGWCTGCTTTTTGGAAATCC	520
Chaq virus_PCR_R	TCCATGTCCTGTHGGGTCTATCTG	

Craigie's hill virus_PCR_a_F	GCAAAATCCGTGGTTCATACCAG	1200
Craigie's hill virus_PCR_a_R	CTTAACAGGACGCTCCAAGTGGAT	
Craigie's hill virus_PCR_b_F	CCTATCTGTCAAGCTGTWCTGCCAAC	1400
Craigie's hill virus_PCR_b_R	GTGTGCCAACTAGGCTCAGGAG	
Dansoman virus_PCR_F	GCGCAGACGGAGGACGGCA	1300
Dansoman virus_PCR_F	ARCGGKGTCACWCGCGGCTC	
Galbut virus_PCR_F	GATCGAGATGGAACTCCRCTCTC	580
Galbut virus_PCR_R	GCCKCATACTTGGTGCTGCCAACTG	
Kallithea virus_PCR_F	CGACATCACATTCGACCCATATCC	970
Kallithea virus_PCR_R	TCCCATAAAGTGCGATCCCATAG	
La Jolla virus_PCR_F	GTGGAGTAAAGCAACGACTTGG	1300
La Jolla virus_PCR_R	CAACTGCRTGTTTGAGTTCCCAACGA	
Motts mill virus_PCR_F	AATCGCTCCAMYCCAGGCACTAC	920
Motts mill virus_PCR_R	TGGTAGCTGTYTTCTGRGCAGC	
Newfield virus_PCR_F	GGCTGTTACGGTGATGATGG	1057
Newfield virus_PCR_R	CCGCTGAAAATACCGCTCA	
DMELSV_PCR_F	GGATTCAAAACCCTTTAATATCTGGCCT	600
DMELSV_PCR_R	CCTGACATCAAGACGTAAACCTCTGA	
Thika virus_PCR_F	CTTCGAAGCATCYCTGCATCGTAAAG	900
Thika virus_PCR_R	GCACCCACAGCTAGCATRTCTGG	
Torrey pines virus_PCR_F	GACGTCVTACATCAACGCTAACACGG	480
Torrey pines virus_PCR_R	CACGACTGCAGGAGCATCATTAAC	
Twyford virus_PCR_F	CGCAGTCAGTTTGCATCAGG	900
Twyford virus_PCR_R	CTCAGCTAAGGAGCCTTCCAT	
Berkely_Virus_PCR_F	TTCCCGGCTGATTCAACTAC	517
Berkely_Virus_PCR_R	TTCCTGGTAACAAGCCCAAC	
Brandeis_Virus_PCR_F	GCTCCCCAGAAATCATTGAA	593
Brandeis_Virus_PCR_R	GCGTTGGTATGTGACGTTTG	
Charvil_Virus_PCR_F	CAGAGCGGTGATGAGACAAA	554
Charvil_Virus_PCR_R	AGCCCTCTGGTGTGGTATTG	
Kilifi_Virus_PCR_F	CCACTCAGCATTCAAGCAAA	591

Kilifi_Virus_PCR_R	GGATTGGGATGGAAGGTTTT	
Bofa_Virus_PCR_F	AAAGCCGTCGCTCAATTATG	511
Bofa_Virus_PCR_R	CTATTTTGCTTGGCCCTTCA	
DmelBunyalike_PCR_F	AACCCATTGACTGCCAACTC	574
DmelBunyalike_PCR_R	GTTGGCCACAAGGTATGCTT	
DmelFlaviLike_1_PCR_F	CCCACACCCCGATACATTAC	579
DmelFlaviLike_1_PCR_R	CTTGTATGGCCATTCCGTCT	
DmelFlaviLike_2_PCR_F	CTTGCATACAAACCGGAGGT	509
DmelFlaviLike_2_PCR_R	GCCATTCGTTCGTCGTAAAT	
DmelPartitiLike_6_PCR_F	ATCAACCATGATGGGATCGT	592
DmelPartitiLike_6_PCR_R	TGGTCAGAAGTCCTGTCACG	
Ds_Teise_seg1_00562_F	GCTATGGTTCCGGGCTCAAG	713
Ds_Teise_seg1_01274_R	TCCTTTCCGCCGACATCAAC	
Ds_Teise_seg2_00570_F	TGATCTTGGTCCCGGAAAGC	636
Ds_Teise_seg2_01205_R	GCGTCCATGCGTCCAAATAC	
Motts_like_bridgeB_02798_F	CACTTGCCAGAAACCTTCCG	729
Motts_like_bridgeB_00584_R	CTCCAGTCACGACAACACAC	
Dsuz_Medway_01065_F	AACGTCGGACTTCATCGAGC	502
Dsuz_Medway_01567_R	TCCAACATGTACGGCAAGCC	
Ditton_pol_03095_F	TGATTGCAGGATTGGATGG	713
Ditton_pol_03807_R	CAGCATATCGGTTACAGAGC	
DS_nido-like_03979_F	ATACATGGAATCTGTGGAGGC	686
Ds_nido-like_04665_R	AGCATAATTGACAGTCGAGC	
KL224_Ds_Snodland_01372_R	AGCACACTTGGTTCGTTGC	741
Ds_Snodland_00632_F	TCGGTTCACAGCCAGTATCC	
Larkfield_02095_F	TCAATCAAGCGAGTATCCACC	749
Larkfield_02843_R	ACGATGTTCAATGTCCTAGGC	
Ds_Tama_C_01710_R	AGGTTGATGGGATCGGATGG	645
Ds_Tama_C_1066_F	CATATGTTGACACTGGCTGCG	
Ds_Mogami_02095_F	CCGTGGCGATATGTACTTGC	620
Ds_Mogami_02714_R	TCGGACACTGTAGACTGAGC	

Ds_Nora_Virus_02975_F	ACCACCTGAGAACCTATGGC	804
Ds_Nora_Virus_03778_R	AAGTCGAGTCCTCTACCAACC	
Ds_Barming_04850_F	GTTGCCAATAAGCCTCCATTCC	686
Ds_Barming_05536_R	CCTGTTCTACTGCAGCTTGG	
Ds_Cyril_B_01230_R	TGCTAGTTACTGGGTCACGC	918
Ds_Cyril_B_313_F	ATCCTCAGTTGGCTCGTCG	
Ds_Cyril_A_00954_R	ATTTGCCGCTCTCAACATCG	649
Ds_Cyril_A_00306_F	GCTTCACATCCTCAGTTGGC	
Ds_Naganuma_Virus_00173_F	ATTCCAAGCCGAGACGACC	894
Ds_Naganuma_Virus_01066_R	TCGTTGGAGTCACATCCACC	
Ds_Beult_05955_F	CAGTTGAACGTGTCTCTTAGGTG	836
Ds_Beult_06790_R	CCATCCGTGTATCCATTGGC	
Ds_Saiwaicho_03698_R	TCTTGCACTTACCGACGAAG	995
Ds_Saiwaicho_02703_F	CTGCTGAGAACCGACGATTC	
Ds_Saiwaicho_03300_R	TATCTTCTTGACCGACCGCC	678
Dsuz_Saiwaicho_02622_F	GTGCTTCAACGTACGATCCG	
Dsuz_Broad_00442_F	AGATGTCTAGTGCCGTGCC	867
Dsuz_Broad_01308_R	TCAGCATGTCCAGTTGTTGC	
Eccles_virus_02305_F	TACTATGCTACTCGTCGCGC	745
Eccles_virus_03050_R	CAATTGTTAGCTCCACCCGG	
Ds_Kiln_Barn_03045_F	TGTCGATCCAACCTCTCAACC	671
Ds_Kiln_Barn_03715_R	CGCTGCCATATTCCGTATAGC	
Ds_Notori_02969_F	CAGAAGGAGACAACTTACGG	790
Ds_Notori_03758_R	GGTAATCTTGAAGGCCATCC	
Ds_Murles_01176_F	ATACGCGTGAATTGCAGTCG	782
Ds_Murles_01958_R	GAGTTCGCAGCATGAAGACG	
SIGMAV_qPCR_F	ATGTAACTCGGGTGTGACAG	154
SIGMAV_qPCR_R	CCTTCGTTCATCCTCCTGAG	

*Cycling conditions as described in Supplementary Table 1.

Table S 3. Primers used for LJV Sanger sequencing. For details, see page 23. **F**: Forward primer; **R**: Reverse primer.

Oligo name	Sequence (5'-3')	Position
LJV_Part_1_F	GCTCTGAAGGCCTTGGAAAC	22 - 41
LJV_Part_1_R	ACGTATCGGGTTCTGTCTCC	721 - 702
LJV_Part_2_F	GTGTGTGGCGTTACGATTCT	639 - 658
LJV_Part_2_R	TGCGAGGTCCATCATAACATG	1334 - 1314
LJV_Part_3_F	TCATACCAAATCGATACACTCCG	1260 – 1282
LJV_Part_3_R	ATGTACTCAAGAGGCGTCGG	1960 – 1941
LJV_Part_4_F	GAGGAACCGTGATAGCTGGG	1913 – 1932
LJV_Part_4_R	ATCTTCGAGTCAGGAGCAGT	2660 – 2641
LJV_Part_5_F	CCTGGAGCTGTGCAAGTTTC	2500 - 2519
LJV_Part_5_R	AAAGTAACCGTGCCGCAATT	3341 - 3322
LJV_Part_6_F	TTCCGATTTTGGCGTGGTTC	3223 - 3242
LJV_Part_6_R	CGCCCCATGTTTGTAAGCAA	4019 - 4000
LJV_Part_7_F	TCAACAGGCCTTAACATCACT	3940 - 3860
LJV_Part_7_R	CTGGCGAACACCTTAAGTCG	4696 - 4677
LJV_Part_8_F	CGGCGTGTTATTGATCTTCCA	4585 - 4560
LJV_Part_8_R	TTCGACGTTCTTGGTTGTCA	5323 - 5304
LJV_Part_9_F	TGAGTGACGAAGAGAGTTTGTC	5168 - 5189
LJV_Part_9_R	TGGGACATTACAAGGACGGG	5870 - 5851
LJV_Part_10_F	CCAATCAGTAGGTCCGTGGT	5802 - 5821
LJV_Part_10_R	TCCGAGTCATTCTGTGCTGT	6527 - 6508
LJV_Part_11_F	GCACGTATTCCACCTACAGC	6493 - 6512
LJV_Part_11_R	TCAGGTGACGCTCATTTCCT	7303 - 7284
LJV_Part_12_F	TCCCGGCATCTCAAAGTGAA	7199 - 7218
LJV_Part_12_R	AGGTAAGTGCATTTTGGCCG	7947 - 7947
LJV_Part_13_F	TGAAGTTTCCGGTAAGTGCG	7869 - 7888
LJV_Part_13_R	AAAGGAGATGCGCAGAACAC	8576 - 8557
LJV_Part_14_F	TCCTGAAGTTGCGAACCAGA	8421 - 8440
LJV_Part_14_R	TCGCCGTAAACATACATGCA	9134 - 9115
LJV_Part_15_F	GGTGAAGGAAAATGGCGTGA	9079 - 9098
LJV_Part_15_R	TGGATGTGGCACGAAATTACA	9312 - 9292

LJV_RACE_1_F	GGATTCCAAGAGGTAGTCCCGT-GAAC	241-216
LJV_RACE_2_R	CTTTTAGGTGTGGTAGAGTATCATG	153-129
T1	GGCCACGCGTCGACTAGTACTTTT-TTTTTTTTTTTT	Oligo(dT)
T2	GGCCACGCGTCGACTAG-TACGGGGGGGGGGGGG	Adapter
T22	GGCCACGCGTCGACTAGTAC	Adapter

Table S 4. Primers designed for the RT-PCR detection of virus loads. **F**: Forward primer; **R**: Reverse primer.

Oligo name	Sequence (5'-3')
LV_qRT_570_F	AAGGCCTTGGAAACCTTCATCTC
LV_qRT_664_R	CAAATACTACACAGCCGACCTCCA
LVqRT_probe_630	Fam-TCGTATATGATGATCACAAGGTTGCTCACACC

Temp	Time	Description
Step 1		**Activation**
55°C	10 min	
95°C	1 min	
<u>40 cycles</u>		**Amplification and fluorescence detection step**
Step 1		
95°C	10 s	
Step 2		
60°C	30 s	

Table S 5. Primers designed for RT-PCR detection during passage experiments. For details, see in page 27. **F**: Forward primer; **R**: Reverse primer

Oligo name	Sequence (5'-3')	Product size (bp)
La_Jolla_virus_qPCR_F	AATAAATCATAAGCTCTGATATCCCGGCC	136
La_Jolla_virus_qPCR_R	AATAAATCATAAGCAGCTTTTGAACCATA-TTGTG	

Temp	Time	Description
Step 1		**Holding stage**
55°C	10 min	
40 cycles		**Cycling stage**
Step 1		
95°C	10 s	
Step 2		
60°C	60 s	
Step 1		**Melt curve stage**
95°C	15 s	
Step 2		
60°C	60 s	
Step 3		
95°C	15 s	
0.5°C		**Temp. increment (melt curve)**

Table S 6. Primers designed for RT-PCR detection after viral infection. For details see page 27. **F**: Forward primer; **R**: Reverse primer

Oligo name	Sequence (5'-3')	Product Size (bp)
RpL32_F	GCCGCTTCAAGGGACAGTATCTG	78
RpL32_R	AGACGCGGTTCTGCATGAG	
*drsl6*_6667_F	ATGATGCAAATCAAGTTTCTATTC	79
*drsl6*_6667_R	CAGCATCGGCCTCTTTGC	
*drsl6*_7846_F	ATGATGCAAATCAAGTTCCTGTTC	78
*drsl6*_7846_R	AGCATCGGCCTCTTTGCCTC	
*Vir1*_F	GAGAAGAGGGAGCGTGAAGA	136
*Vir1*_R	TCCTCATCCTGATCCTGGTC	
*upd3*_F	GAACAAAACAAGACGCTGCC	203
*upd3*_R	ACCTGTAGTGTACCCTGTGC	
*socs36E*_F	CCTGCGTGATGTTCTTCGAG	210
*socs36E*_R	GTGTGTTCTGATCGATGGGC	

*Cycling conditions as described in Supplementary Table 5.

Table S 7. List of isolates obtained from naturally infected larvae. For details, see page 20.

	Isolate name	Virus found			
1	KN8				
2	KN29		Teise Virus		
3	KS4	MMlV	Teise Virus		
4	KS5	MMlV	Teise Virus		
5	KS6.1	MMlV	Teise Virus		
6	KS6.2				
7	KS9.1		Teise Virus		
8	KS9.2		Teise Virus		
9	KS9.3		Teise Virus		
10	KT1				
11	KT2	MMlV			
12	KT3	MMlV			
13	KT4	MMlV	Teise Virus		
14	KT5		Teise Virus		
15	KT6				
16	KT7		Teise Virus		
17	KT8		Teise Virus		
18	KT9				
19	KT10				
20	KT11				
21	KT12				
22	KT13-19				
23	GI6				
24	GI16				
25	GI17			LJV	
26	OS37		Teise Virus		
27	OS71				
28	OS59	MMlV	Teise Virus		
29	OS61		Teise Virus		
30	OS63				
31	OS73				
32	OS57		Teise Virus		
33	OS55		Teise Virus		
34	OS41		Teise Virus		

35	OS39		Teise Virus		
36	OS35			DAV	
37	OS31				
38	OS29		Teise Virus		
39	OS28				
40	OS27			DAV	LJV
41	OS26		Teise Virus	DAV	LJV
42	OS25		Teise Virus		
43	OS24	MMlV			
44	OS23				
45	OS21		Teise Virus		
46	OS20				LJV
47	OS19B		Teise Virus		
48	OS19A				
49	OS18		Teise Virus		
50	OS17		Teise Virus		
51	OS12		Teise Virus		
52	OS10		Teise Virus		
53	OS8				
54	OS7				
55	OS3				
56	OS1	MMlV			
57	OS69				
	Total	9	27	3	4

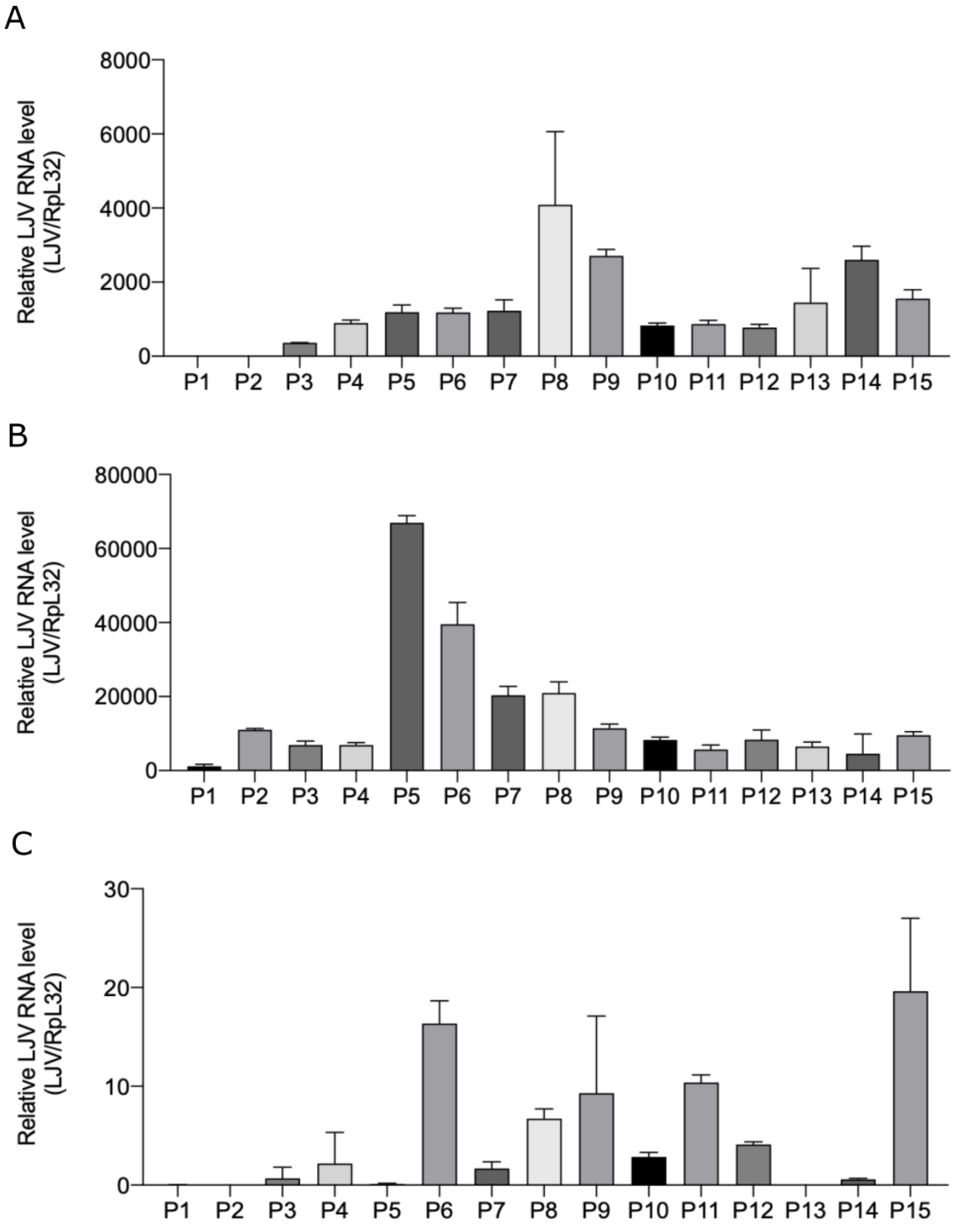

Figure S 1. Biological replicates of the serial passages with the LJV-isolates. Each line represents one of the passages carried out for OS27 (A), OS26 (B) and Gi17 (C). **P**: Passage.

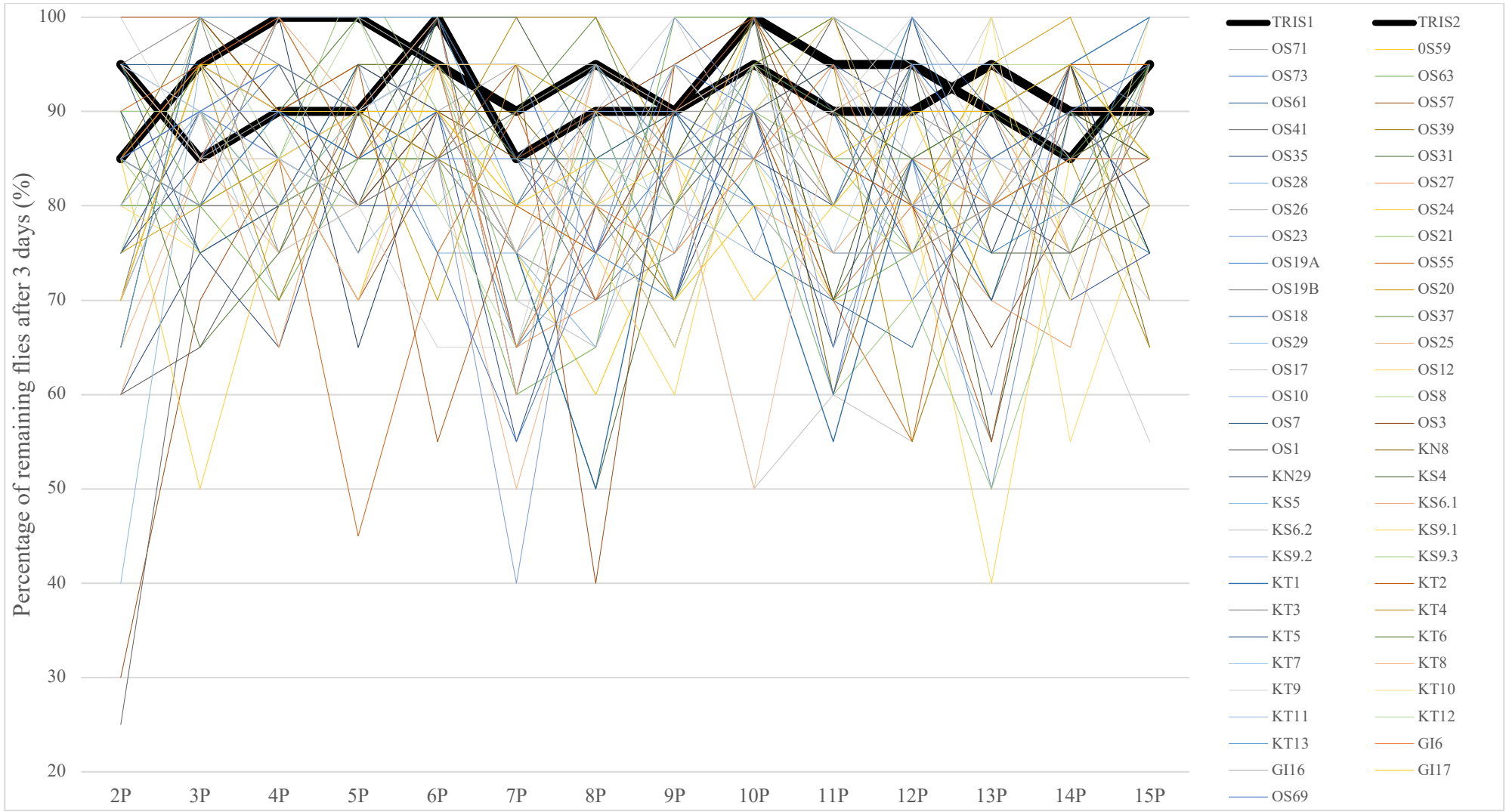

Figure S 2. Passage carried out with the 57 isolates obtained during sampling. Survival rate was noted at the recovery time.

Selbstständigkeitserklärung

Hiermit versichere ich, die vorgelegte Thesis selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt zu haben, die ich in der Thesis angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Thesis erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der ‚Satzung der Justus-Liebig-Universität zur Sicherung guter wissenschaftlicher Praxis' niedergelegt sind, eingehalten. Gemäß § 25 Abs. 6 der Allgemeinen Bestimmungen für modularisierte Studiengänge dulde ich eine Überprüfung der Thesis mittels Anti-Plagiatssoftware.

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