Felix Briem

Institut für Pflanzenschutz in Obst- und Weinbau

Abiotic and biotic factors influencing the spatio-temporal dispersal and population dynamics of *Drosophila suzukii*, the Spotted Wing Drosophila, in Southwest Germany

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Kontakt | Contact:
Felix Briem
JKI - Institut für Biologischen Pflanzenschutz
Heinrichstraße 243
64287 Darmstadt

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Abiotic and biotic factors influencing the spatio-temporal dispersal and population dynamics of *Drosophila suzukii*, the Spotted Wing Drosophila, in Southwest Germany

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“It is not the strongest of the species that survives,
not the most intelligent that survives.
It is the one that is the most adaptable to change.”
— Charles Darwin

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ABSTRACT

The Spotted Wing Drosophila, *Drosophila suzukii* Matsumura, is an invasive polyphagous insect pest. Indigenous to Southeast Asia it overcame its natural barriers in 2008 and has spread to southern Europe (Italy and Spain) and North America (California, USA). Through its adaptability to different climate conditions and the annidation to a hitherto vacant ecological niche, it was able to establish and spread rapidly through Europe and the Americas (South America, North America). In the following, it was first recorded in Germany in 2011. Since summer 2014, it is known from the entire federal territory. The mild winter of 2013/2014 supported *D. suzukii* to finally establish in south-western Germany, the same year when enormous crop loss occurred for the first time.

In anticipation of a forecasted immigration of *D. suzukii* to Germany, the Institute for plant Protection in Fruit Crops and Viticulture of the Julius Kühn-Institut (JKI) started to establish a monitoring program in southwest Germany in 2011. In late summer of the same year, the first individuals were captured. In the following years the monitoring program was enlarged. Traps were installed at sites believed to be suitable habitats such as forest edges, forests, and open agricultural landscape as well as in fruit crops and vineyards. Based on this monitoring, the distribution and flight activity of *D. suzukii* was examined and statistically analysed along the years. On the basis of the obtained monitoring data, the year was divided into five "*Drosophila* seasons": early spring, late spring, summer, autumn and winter. Statistical analysis of the data revealed that both hot and dry phases in late spring and summer and low precipitation during these seasons are negatively correlated with the activity of *D. suzukii*. Frost days in winter were also negatively correlated with the flight activity and thus the survival rate of the individuals. As part of the monitoring, additional traps were installed in the canopy of various tree species at different forested sites. By examination of the canopy of pine trees (*Pinus sylvestris)*, especially in winter and spring, it was found that crown-dwelling mistletoe (*Viscum album*) represents one of the first host plants in early spring for *D. suzukii* in Southwest Germany. In laboratory assays *V. album* was identified as a host for nutrition and development. The identification of additional host plants, especially in winter and spring, proved to be difficult, thus, a method to analyse ingested food resources molecularly was adapted for the first time to *D. suzukii*, a sponging-feeding Brachycera, to facilitate future analysis of the nutrition spectrum. The optimized molecular nutrition analysis promises to identify new winter retreats and resources, which may be helpful for developing management strategies. The results presented here will improve the timing of future management strategies through increased knowledge of activity and food intake of *D. suzukii*.

Keywords: Drosophilidae; host plants; insect traps; insect behaviour; invasive species; molecular gut content analysis; overwintering; population dynamics;

ZUSAMMENFASSUNG

Die Kirschessigfliege, *Drosophila suzukii* Matsumura, ist ein polyphages, invasives Schadinsekt. Ursprünglich autochthon im Südosten Asiens, konnte sie ihre natürlichen Ausbreitungsbarrieren überwinden und im Jahr 2008 erstmals in Südeuropa (Italien und Spanien) und Nordamerika (Kalifornien, USA) nachgewiesen werden. Durch ihre Anpassungsfähigkeit an unterschiedlichste klimatische Bedingungen und das Einnehmen einer bisher unbesetzten ökologischen Nische, hat sie rasch stabile Populationen in Europa sowie Nord- und Südamerika aufgebaut und sich weiter ausgebreitet. Bereits 2011 wurde *D. suzukii* in Süddeutschland und 2014 im gesamten Bundesgebiet nachgewiesen. Der milde Winter 2013/2014 verhalf *D. suzukii*, sich endgültig in Südwestdeutschland zu etablieren. Im selben Jahr wurden erstmals große Schäden durch Ertragsausfälle im deutschen Obst- und Weinbau.

In Erwartung einer möglicherweise bevorstehenden Einwanderung der Kirschessigfliege in Deutschland baute das Institut für Pflanzenschutz in Obst- und Weinbau des Julius Kühn-Instituts (JKI) ab 2011 ein Monitoringprogramm in Südwestdeutschland auf. Bereits im Spätsommer desselben Jahres konnten die ersten Individuen gefangen werden. In den folgenden Jahren erfolgte eine Ausweitung des Monitorings. In Habitaten, die als geeignet empfunden wurden, wie beispielsweise an Waldrändern, in Wäldern sowie offener Agrarlandschaft ebenso wie in Obstanlagen und Weinberge, wurden Monitoringfallen installiert. Anhand dieses Monitorings konnte die Verbreitung und die Flugaktivität von *D. suzukii* im Jahresverlauf untersucht und statistisch analysiert werden. Anhand der gewonnenen Monitoringdaten wurde das Jahr in fünf „Drosophila-Jahreszeiten“ eingeteilt: Früher Frühling, später Frühling, Sommer, Herbst und Winter. Statistische Analysen der Daten zeigten, dass heiße und trockene Phasen im späten Frühling und im Sommer ebenso negativ mit der Aktivität der Kirschessigfliege korrelierten wie geringer Niederschlag zu diesen Jahreszeiten. Frosttage im Winter hatten ebenfalls einen negativen Einfluss auf die Flugaktivität und folglich auf die Überlebensrate der Individuen. Im Rahmen des Monitorings wurden an verschiedenen Waldstandorten zusätzlich Fallen in Kronen verschiedener Baumarten installiert. Durch erhöhte Fangzahlen in Kieferkronen (*Pinus sylvestris*) im gesamten Jahresverlauf, aber vor allem im Winter und Frühjahr und die daraufhin durchgeführte Untersuchung der Kronen, konnte festgestellt werden, dass kronenbesiedelnde Misteln (*Viscum album*) eine der ersten Wirtspflanzen im zeitigen Frühjahr für

D. suzukii darstellen. Des Weiteren wurde *V. album* als Nahrungs- und Entwicklungswirt identifiziert. Da sich der Nachweis weiterer Wirtspflanzen, vor allem im Winter und Frühjahr als schwierig erwies, wurde die Methode der molekularen Nahrungsanalyse erstmalig auf tupfend-saugende Brachycera am Beispiel von *D. suzukii* angepasst und damit ein zukünftiges Analysieren des Nahrungsspektrums erleichtert. Die optimierte molekulare Nahrungsanalyse ermöglicht Rückzugsgebiete im Winter zu identifizieren und hilft so bei der Entwicklung neuer Bekämpfungsstrategien. Die in dieser Arbeit vorgestellten Ergebnisse werden den Einsatz zukünftiger Bekämpfungsmaßnahmen durch ein erweitertes Wissen zur Aktivität im Freiland und der Nahrungsaufnahme verbessern.

Schlagwörter: Drosophilidae; Insektenfallen; Insektenverhalten; invasive Arten; molekulare Nahrungsanalysen; Populationsdynamik; Überwinterung; Wirtspflanzen;

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DEFINITIONS AND ABBREVATIONS

ACV	Apple cider vinegar
°C	Degree Celsius
Cfb	Temperate oceanic climate
CRISPR/Cas	Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated genes
DNA	Deoxyribonucleic acid
eDNA	Environmental Deoxyribonucleic acid
€	Euro
GMOs	Genetically modified organisms
IAS	Invasive alien species
IPM	Integrated pest management
JKI	Julius Kühn-Institut
MGCA	Molecular gut content analysis
MM	Million
NGS	Next generation sequencing
RNA	Ribonucleic acid
SIT	Sterile insect technique
ssp.	Subspecies
US$	United States dollar
USA	United States of America
VOC	Volatile organic compounds

1 GENERAL INTRODUCTION

Increasing world trade, travel and tourism during the last decades have resulted in numerous novel pathways for organisms to break down biogeographic barriers isolating populations for millions of years and spread outside their regions of origin (Essl et al. 2015; Hulme 2017; Padayachee et al. 2017). Once introduced into a new region, some of the non-indigenous or alien species can become invasive. Those invasive species are a leading threat to native wildlife, human health and food production in the newly invaded areas with associated economic impacts estimated in hundreds of billions US$ each year, worldwide (Kenis et al. 2009; Pimentel et al. 2005; Pysek and Richardson 2010).

While some invasive alien species (IAS) can have an impact on health for humans, others can threaten indigenous species. Furthermore, the economic impact of IAS may be considerable (Pimentel et al. 2005). IAS can become a major cause of crop loss in agriculture and can adversely affect food security (Cook et al. 2011; Paini et al. 2016). In recent years, an increasing number of IAS endangering agriculture have occurred and already caused immense economic impacts. During the last decade, a new IAS was dramatically threatening fruit crop production and viticulture worldwide. The vinegar fly *Drosophila suzukii* Matsumura (1931) (Diptera: Drosophilidae), commonly known as Spotted Wing Drosophila, is a highly polyphagous IAS mainly infesting thin-skinned fruits, such as soft and stone fruits, but numerous wild and ornamental fruits, too (Asplen et al. 2015; Briem et al. 2016; Cini et al. 2012; Lee et al. 2011a; Poyet et al. 2015; Vogt 2017). In the last decade, *D. suzukii* has become a cosmopolitan and a major problem in fruit production and viticulture, leading up to 100 % infestation and thus to substantial economic losses (Mazzi et al. 2017).

Native to Southeast Asia *D. suzukii* has overcome its natural barriers most probably supported by global trade, and reached southern Europe (Italy and Spain) and the USA (California) in 2008 (Asplen et al. 2015; Cini et al. 2012; Hauser 2011). Subsequently, enormous crop losses occurred in many regions. Since *D. suzukii* infests ripening and ripe fruit close to harvest, chemical plant protection is critical and has to be applied with caution.

1.1 Description and phylogenetics

The earliest records of *D. suzukii* date from 1916 in Japan followed by Korea and China from 1930/1931. The species was at first described in 1931 by Dr. Shounen Matsumura as *Drosophila suzukii*, commonly named cherry drosophila (Kanzawa 1936). Today, *D. suzukii* belongs to the *melanogaster* species group of the subgenus *Sophophora*. The *melanogaster* group is further divided into species subgroups, one of which (the *suzukii* subgroup) comprises, together with six other subgroups, the oriental lineage (Kopp and True 2002; Schawaroch 2002). Phylogenetics of these subgroups are still unresolved. Recent papers suggested *D. biarmipes* as sister taxon of *D. suzukii* (Chiu et al. 2013; Ometto et al. 2013; Rota-Stabelli et al. 2013; Yang et al. 2012); whereas others (van der Linde and Houle 2008; Prud'homme et al. 2006) suggest *D. subpulchrella* as sister species of *D. suzukii*. Genome sequencing might prospectively help to understand the phylogenetics of Drosophila.

1.2 Morphology and biology

Adult individuals have a body length of 2 to 3 mm. The eyes are red, their thorax is yellowish brown to pale brown, the abdomen has black transverse stripes, and the antennae are short with branched arista (Figure 1). Sexual dimorphism is obvious since males have a black spot on each wing tip (Figure 1a), whereas females have no wing spots, have larger bodies (Figure 1b) and possess a large serrated, saw-like ovipositor (Figure 1e). The eggs are oblong with a length of 0.5 - 0.6 mm, milky-white and possess two respiration extensions of 0.5 - 0.6 mm length apically (Figure 1f). The larvae are white with visible internal organs and black, sclerotized mouthparts. The pupae are spindle-shaped, reddish brown possessing two stalks with small star-like projections for respiration (Kanzawa 1936) (Figure 1c).

The main distinguishing features of both sexes are also present in other Drosophilidae, potentially leading to miss-identification with similar species (Calabria et al. 2012). The dark spot on the males' wing tips plays an important role during mating (Revadi et al. 2015). They are nearly identical in shape and position to those of *D. subpulchrella* making species identification challenging in areas they are sympatric (Cini et al. 2012; Takamori et al. 2006). Furthermore, the lack of the wing spots directly after hatching can lead to misidentification with other closely related Drosophilidae (Cini et al. 2012). Consequently, other characteristics such as the sex combs on the fore tarsi (Figure 1d) should additionally be used for correct identification of the males (Kanzawa 1939; Vlach 2013). Within females, the shape and size of the serrated ovipositor can be used to distinguish *D. suzukii* from most Drosophilidae (Hauser 2011); but not to distinguish *D. suzukii* females from *D. immigrans* or *D. subpulchrella* as those species possess a quite similar ovipositor (Hauser 2011). A dichotomous key for identification of adult *D. suzukii* has been developed (Vlach 2013).

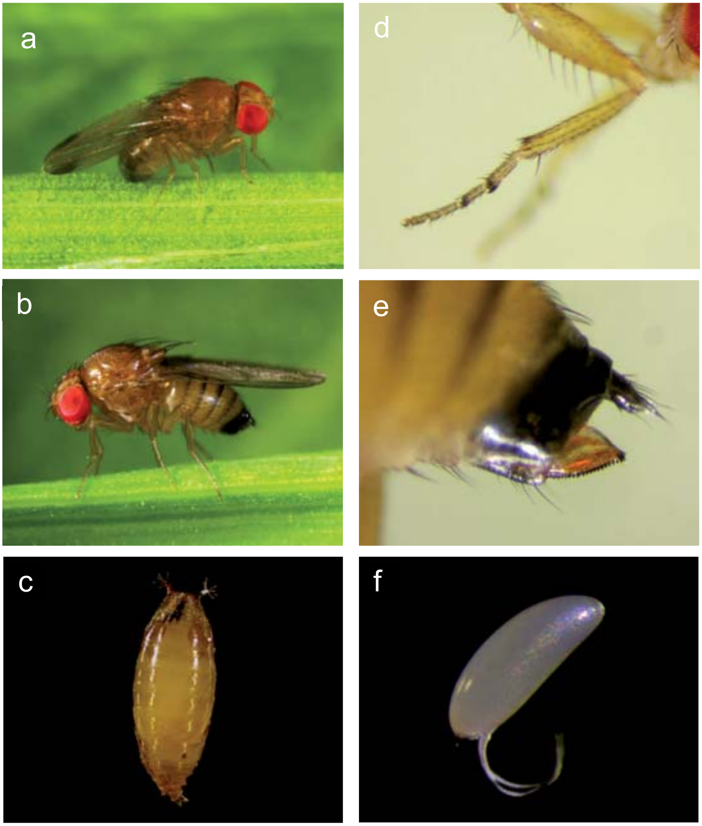

Figure 1. Developmental stages and characteristics of *Drosophila suzukii*: a) adult male; b) adult female; c) spindle-shaped pupae; d) fore tarsus of males with sexual combs ; e) serrated, saw-like ovipositor, f) mature egg with respiration extensions.

The determination of the preimaginal stages (eggs, larvae and pupae) is extremely challenging, as no species specific morphological diagnostic features have been identified so far (Hauser 2011). Recently, Kim et al. (2014) developed a molecular diagnostic method to identify field-collected individuals from various sources and specimens regardless of their stage. This tool could be applied when specimens are poorly preserved for proper morphological identification or when only immature stages are available.

In contrast to most other Drosophilidae, female *D. suzukii* oviposit into intact ripening and ripe fruits close to harvest using their serrated ovipositor (Bellamy et al. 2013; Cini et al. 2012; Hauser 2011; Mitsui et al. 2010). Females cut the skin of the fruit with their ovipositor and deposit their eggs directly underneath. One to more than 10 eggs are laid per fruit, depending on the availability of susceptible fruits (Kanzawa 1939). Egg-laying activity can vary from 10 to 65 days with more than 25 eggs per day (Kinjo et al. 2014). Each female can lay more than 300 eggs during its lifetime; reports of exact numbers are inconsistent (Kanzawa 1939; Tochen et al. 2014). The larval hatch starts within the first 24 hours after oviposition. The larvae develop through three instars and may leave the host fruit for pupation on the fruit surface or in the soil, but pupation inside the fruit is more common (Walsh et al. 2011). However, a recent study found more pupae in the soil in wild blueberries than in the fruits (Ballman et al. 2017). The whole life cycle from oviposition to adult emergence takes 8 to 25 days, depending on ambient temperature (Kanzawa 1939; Lee et al. 2011b; Tochen et al. 2014). This short generation time in combination with high reproductive potential causes rapid population growth and increasing pest pressure throughout the season (Wiman et al. 2014).

Based on the wide geographical range in Asia, Kanzawa (1939) already assumed that the number of generations per year could range from 3 to 13 depending on the prevailing climatic conditions. Kimura (pers. communication) argued that the number of generations might be overestimated assuming 5 to 8 generations a year, up to 7 generations are assumed in southern Germany.

The lifespan of adults during the summer period is 20 to 56 days, but overwintering adults may survive for more than 200 days (Kanzawa 1936). However, Shearer et al. (2016) showed that also summer individuals can survive up to 200 days if kept at lab-conditions at 5 °C whereas winter morphs can even live much longer. They further showed that *D. suzukii* is overwintering as adult winter morph with darker coloration than summer morphs and have an increased wing size. The lifespan of overwintering adults is considerably longer than non-overwintering adults and many survive until the following May or June, starting reproduction in spring as early as temperatures are appropriate (Kanzawa 1939; Shearer et al. 2016). Overwintering individuals are mobile above 5 °C, when they need food resources. Adults usually feed on fruits that have been damaged e.g. by birds or during oviposition, on microorganisms and yeasts growing on the surface of plants (Fountain et al. 2018) or on sap from trees (Bellamy et al. 2013; Kanzawa 1939; Lee et al. 2011b). The main activity lies between 20 °C and 25 °C whereas temperatures above 30 °C reduce activity and survival probability rapidly (Hamby et al. 2013; Kanzawa 1939; Tochen et al. 2014). Summer morphs avoid hot and dry temperatures and direct sunshine, thus they are most active during dusk and dawn while winter morphs are more active during the middle of the day (Briem et al. 2018b; Hamby et al. 2013; Shaw et al. 2018).

Oviposition generally occurs from summer morphs between April and the end of October into ripening and ripe fruits (Briem et al. 2016; Kanzawa 1939; Panel et al. 2018; Shearer et al. 2016; Zerulla et al. 2015) whereas winter morphs were reproductively immature in autumn and start reproduction and oviposition in spring of the following year (Vogt et al. 2018), suggesting a winter reproductive diapause while overwintering as adult fly (Dalton et al. 2011; Mitsui et al. 2010).

1.3 Origin, movement and current distribution

Little is known about the geographical origin of *D. suzukii*. It was first identified in 1916 in Japan followed by Korea and China in 1930/1931 (Kanzawa 1936) and is thought to be a native of eastern and south-eastern Asia, including China and Korea (Walsh et al. 2011). *D. suzukii* is not native to Japan but introduced into the country at the turn of the last century (Hauser 2011). In 1980, *D. suzukii* was first recorded outside of its native region on Hawaii, occurring even at higher elevations in rainforests (Hauser 2011; O’Grady et al. 2002). This population originates most probably from Japan (Fraimout et al. 2017). Since 1980, several reports of *D. suzukii* recorded at other places of the world could not be verified until its first records in California (USA) and southern Europe (Italy and Spain) in 2008 (Asplen et al. 2015; Calabria et al. 2012; Cini et al. 2012; Hauser 2011; Langille et al. 2017). Afterwards, it rapidly spread through the continents and became a cosmopolitan, now present in most regions of the Nearctic, the Neotropical, the Oriental, the Palearctic and the Sino-Japanese realm (Asplen et al. 2015; Cini et al. 2014; Depra et al. 2014; Rego et al. 2017). While western North America is most probably invaded by specimens from Southeast China and Hawaii, it is thought that eastern North America was invaded later by individuals from western North America. European populations are more homogenous and originate mainly from northeast China, with limited gene flow from eastern North America (Fraimout et al. 2017).

In late summer 2011, *D. suzukii* was recorded for the first time in southern Germany (Heuck 2012; Vogt et al. 2012a; Vogt et al. 2012b) and has spread rapidly throughout the country. Since summer 2014, it is present in all Federal States (Asplen et al. 2015; Köppler and Vogt 2015; Vogt and Briem 2015), often causing severe economic damage. After the rapid spread in Europe and the Americas during the last decade, it seems that *D. suzukii* will continue to expand its range, since not all regions, such as southern Africa or Australia/New Zealand, with the preferred or at least tolerated climate conditions of *D. suzukii,* are currently occupied by the pest (Figure 2).

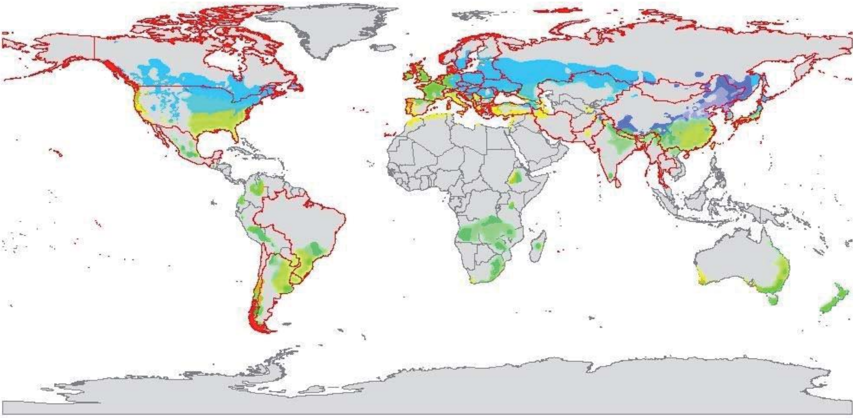

Figure 2. Map showing the worldwide distribution of *D. suzukii* and indicating its preferred (blue/ purple = Continental climate with dry winter, green = warm temperate climate, wet all year/with dry winter) and tolerated (yellow = warm temperate climate with dry summer) climates of the Köppen-Geiger climate classification. Countries with reported *D. suzukii* occurrence are outlined in red (data taken from CABI (2019), status December 2018), overlaid with a Köppen-Geiger climate classification map adapted from Beck et al. (2018) (CC-BY 4.0, http://creativecommons.org/licenses/by/4.0/).

Rota-Stabelli et al. (2013) already suggested that first records of *D. suzukii* in the USA and Europe both occurred close to ports. Indeed, Cini et al. (2014) identified the area around Avignon in Southern France as the most likely spreading centre. This region imported three to ten times more fruit, some of which may have been infested, at this time than Italy and Spain, where *D. suzukii* was first reported in Europe. The area around Avignon is not far away from the port of Marseille. The pest could have been undetected and first infestations could have happened unnoticed already before 2008. Indeed, it was suggested that *D. suzukii* has a long-range dispersal of about 1,400 km per year which could have been facilitated by human transportation of fruits and flowers (Calabria et al. 2012; Hauser 2011; Kanzawa 1936). It is not clear whether the high dispersal was active or passive via infested fruits (Cini et al. 2014). Kiss et al. (2013) supported the thesis of passive dispersal via infested fruits suggesting that the isolated *D. suzukii* presence near a highway resting area in Hungary could have happened due to infested fruit that was negligently thrown away.

1.4 Relevance in fruit production

Most Drosophilidae associated with humans are considered nuisance, household and fermentation industry pests. Thus, *D. melanogaster* is usually attracted to fermenting volatiles of overripe or spoiled fruits (Asplen et al. 2015; Landolt et al. 2012b). In contrast, female *D. suzukii* oviposit into ripening and ripe soft-skinned fruits, occupying its own hitherto vacant niche preparing the fruits for secondary pests such as pathogens (e.g., bacteria and yeasts) (Cini et al. 2012; Hamby et al. 2012; Ioriatti et al. 2015; Lee et al. 2011a). Once the egg is deposited directly underneath the fruit skin, the larvae can hatch within the first 24 hours post-oviposition and start their destructive feeding on the fruit pulp. The infested fruit begins rapidly to collapse and fruits become unmarketable (Bolda et al. 2009; Hauser 2011).

The host range of *D. suzukii* varies widely and includes numerous cropped, wild and ornamental hosts (Briem et al. 2016; Lee et al. 2011a; Poyet et al. 2015). Kanzawa (1936) already reported that it infests cherries, grapes, gooseberries, raspberries, peaches, plums, persimmons, tomatoes, olives, mulberries, and loquats. Among cropped fruits mainly thin-skinned berries (e.g. caneberries, blueberries, strawberries) and stone fruits (e.g. cherries, peaches, apricots, plums) are susceptible to infestation (Bellamy et al. 2013). Certain fruits such as apples, pears or grapes can also be infested if split, previously damaged or when fruits are in bad conditions (Entling et al. 2018; Lee et al. 2011a). However, *D. suzukii* is not a significant pest of these crops. As already mentioned, many wild and ornamental fruits can serve as potentially important host such as wild *Rubus* spp., *Viscum album*, *Prunus cerasifera*, among many others (Briem et al. 2016; Cini et al. 2012; Lee et al. 2015a; Lee et al. 2015b; Mitsui et al. 2010; Poyet et al. 2015). Considered as minor pest in China, *D. suzukii* caused significant damage on bayberry (*Myrica rubra*) orchards in the Yunnan Province (Vogt 2017). Increased infestation was reported only recently, mainly from late cherry varieties (Haye et al. 2016).

Already in 2008, the year when *D. suzukii* was first recorded in mainland USA and Europe, economic losses of fruit production for California, Oregon and Washington were estimated at 40 % for blueberries, 50 % for caneberries, 33 % for cherries and 20 % for strawberries and assumed to cost more than 500 MM US$ annually (Bolda et al. 2009; Goodhue et al. 2011). In the Italian part of Trentino province, both first oviposition on wild hosts (*Vaccinium*, *Fragaria* and *Rubus* spp.) and economically significant damage on several species of cultivated berries were reported (Cini et al. 2012), a potential damage to strawberry, raspberry, blueberry, blackberry and cherry production of more than 3 MM € in 2011 was assumed (De Ros et al. 2013).

One has to consider that determining the economic assessment of the impact of *D. suzukii* is highly complex. The most detailed studies are focused on single regions such as California, USA (Bolda et al. 2009; Goodhue et al. 2011) or the Trentino region in Italy (De Ros et al. 2013) and do not comprise lager regions or whole continents. Consequently, the economic impacts of *D. suzukii* are probably enormous as they can cause up to 100 % crop loss (Mazzi et al. 2017). The infestation level can vary between farms and depends strongly on cultivar, location, microhabitats, vegetation structure, cultivation measures and weather conditions (Diepenbrock et al. 2016; Mazzi et al. 2017; Van Timmeren and Isaacs 2013).

1.5 Control strategies

Current control strategies of *D. suzukii* are mainly based on insecticides which can reduce damage of agricultural crops below the economic injury level, however, these increase production costs (Cini et al. 2012; Goodhue et al. 2011; Mazzi et al. 2017; Schetelig et al. 2018; Tochen et al. 2016). Infestations of *D. suzukii* also increases economic costs depending on the crop, beyond high yield loss to include other costs associated with monitoring infestation levels (Farnsworth et al. 2017; Goodhue et al. 2011; Mazzi et al. 2017).

The most widely adopted strategy to control *D. suzukii* in crop production is using insecticides for plant protection (Cini et al. 2012; Haye et al. 2016; Mazzi et al. 2017). Conventional insecticides such as pyrethroids, organophosphates and spinosyns were proven to be effective against this pest (Beers et al. 2011; Cowles et al. 2015; Van Timmeren et al. 2018). Unfortunately, currently used broad-spectrum products are mostly incompatible with integrated pest management (IPM) programs (Beers et al. 2011; Haye et al. 2016). Neonicotinoids have not been used much as a management tool because they were perceived to be less effective (Bruck et al. 2011). In general, the evaluation of the efficacy of these insecticides was done for almost all of the major fruit growing regions where *D. suzukii* is distributed. These products mainly target adult *D. suzukii*. Given the high reproductive capacity of the fly, its overlapping generations and repeated application of these insecticides, the likelihood of insecticide resistance development is a big concern (Bruck et al. 2011). So far, no insecticide selectively active against *D. suzukii* has been developed. Chemicals currently used can have non-target effects on natural enemies and other beneficial organisms such as pollinators, possibly reducing biodiversity in an environment already challenged by the decline of insects (Haye et al. 2016; Roubos et al. 2014; Schetelig et al. 2018).

Currently, most sustainable IPM strategies include sanitary measures such as defoliation in viticulture, regular mulching of ground covering vegetation, complete picking, removal of dropped or leftover fruit in order to reduce reproduction and food resources (Cini et al. 2012; Vogt 2017; Walsh et al. 2011). Further, it was recommended to harvest even infested fruits and treat them with heat (solarization) or cold, to kill eggs, larvae and pupae and thus prevent emergence of adults (Lee et al. 2011b; Schetelig et al. 2018; Vogt 2017).

The most promising alternative to insecticide application is the exclusion of *D. suzukii* with the use of nets. This has recently been proven to be highly efficient with significant reduction of the infestation level (Boehnke et al. 2018, Cormier et al. 2015; Haye et al. 2016; Leach et al. 2016; Rogers et al. 2016; Schattmann et al. 2015). The timing when the nets are closed is very important. When they are closed too late, *D. suzukii* might already be present in the plantation, causing severe damage (Boehnke et al. 2018, Rogers et al. 2016). When the nets are closed too early pollinators might be excluded before crops are pollinated (Leach et al. 2016). Leach et al. (2016) already assumed that nets will pay for itself through savings in insecticides and a guaranteed higher yield, but acquisition costs for the nets and a stable framework are quite high.

Where exclusion netting is not applicable, favourable microhabitat conditions could be impaired using wider tree spacing for improved ventilation, mulching or a better drainage (Haye et al. 2016; Vogt 2017). Unfortunately, most of these procedures are labour or cost intensive, leading to higher additional costs and thus to lower profitability (Vogt 2017). Currently, only exclusion netting has been highly efficient and sustainable to avoid *D. suzukii* infestation until the day of harvest (Boehnke et al. 2018, Leach et al. 2016; Schetelig et al. 2018).

IPM strategies such as mass trapping or attract-and-kill strategies require greater species-specify and efficiency of attractants used (El-Sayed et al. 2006; Schetelig et al. 2018). Without highly efficient baits, specimens are attracted to the vicinity of the traps but are not directly lured into and killed by the trap. This circumstance might end up with higher infestation in the direct vicinity of the trap (Hampton et al. 2014).

The larvae or pupae of Drosophilidae are generally attacked by several parasitic wasps (Carton 1986). However, *D. suzukii* has evolved an extremely potent cellular immune response, making it extremely challenging for common larval parasitoids such as *Amazonides tabida*, *Leptopilina boulardi*, *L. heterotoma*, to parasitize the fly (Poyet et al. 2013). Among several recently tested Asian and European larval parasitoids (*Leptopilina* ssp., *Asobara* ssp. and *Ganaspis* ssp.), very few can utilize *D. suzukii* as host (Girod et al. 2018a; Girod et al. 2018b; Rossi Stacconi et al. 2015; Schetelig et al. 2018). Nomano et al. (2015) described an *Asboara* species (*Asobara* sp. TK1) unique to *D. suzukii*. Further, two pupal parasitoids *(Pachycrepoideus vindemmia*, *Trichopria drosophilae*) can successfully parasitize *D. suzukii*. The success of these pupal parasitoids is determined under laboratory conditions, but in-field parasitation levels need still to be determined (Mazzetto et al. 2016; Schetelig et al. 2018). Larval parasitoids would be most interesting as they interfere much earlier than pupal parasitoids. Besides parasitoids, predatory arthropods might reduce *D. suzukii* population by feeding on eggs, larvae, pupae and adults. Unfortunately, as generalists they can provide only low-level background control (Symondson 2002; Wolf et al. 2018). Entomopathogenic fungi such as *Isaria fumosorosea* and *Metarhizium* *anisopliae* are also being assessed as potential biological control agents (Naranjo-Lazaro et al. 2014).

Prospective new biotechnology-based control strategies such as RNA interference and CRISPR/Cas genome editing might be interesting technologies and were already evaluated in *D. melanogaster*, but they still need further research for application on *D. suzukii* (Schetelig et al. 2018). In general, the safety of these methods still needs clarification. The regulation for genetically modified organisms (GMOs) is still difficult in most countries, whereas in single countries (e.g. Brazil, the USA or Panama), first transgenic insects (mosquitoes and screwworms) have already been released (Schetelig et al. 2018). Another technique for the control of pest insects such as *D. suzukii* might be the Sterile Insect Technique (SIT). However, it has a lot of constraints regarding costs, efficiency, fitness of the insects, and logistics or mass rearing. This applies in particular when to be used to control such a wide-spread insect with an enormous population growth. For all biotechnology approaches, more research is needed and it takes time until these methods are efficient, ready to use and adopted.

For the above-mentioned reasons, an effective and guaranteed long-term control is still not possible. The combination of cultural measures, insecticides, netting and parasitoids/predators offer a suitable alternative in most fruit crops for the moment (Haye et al. 2016; Mazzi et al. 2017).

1.6 Monitoring the pest insect

Monitoring insects generally allows the observation and analysis of populations and is a necessary and important method to gather information on species dynamics and in-field behaviour (Kuno 1991). This knowledge is necessary for any successful IPM and possibly supports the development of efficient control strategies to minimize crop loss (Cha et al. 2018; Cini et al. 2012; Cohnstaedt et al. 2012). Sampling of the population can be carried out via active sampling (Hammack et al. 2003) or trapping with and without attractants (Boiteau 2000; El-Sayed et al. 2009; Landolt et al. 2012b).

Fermented products were already known to play an important role in the monitoring of *D. suzukii* (Kanzawa 1939; Walsh et al. 2011), when it started to spread through the Americas and Europe in 2008. Therefore, *D. suzukii* was mainly monitored using homemade baits, based on fermenting products such as apple cider vinegar (ACV), baker’s yeast or wine (Kleiber et al. 2014; Lee et al. 2012; Lee et al. 2013).

Among the baits used, ACV has been the most widely recommended one as it is affordable, convenient and trapped flies can be determined easily (Lee et al. 2012). Unfortunately, baits based on fermenting products are not species-specific as they are attractive to a wide range of non-target insects mainly other Drosophilidae (Cha et al. 2013; Lee et al. 2012), making species determination labour intensive.

Limited information on *D. suzukii* trapping in terms of baits, pheromones and physical trap designs were available simultaneously to the invasion of *D. suzukii* in 2008. In the following, immense effort was put into alternative trap designs in terms of physical features such as trap size, shape, colour or size of the entry holes (e.g. Basoalto et al. 2013; Iglesias et al. 2014; Kirkpatrick et al. 2018; Lee et al. 2013; Renkema et al. 2014; Rice et al. 2017). Other studies focused on developing effective and species-specific lures (e.g. Burrack et al. 2015; Cha et al. 2012; Cha et al. 2013; Cha et al. 2014; Cha et al. 2015; Cha et al. 2017; Feng et al. 2018; Landolt et al. 2012a).

Most of the newly developed and commercially available attractants have shown higher efficacy in detecting and trapping *D. suzukii* than ACV-based traps (Cha et al. 2018). Unfortunately, these products are as unspecific as the homemade fermenting baits mentioned above. Following non-target insects are still attracted into traps making accurate identification of *D. suzukii* still labour intensive (Cha et al. 2014; Cha et al. 2018; Iglesias and Liburd 2017). For these reasons, and to ensure a standardized and comparable data set through the years, the field presence of *D. suzukii* in Southwest Germany was examined using traditional “fly traps” baited with ACV.

1.7 Molecular gut content analysis

Like most other Brachycera, *D. suzukii* is well adapted to feed on juice from fruit using its haustellum. This circumstance in combination with the small body size of 2 to 3 mm makes it extremely challenging to define food resources of *D. suzukii* only by observation, especially when population densities are low in winter and spring. So far, the main knowledge of host plants of *D. suzukii* is mainly based on studies dealing with field-collected fruit samples, laboratory-based feeding and oviposition experiments of larvae and the emergence of adult flies based on egg laying and developmental success in fruits. Only a few studies reported from field observations of *D. suzukii* feeding on tree or fruit sap and on extra-floral or floral nectaries (Kanzawa 1939;

Tanabe 2002; Tochen et al. 2016; Toda 1992). Hence, the knowledge on host or food plants is scarce and has to be determined more precisely.

Drosophila suzukii belongs to the group of sponging-feeding insects, thus, studying its diet by determination of the gut content morphologically is impossible. In such a case, where the knowledge on the feeding ecology of a species is lacking, the molecular gut content analysis offers an effective approach to circumvent these problems and to study the feeding activity as it is applicable to visually undiscernible prey remains (Staudacher et al. 2016; Symondson and Harwood 2014; Wallinger et al. 2012). Thus, the DNA based molecular gut content analysis can serve as tool to obtain correct information of ingested plant DNA (Avanesyan and Culley 2015; De la Cadena et al. 2017; Wallinger et al. 2015; Wallinger et al. 2013).

However, a greater knowledge on the year-round food choice of *D. suzukii* at different sites is crucial to interpret the spatio-temporal dispersal and to develop precise risk assessments. This knowledge could further support the development of more effective biotechnological control strategies such as “attract and kill” or “push and pull” (Alnajjar 2017; Renkema et al. 2016).

1.8 Aim of the work

As *D. suzukii* has an enormous economic impact on soft-skinned fruit production, sustainable strategies to control the pest should be improved. Due to its unique niche to oviposit into healthy fruits close to harvest, *D. suzukii* has caused severe yield loss and increased costs for soft- and stone-fruit production worldwide. Only limited knowledge was available on the biology of *D. suzukii* when it started to spread through the Americas and Europe in 2008. More knowledge on population behaviour, especially with regard to low temperatures during winter or spring and during hot and dry weather conditions in summer, was urgently needed. The in-field behaviour, such as movement and migration as well as host plant utilization during the seasons, is an additional important knowledge gap that should be closed.

Consequently, the present thesis is concerned with (I) the identification of the in-field behaviour of *D. suzukii* in Southwest Germany. This area is a landscape with small structures, offering several suitable habitats and retreats in the course of the year. A better understanding of the appearance, movements, preferred habitats and host plants in correlation with climate conditions and land use should help to improve the efficacy of control strategies. (II) *D. suzukii* overwinters in a reproductive diapause showing flight activity on mild days during the cold season. As it was assumed that *D. suzukii* needs nutrition during the cold period, monitoring data should be used to figure out hot spots where *D. suzukii* can find this nutrition in the cold period when fruits are missing. This was observed in order to get more knowledge about such resources, offering a basis for developing strategies to prevent access to those resources. (III) The survival of hot and dry periods in summer was another knowledge gap that should be closed since *D. suzukii* was thought to become sterile due to extreme temperatures, and individuals undergoing such extreme weather conditions are not able to recover completely. (IV) The last issue of this study was to close the knowledge gap on the feeding ecology of the pest. Several studies focused on oviposition and the emergence of adult individuals from sampled fruits, but the direct feeding is not well-studied because *D. suzukii* soaks up nourishment in liquid form. Studying its diet by dissecting the flies is impossible as no microscopically identifiable food remains can be found within the gut content. Further, tracking the food uptake of such a small insect, especially when population densities are low in winter and spring, to figure out the food resources utilized, is extremely challenging. Hence, a molecular based method needed to be adapted for this sponging-feeding insect to provide a tool to identify the ingested plant-DNA in the guts of *D. suzukii*, even around the year.

2 RESULTS // ORIGINAL PAPERS

Results are represented by publications originated during this PhD thesis covering the identification of the occurrence of *D. suzukii* in Southwest Germany and in-field behaviour in terms of its occurrence the year round, overwintering sites, identification of host plants and the robustness to extreme weather conditions.

2.1 Explorative Data Analysis of *Drosophila suzukii* Trap Catches from a Seven-Year Monitoring Program in Southwest Germany

This publication analyses a seven-year monitoring program of the Institute for Plant Protection in Fruit Crops and Viticulture (Dossenheim and Siebeldingen) and the Institute for Biological Control (Darmstadt) of the Julius Kühn-Institut, the Federal Research Centre for Cultivated Plants. S. 16 ff.

2.2 An invader supported by a parasite: Mistletoe berries as host for food and reproduction of Spotted Wing Drosophila in early spring

In this article, mistletoe (*Viscum album*) was identified as an important early season host for *D. suzukii*, both nutrition for adult individuals as well as developmental success of oviposited eggs until the adult stage was shown. S. 33 ff.

2.3 Response of *Drosophila suzukii* (Diptera: Drosophilidae) to extreme heat

This article deals with a laboratory-based study simulating in-field weather conditions on four days in a row, analysing the behaviour and survival of male and female *D. suzukii* during extreme weather conditions which occurred during the hot summer 2015 in southwest Germany. S. 45 ff.

2.4 Identifying plant DNA in the sponging-feeding insect pest *Drosophila suzukii*

This article is the first approach to identify ingested plant DNA in the gut content of sponging-feeding Brachycera molecularly using *D. suzukii* as model species.

S. 55 ff.

2.1 Explorative Data Analysis of *Drosophila suzukii* Trap Catches from a Seven-Year Monitoring Program in Southwest Germany

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Explorative Data Analysis of *Drosophila suzukii* Trap Catches from a Seven-Year Monitoring Program in Southwest Germany

Felix Briem [1,*], Anto Raja Dominic [2], Burkhard Golla [2], Christoph Hoffmann [3], Camilla Englert [4], Annette Herz [4] and Heidrun Vogt [1]

[1] Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Straße 101, 69221 Dossenheim, Germany; heidrun.vogt@julius-kuehn.de

[2] Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Strategies and Technology Assessment, Stahnsdorfer Damm 81, 14532 Kleinmachnow, Germany; anto.raja@julius-kuehn.de (A.R.D.); burkhard.golla@julius-kuehn.de (B.G.)

[3] Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Geilweilerhof, 76833 Siebeldingen, Germany; christoph.hoffmann@julius-kuehn.de

[4] Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Biological Control, Entomology and Beneficial Insects, Heinrichstraße 243, 64287 Darmstadt, Germany; camilla.englert@julius-kuehn.de (C.E.); annette.herz@julius-kuehn.de (A.H.)

* Correspondence: felix.briem@julius-kuehn.de; Tel.: +49-(0)6221-86805-33

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Abstract: Over the last decade, *Drosophila suzukii* Matsumura, an invasive pest of soft-skinned fruits, gradually established itself in Europe, often resulting in significant economic losses. In 2011, when *D. suzukii* was first described for Germany, the Julius Kühn Institut (JKI) started a monitoring program in southwest Germany to study the occurrence and activity of the fly. Capture data from late 2011–early 2018 from 100 traps were analyzed for the effect of weather and immediate habitat on trap captures at different times of the year. We identified five phases in the annual population development cycle of *D. suzukii*. We found that the mild winter of 2013/2014 helped the thorough establishment of *D. suzukii* in Germany. Habitat types in the immediate vicinity of the trap and local weather conditions had a strong influence on trap captures. Forest borders and hedges were found to provide adequate overwintering shelter for the flies. Trap captures in forests and hedges were generally higher than those of vineyards and orchards, even during the fruiting seasons. Summer capture rates were correlated with the number of heat days and precipitation. We also discuss briefly the limitations of using trap captures as representative of fly density in the field.

Keywords: Drosophilidae; host plants; insect behavior; insect traps; invasive species; online DB; population dynamics

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1. Introduction

Drosophila suzukii Matsumura 1931 (Diptera: Drosophilidae), commonly called spotted wing *Drosophila*, is an invasive insect pest of soft-skinned fruits native to Southeast Asia. As a highly polyphagous pest species, adults are attracted to a wider range of odors of ripening and ripe fruits than most drosophilids [1]. In contrast to most Drosophilidae, female *D. suzukii* possess a saw-like sclerotized ovipositor, enabling oviposition into healthy, ripening and ripened fruits [2,3], resulting in heavy economic losses to fruit growers [4,5]. Many economically important soft and stone fruits (e.g., raspberries and blackberries: *Rubus* spp., strawberries: *Fragaria ananassa*, cherries: *Prunus avium*, *P. cerasus*, plums: *P. domestica*, elderberry: *Sambucus* spp.), some susceptible grapevine varieties, as well as numerous wild or ornamental fruits (e.g., *P. serotina*, *Viscum album*, *Rubus* spp., *Basella alba*, [6–11] are known hosts. While a high number of reproduction hosts is known from fruit sampling and lab studies, the complete feeding ecology is yet to be described. Early attempts to identify the ingested plant DNA and microbes in the guts of adult individuals have been successful [12,13]. Further studies using these techniques will help to understand the whole feeding ecology of this pest species.

Continental USA (California) and Southern Europe (Italy and Spain) recorded *D. suzukii* in 2008 for the first time [2,14,15]. It spread rapidly the following years, with periodic reports of economic damage in cultivated soft and stone fruits around the world [4–6].

In 2011, the Julius Kühn-Institut (JKI) established a monitoring program in southwest Germany in the vicinity of the JKI in Dossenheim, Siebeldingen and Darmstadt expecting the arrival of *D. suzukii*. Monitoring traps were randomly installed in different fruit crops, forested areas and hedges, in experimental fields and in the direct vicinity of the institutes. First recorded observations of *D. suzukii* in Germany date from late summer 2011 from private observations in Bavaria and from the JKI monitored traps [16,17].

In spring 2016, the JKI launched the online platform DrosoMon (http://drosomon.julius-kuehn.de/), which serves as a database and visualization platform for monitoring the occurrence and activity of *D. suzukii*. Currently, several other institutes and plant protection services from all over Germany, as well as institutions from other European countries have joined this project. DrosoMon helps with the harmonization of the datasets and simplifies the comparability of trap data between monitoring systems. DrosoMon started with the data of the three JKI sites located in the northern part of the upper Rhine valley, an important fruit- and wine-growing region. Many of the fruit crops are susceptible to *D. suzukii* infestation, and the resulting damages lead to substantial economic losses. This region is characterized by small-scale agriculture thriving under the humid Atlantic climate with warm summers and moderate winters [18,19] and provides diverse habitats and retreats for *D. suzukii*. The climate differs significantly from that of other fruit- and wine-growing regions like the Pacific Northwest with hot dry summers and cold winters or the coastal climates of North America with their mild winters [20–25]. However, it is quite similar to the moderate summer and winter climate of Northern Italy, where *D. suzukii*'s presence since 2009 has been related to episodic damages to fruit harvests [25–28].

When *D. suzukii* was first recorded in Europe, it was suggested that cold winters and hot dry summers are unsuitable for this species [15,21]. Consequently, it was supposed that only low numbers of individuals might overwinter at sheltered sites [3,6]. Previously published studies already showed that *D. suzukii* is well adapted to various weather conditions [29] and defined weather indices for their development, mortality and population dynamics [20,27,30,31]. Seasonal dimorphism of winter and summer morphs has been put forth as an adaptation to survive hot and dry summers and overwinter colder winters [6,32].

In the present study, a number of the above-mentioned weather indices were evaluated for their impacts on *D. suzukii* trap captures in the upper Rhine Valley. We describe for the first time the seasonal trap-activity of *D. suzukii* in various habitats of the highly diverse and fragmented upper Rhine valley.

The objectives of this study were (i) to study the spatio-temporal trap activity of *D. suzukii* in cropped and wild habitats, (ii) the relationship between land use and the seasonal and annual capture rates and (iii) the impact of weather on capture rates.

2. Materials and Methods

2.1. Study Area: Topography and Weather

The monitored areas were clustered around the "Institute for Plant Protection in Fruit Crops and Viticulture" with its sites in Dossenheim (JKI Dos) and Siebeldingen (JKI Sie) and the "Institute for Biological Control" in Darmstadt (JKI Dar), covering an area of ~75 km^2, in southwest Germany (Figure 1). This region is characterized by the central Rhine rift valley with the Palatinate Forest to the west and the Forest of Odes to the east. About half of the agricultural area in the upper Rhine valley is dedicated to specialized crops (e.g., asparagus, blackberries, cherries, corn, plums, strawberries, raspberries, hops, tobacco, various vegetables and wine). It is also home to the three largest wine-growing regions in Germany: Baden, the Palatinate and Rhine-Hesse.

The upper Rhine falls under the 'humid, warm temperate' climate (Cfb) of the Köppen and Geiger climate classification: oceanic with warm summers and mild winters, with rainfall distributed throughout the year [18,19]. During the monitoring period (2011–2018), the mean annual temperature was 11 (±7.3) °C, and the mean annual precipitation was 746 (±103) mm. This period also recorded some of the hottest summers, with 2015 being the hottest ever recorded in Germany (German Weather Service, DWD).

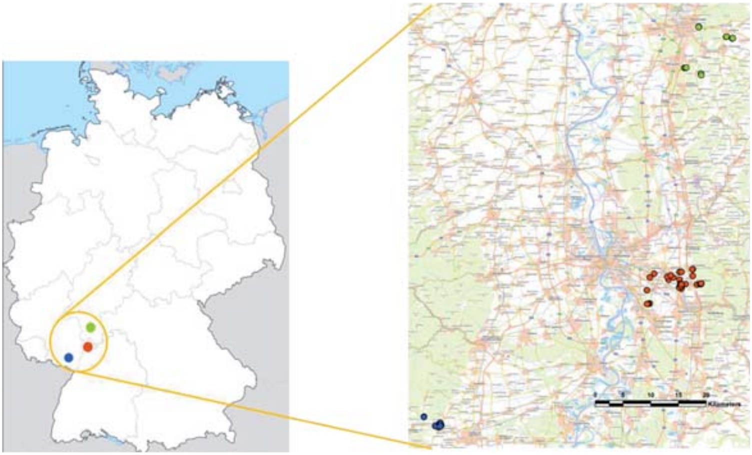

Figure 1. Map of Germany (left) showing the monitoring area (orange inset) and the location of the monitoring traps belonging to each of the three Julius Kühn-Institut (JKI) work groups (red = JKI Dossenheim (Dos), blue = JKI Siebeldingen (Sie) and green = JKI Darmstadt (Dar)). Source: © GeoBasis-DE/BKG 2018.

2.2. Trap Designs and Placement

The traps of the three sites (Figure 2) were quite similar in design and bait used, following the guidelines from the JKI's *D. suzukii*-website (https://drosophila.julius-kuehn.de) with minor variations in the size and shape of the cups. The JKI Dos trap [33] (Figure 2 and Figure S1) was a clear plastic cup (JETB 850, Jokey Plastik Wipperfürth GmbH, Wipperfürth, Germany) with an airtight lid. A stencil (Figure S1b) was used to drill holes uniformly (21 holes in two rows, Ø = 2.5 mm, distance between the holes = 20 mm) into the upper third of the cup, leaving 1/3 of the cup circumference unperforated to allow complete decanting of the trap contents. The traps were filled with 200 mL of unfiltered apple cider vinegar (ACV) (acetic acid content 5%, K-Classic, Kaufland, Neckarsulm, Germany), diluted to 40% with tap water. Finally, a drop of odorless detergent (1L = 0.025%) was added to the mixture to reduce surface tension. When temperatures were close to freezing, 5% NaCl was added to the trap mixture to avoid the freezing of the bait liquid. Bioassay tests showed no statistical differences between 0% and 5% NaCl in the attractiveness of the mixture, whereas differences were significant between 0% and 10% NaCl for both females (W = 133, p-value < 0.001) and males (W = 133, p-value < 0.001).

The JKI Sie trap (Figure 2) (Econo Plastic cup 500 mL, Huthamaki, Alf, Mosel, Germany) had 33 holes (Ø = 2.0 mm, distance between the holes = 5 mm) burned with a soldering iron into the upper third of the cup, leaving 3/4 of the cup circumference unperforated. The perforated area was reinforced with red tape before burning holes. The traps were filled with the same ACV mixture (200 mL) as the JKI Dos trap. The JKI Dar trap (Figure 2) was an air-tight, clear plastic cup (polypropylene, 1000 mL, H. Hermann Rotert GmbH and Co. KG, Bad Iburg, Germany) with 10 holes (Ø = 2.5 mm, distance between the holes = approximately 28 mm) drilled on the side in the same fashion as the JKI Dos trap. The baiting liquid was unfiltered ACV (acetic acid content 5%, Alnatura, Bickenbach, Germany) diluted to 40% with tap water and a drop of detergent (Elina, Karlsbach, Germany). The traps were filled with 100 mL of the mixture.

Trap sites were chosen randomly to represent a wide range of host plants and/or potential overwintering grounds in the immediate vicinity. The traps were installed in semi-natural habitats, mainly hedges with wild host plants of *D. suzukii*, forested, agricultural and urban areas, in the vineyards of JKI Sie and in the experimental orchards of JKI Dos and JKI Dar.

The JKI Dos traps where changed fortnightly, JKI Sie traps weekly during summer, spring and autumn and fortnightly in December and January. JKI Dar traps were changed weekly between March and September and fortnightly during the rest of the year. This schedule was followed as much as possible with minor variations depending on weather conditions and trap accessibility.

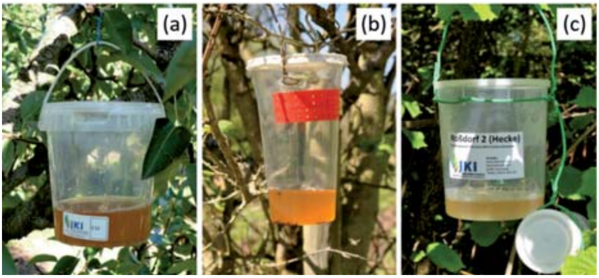

Figure 2. Monitoring traps: (**a**) JKI Dos, (**b**) JKI Sie, (**c**) JKI Dar. Photos: F.B., C.H., C.E.

2.3. Species Identification

The trap content was decanted onto a sieve (mesh size <0.5 mm) to filter out the flies from the liquid. Larger insects were manually separated and the rest transferred into petri dishes where they were identified using an identification key [34] and counted under a stereo microscope (Stemi 2000, Zeiss, Oberkochem, Germany). When the catch was >5 mL with over ~80% estimated *D. suzukii*, the contents were transferred to petri dishes of a diameter of 6 cm or 14 cm depending on the volume of the catch (less than or greater than 15 mL, respectively). The smaller petri dish was split into 6 equal segments and the bigger one into 8 with metal wires attached to a plastic ring insert (Figure S2). *D. suzukii* counting was carried out for two randomly selected diametrically opposed sections and then extrapolated to the whole petri dish. At JKI Sie and JKI Dar, every single fly was checked and counted.

2.4. Monitoring Data from DrosoMon

The following trap-specific characteristics were entered in the online database DrosoMon: coordinates of the trap location, trap design, bait used, plant on which the trap is hung and the habitat type in the direct vicinity of the trap. Traps were placed on 56 different host plants, located in six different habitat types (Table 1) [35]. Capture data for each trap deployment were entered as start and stop dates and the number of male and female *D. suzukii* captured. Based on the capture rates in different periods of the year and the current knowledge on the phenology of *D. suzukii* [6,20,22,36], the captures were categorized into five phenology/seasonal classes (Table 2) representing either their activity patterns or the annual population phases of the fly. Thus, a typical *D. suzukii*-year in this region starts on the 12 March of each year (Table 2). Though each institute set a fixed trap deployment period, they varied over the monitoring period and between institutes. The median collection period for the trap contents for JKI Dos, JKI Sie and JKI Dar was 14 (range: 4–28), 7 (range: 4–44) and 7 (range: 5–48) days, respectively.

Table 1. Total number of traps per JKI site in each habitat type and the marginal sums.

Institute	Forest	Forest Border	Hedge	Orchards	Vineyard	Urban Area	Total
JKI Dos	33	2	18	15	0	0	68
JKI Sie	1	1	5	6	4	2	19
JKI Dar	3	1	3	4	0	2	13
Total No. of traps	37	4	26	25	4	4	100

Table 2. Definitions of the five phenology classes representing the activity patterns of adult *D. suzukii*.

Seasonal Classes	Start-End	Day of Year	Observation
Early spring	March 12–May 15	71–135	Lowest capture rate
Late spring	May 16–July 29	136–210	Increase in trap captures, emergence of summer morph
Summer	July 30–October 7	211–280	First peak in number of captures
Autumn	October 8–December 31	281–366	Second peak in number of captures, emergence of winter morph
Winter	January 1–March 11	1–70	Winter decline of captures

2.5. Exploratory Statistical Analysis

Fly captures were standardized by calculating the sum of females and males captured per day per trap for each trap deployment. In all subsequent analysis, the sum of individuals/day/trap has been used unless indicated. The daily captures per trap were summarized by sites, host plants and sampled habitat type over years and seasons independently and visually explored for patterns and variations. Annual population dynamics were described by seasons and sampled habitat types. Based on the typical capture patterns over the year, we consider the period from March 12–March 11 of the following year as one annual population cycle, referred to as *D. suzukii*-year hereafter. Traps were sorted in order of their highest capture rates, and the top five traps were identified and manually checked for location effects by way of surrounding habitats/land use types to explain their disproportionately high captures. The numbers of females and males were converted to percentages for comparison. Seasonal sex ratios were compared and tested for significant differences using negative binomial regression from the R: MASS package [37].

Daily gridded weather data from the DWD was mapped to the location of the traps, and a number of weather indices shown to affect *D. suzukii* growth and development [20,27,30–32,38,39] were calculated for each trap deployment (Table 3). The capture rates were regressed against the weather indices using negative binomial regression to account for the extreme overdispersion in the dataset. Hot days and optimal development days were regressed against late spring and summer captures. Frost, ice and winter mortality days were regressed against early spring, autumn and winter captures. The mean of daily mean temperature was regressed against captures from all seasons. All analyses were performed in R 3.3.3 (R Development Core Team 2016), and graphs were done using the ggplot2 package [40].

Table 3. Weather indices created to test the relationship between weather and capture rates of *D. suzukii*. All parameters were calculated for the 30-day period before the trap contents were collected. Expected relationships are coded '−' as unfavorable and '+' as favorable to *D. suzukii*.

Weather Indices	Description	Expected Effect on *D. suzukii*
$T_{max} < 0$ °C	Ice days	−
$T_{min} < 0$ °C	Frost days	−
$T_{max} \leq 8$ °C	Cold days	−
20 °C $\leq T_{mean} \leq$ 30 °C	Optimal development range	+
$T_{max} > 30$ °C	Hot days	−
T_{mean} °C	Average temperature	Varying with season

3. Results

The number of traps monitored varied across the years from 25 in 2011 to 93 in 2016–2017 (Table 4). The traps were exposed for a total of 116,602 days (min = 4, max = 48) and captured 756,768 adult *D. suzukii* individuals. The first captures of *D. suzukii* during the monitoring were between September and November, 2011 in a plum orchard, in the experimental field in Dossenheim (10 ♂, 1 ♀) and in a vineyard in Siebeldingen (3 ♂, 1 ♀). The JKI Dar captured the first individuals (12 ♂, 6 ♀) in August 2013 in a commercial cherry orchard near Nieder-Beerbach, Germany.

Table 4. The number of traps monitored by each institute for each *D. suzukii*-year. Given in brackets are the number of traps that were not included in this study, as these traps were in operation before the first catch of *D. suzukii*.

Operator	2011–2012	2012–2013	2013–2014	2014–2015	2015–2016	2016–2017	2017–2018
JKI Dar	(0) 0	(8) 0	(10) 3	10	13	13	6
JKI Dos	(5) 1	11	23	60	56	61	45
JKI Sie	(19) 0	16	17	17	19	19	17
Total	(24) 1	27	43	87	88	93	68

The mean annual trap activity followed population build-up starting in late spring and reaching a peak in summer. Forests and hedges recorded a second peak in autumn, after a brief decline in October (Figure 3). Capture rates steadily declined in winter to the very low numbers seen in early spring and the first half of late spring with a brief increase in April (Figure 3 and Figure S3). They also varied greatly between the sampled habitat types. Traps in forests, hedges and forest borders recorded the highest numbers, in peak seasons, over 1000 flies per day. In contrast, traps in fruit crops and vineyards recorded no more than a maximum of 50–100 flies per day (Figure 4). The plot of all trap captures together showed a distinct two-peak dynamic with higher captures in summer and autumn. Upon closer scrutiny, the two-peak dynamic is to be seen only in the case of forested areas and hedge traps and also not in all years, while those in vineyards and orchards show a decline already from the onset of cold weather (Figure 3). Though the highest number of captures in forested areas and hedges was in autumn, in some years, the median captures in summer were higher than in autumn (Figure 5). In most years, traps in forests and hedges captured consistently more flies in autumn than in summer, whereas traps in orchards captured more flies in summer than in autumn. Hedges and forests accounted for a much higher share of winter captures compared to other locations (Figure 4 and Figure S4).

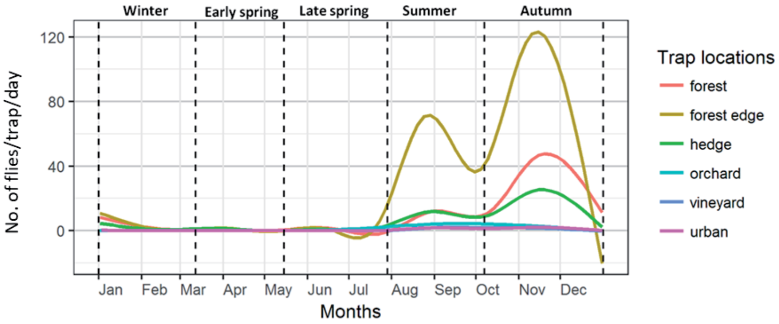

Figure 3. Smoothed LOESS curves of daily trap captures in different habitat types from 2011–2017. The vertical dotted lines mark the phenology phases (top; see Table 2 for a description).

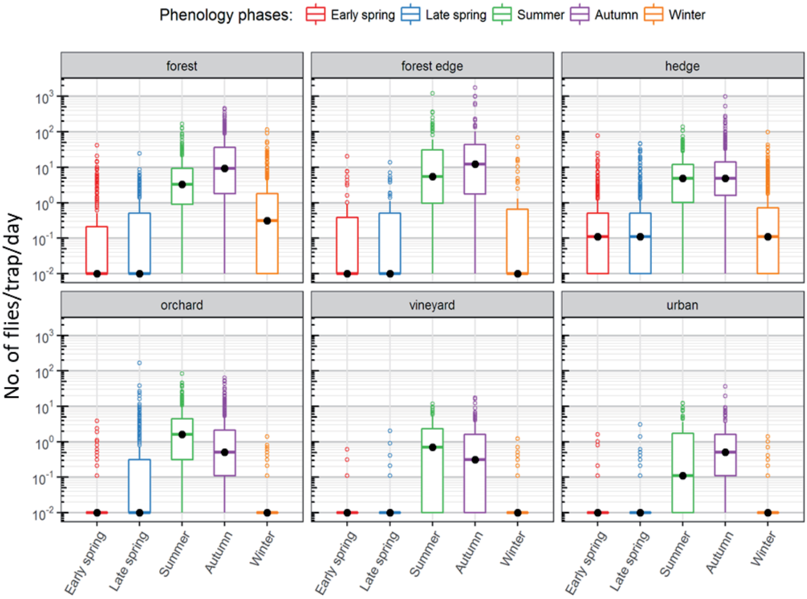

Figure 4. Box plots of daily captures in each sampled habitat type grouped by season. The median values represented by the bold black dots in each box plot highlight the variations in the seasonal dynamics of the captures in each sampled habitat type. The box represents the inter-quartile range (IQR) and the band inside the median. The whiskers represent data that are within 1.5 IQR below or above the first and the third quartiles, respectively. The outliers are represented by dots beyond the whiskers. Note: the Y-axis is log-scaled (captures/day/trap + 0.01).

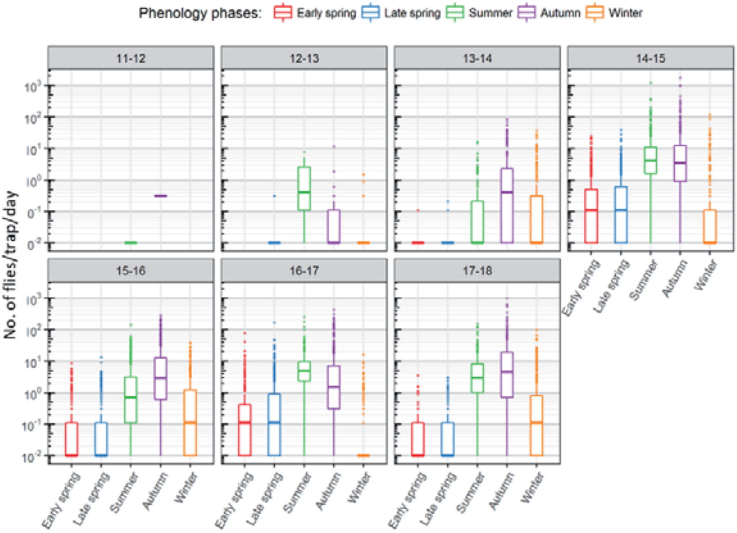

Figure 5. Box plots of captures/trap/day in each *D. suzukii*-year, grouped by SWD phenology. For a description of the box plots, see Figure 4. Note: the Y-axis is log-scaled (captures/day/trap + 0.01).

Five traps with very high capture rates were selected for a detailed analysis of the effects of land use on their capture rates (Figure 6 and Table S1). These traps were located on the borders of the Forest of Odes next to the Rhine valley with the highest captures in 2014–2015 and 2017–2018. Two traps (DO_E21 and DO_E19) were located prominently on a small mountain ledge, close to orchards/vineyards and the forest. Both traps captured between five- and 10-times more individuals over the years than trap DO_E5, which is also close to orchards/vineyards and shrubberies. The trap DO_E1 on the forest border, next to the urban area (Dossenheim) and a quarry captured less individuals than the above-mentioned traps, whereas the trap DO_E2, located deeper in the woods with only trees and no shrubberies nearby, and hence farther than the others from forest borders, orchards or urban areas, captured the lowest numbers.

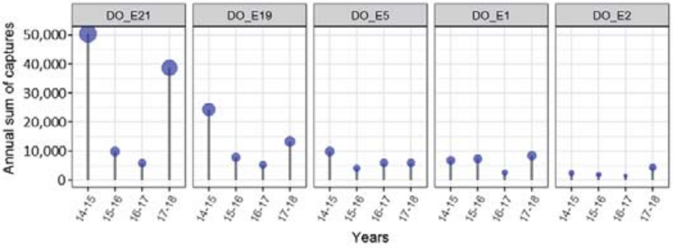

Figure 6. Yearly sum of captured individuals for five traps installed at/near the forest border. Traps DO_E21, DO_E19, DO_E1 and DO_E5 are located near the forest edge, while DO_E2 is located in the forest, 700 m from the forest border.

Overall, an equal number of females and males were captured (50.9:49.1%), with small variations over the seasons. Slightly more females were captured in early spring, late spring and winter compared to summer or autumn (Table 5). The pattern was fairly similar across the different locations except in vineyards where more females than males were captured in summer.

Table 5. Percentages of male and female *D. suzukii* captures in different seasons over the monitoring period.

D. suzukii Phenology	Males%	Females%
Early spring	39.9	60.1
Late spring	44.5	55.5
Summer	52	48
Autumn	55.1	44.9
Winter	40.3	59.7

Though *D. suzukii* was observed already in 2011, the next two years, 2012 and 2013, registered far less captures compared to the years after. Following the winter of 2013–2014, the warmest in the period with zero days below freezing, there was a comparable surge in the captures afterwards, never reaching values as low as those in 2012 and 2013. For studying the relationship between weather and captures, only capture data from 2013 onwards were used as *D. suzukii* appears to have successfully established in the monitored areas during the warm winter of 2013–2014. While the low temperatures in autumn were not accompanied by a corresponding change in capture rates, the higher number of ice days in the winter of 2016/2017 was reflected in the lowest winter captures from this year (Figure 7).

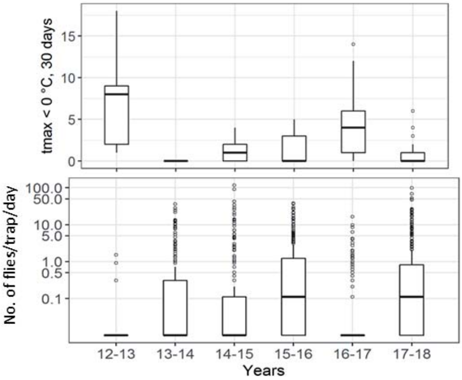

Figure 7. Winter captures: (top) the number of days when tmax < 0 °C in the 30 days before the trap content collection for each trap deployment, (bottom) captures/day from each trap deployment. Both panels are per *D. suzukii*-year. Note: log-scaled y-axis on the bottom panel.

During the monitoring period, the summer of 2017 was the hottest followed by that of 2015, resulting in the low late spring capture rates compared to those of 2014 and 2016. The 2014 and 2016 late springs also received more precipitation than those of 2015 or 2017 (Figure 8). The low summer captures in 2015 also corresponded to the high number of days when maximum temperature exceeded 30 °C this year (Figure 9).

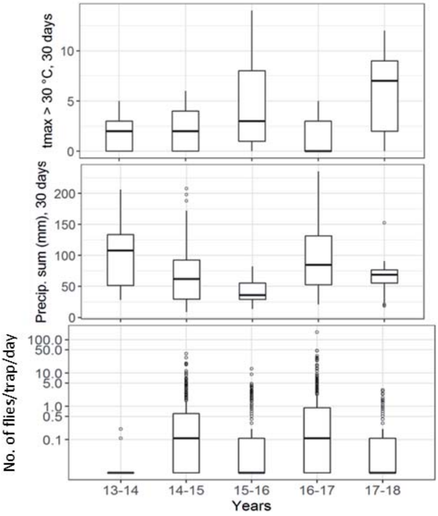

Figure 8. Late spring captures: (top) the number of days when tmax > 30 °C and (middle) precipitation sum (mm) in the 30 days before the traps were emptied for each trap deployment; (bottom) captures/day from each trap deployment. Both panels are per *D. suzukii*-year. Note: log-scaled y-axis on the bottom panel.

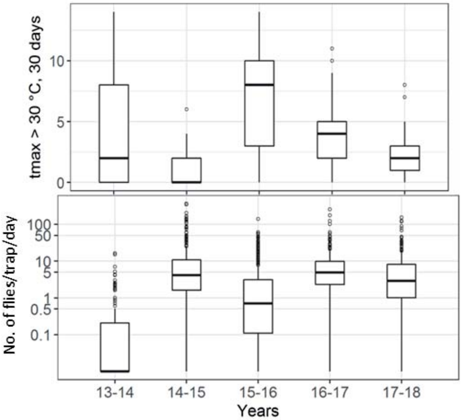

Figure 9. Summer captures: (top) the number of days when tmax > 30 °C in the 30 days before the traps were emptied for each trap deployment; (bottom) captures/day from each trap and trap deployment. Both panels are per *D. suzukii*-year. Note: log-scaled y-axis on the bottom panel.

The significant parameters from regressing seasonal captures against weather parameters are given in Table 6. Both late spring and summer captures were negatively associated with maximum temperatures above 30 °C. Temperatures in the range of 20 °C–30 °C defined as optimal weather for *D. suzukii* were positively associated with captures in late spring, but negatively associated for summer captures. Both frost and ice days were correlated with a decrease in capture rates in the cold seasons, while daily mean temperature (mean) and precipitation (sum) were positively correlated.

Table 6. Results from regression tests of seasonal captures against weather parameters. Shown are only parameters with significant *p*-values. 'coeff' is the regression coefficient, 'se' the standard error.

Season	Weather Parameter	Coeff	se	*p*-Value
Late spring	20 °C ≤ Tmean ≤ 30 °C (days)	0.134	0.008	<0.001
Late spring	Mean of tmean (°C)	0.309	0.020	<0.001
Summer	20 °C ≤ Tmean ≤ 30 °C (days)	−0.045	0.005	<0.001
Summer	Mean of tmean (°C)	−0.163	0.014	<0.001
Summer	tmax > 30 °C (days)	−0.131	0.009	<0.001
Late spring and autumn	tmax > 30 °C (days)	−0.102	0.009	<0.001
Autumn, winter and early spring	tmin < 0 °C (days)	−0.092	0.005	<0.001
Autumn, winter and early spring	Precipitation sum (mm)	0.014	0.001	<0.001
Autumn, winter and early spring	Mean of tmean (°C)	0.110	0.009	<0.001
Autumn, winter and early spring	tmax < 0 °C (days)	−0.350	0.026	<0.001
Autumn, winter and early spring	tmean ≤ 8 °C (days)	−0.026	0.003	<0.001

4. Discussion

In this study, we analyzed the monitoring data of the JKI in the upper Rhine valley in southwest Germany. The study started a few months before *D. suzukii* appeared for the first time in Germany, in August 2011, and followed its establishment in the succeeding years.

Drosophila suzukii has been present since at least late 2011 in southwest Germany. Currently, it is widespread and overwinters successfully in the upper Rhine valley. We suppose that the mild winter of 2013–2014 helped to establish a stable population in this part of Germany. The trap activity of this region was comparable to Northern Italy with a similar climate [27,28].

We split the typical annual population cycle of *D. suzukii* into five phenology phases (early spring, late spring, summer, autumn and winter) to better identify factors that affect the different phases of trap activity. Both weather conditions and habitat type influenced trap activity. The influence of habitat type on the trap captures is manifested in the regular, seasonal changes in the trap activity of *D. suzukii*.

The summer increase of trap captures and the following peak coincide with the ripening of most wild and cultivated host fruits of *D. suzukii* [11,41]. The single annual peak in summer in orchard and vineyard traps suggests that those sites are not suitable overwintering habitats and *D. suzukii* has to re-immigrate each year, whereas forested areas appear to be the favored habitat year-round. The increase of trap captures from late spring onwards until the autumn peak in forests, forest borders and hedges has also been noted in other temperate regions [20,22,24,42]. The single peak in late spring/summer in fruit crops and vineyards is quite similar to the findings in northwestern Italy [28]. We suppose that when attractive food sources are present, the traps are less attractive to the flies, such as in orchards, but in forests, the same baits are more attractive to the flies due to less competition between ACV-baited traps and attractive food sources. Further, forests may provide better habitat conditions when it is too hot in summer or too cold in winter. The presence of berries in hedges or in the forested areas [11,36,43] at various stages of ripeness partly all throughout the year could be an additional factor why forested areas and hedges always recorded very high capture rates and microorganisms growing on the surface of evergreen trees in the cold bottleneck seasons may also serve as food resources, supporting *D. suzukii* populations [13]. Thus, we cannot conclude whether higher trap captures in forests result from a higher *D. suzukii* abundance or a higher attractivity of traps. The winter decline and low captures in early spring have also been noted in California [22,44], northern Italy [28,45] and Canada [20].

The five traps at the forest border of the Forest of Odes suggest a distinct importance of forest borders to *D. suzukii*. Traps with the highest captures, located prominently on a small mountain ledge, were especially attractive in autumn, when most trees in orchards and vineyards have already lost their leaves. We assume that *D. suzukii* migrates from orchards/vineyards in the plains to forested areas in autumn in search of nutrition and overwintering habitats. DO_E21, in particular, offers such habitats since it is located on a *Robinia pseudoacacia* overgrown with *Hedera helix*. Our findings are in agreement with Tait et al. (2018) [46] that *D. suzukii* migrates between different habitats, depending on the season, searching for suitable overwintering habitats. Since DO_E2 captured less individuals than DO_E1, we assume that *D. suzukii* migrates into sheltered overwintering sites such as hedges and forested areas as long as it can find shrubbery. The attractiveness of the traps appears to be strongly influenced by the immediate habitat and the seasonal changes in the habitat with respect to the availability of nourishment and shelter. We recommend taking this into consideration when estimating in-field fly density based on trap captures or when trap capture data are treated as representative of fly density.

Based on our seven-year monitoring data with much higher capture rates in forests than in fruit orchards or vineyards even in the fruiting season, we question the reliability of the estimation of the actual density of *D. suzukii* in the field using ACV traps. When natural food sources are available, the flies seem to prefer the ripe fruits markedly more than the ACV mixture, resulting in an under-representation of the fly density in the fruit orchards and vineyards during the fruiting season. For economic reasons and to have comparable data over the monitoring period, we did not change our trap designs during the study. Our trap designs may have resulted in relatively conservative estimates of the abundance compared to other traps and/or baits that are now available [47–49]. However, it was already suggested that every individual fly captured in an ACV-baited trap represents 198 flies in a 2.7-ha cherry orchard [50]. These findings strengthen the argument that trap captures should be interpreted with caution [5,29,44,51].

We tested the capture data against temperature thresholds that were found to affect *D. suzukii* individuals and populations significantly in field and laboratory studies. The decline in trap captures when tmax > 30 °C in late spring was noted previously [22,30]. Further, it was already suggested that in lab assays, temperatures above 30 °C reduce reproduction or even inhibit it [52,53]. Although our weather data were of quite high resolution (1×1 km^2), it would be impossible to simulate the authentic field conditions that could vary in much smaller units that provide suitable shelters for *D. suzukii*. We also found that the years with high late spring captures correlated with high precipitation. Whether this is due to the effect of precipitation on *D. suzukii* host plants or the higher relative humidity favoring *D. suzukii* was beyond the scope of our study.

Since the climate in southwestern Germany is more akin to that of Northern Italy [28] than to California [22] or Canada [20], we expected *D. suzukii* population dynamics to be similar to that observed in northern Italy. The winter of 2013/2014, with zero days below freezing and only a few cold days, was the mildest during the whole monitoring period. Survival success during this mild winter could have played a key role in the excessive damages observed in the following spring in this region. Next, low temperatures in autumn do not seem to be correlated with low capture rates, whereas ice-days in winter are followed by a decrease in trap captures over a longer period (e.g., winter of 2016/2017). In contrast to previously published findings [20], we did not find any conclusive relationships between mild winter days and earlier fly appearance in the traps the following spring.

Both females and males were captured all throughout the year, but the number of males was slightly higher during the peak seasons (summer and autumn), and previously published studies showed less attractiveness of ACV-baited traps to female *D. suzukii* the more mature eggs their ovaries contained [1,54]. However, we assume that a combination of this and a higher dispersal of males to ensure gene flow results in higher capture rates of males during the peak seasons. More females were captured in the colder seasons when trap captures were lower, suggesting the better adaptability of female winter morphs to colder periods with insufficient food sources. The higher cold tolerance of females was observed in many other studies, as well [20,21,42,55,56]. However, reports are not conclusive. Ryan et al. [52] did not find any difference between sexes in their response to low temperatures, while Enriquez and Colinet [57] even suggested that males are better adapted to survive through winters.

5. Conclusions

Through our monitoring program we were able to record the first appearance of *D. suzukii* in Germany in 2011 and follow its establishment over the following years. Despite its first occurrence in 2011, it was the mild winter of 2013/2014 that helped *D. suzukii* the firm establishment in southwest Germany. The successful establishment in this region is proof of its adaptability to cold winters, hot summers, and fluctuating temperatures, in a small structured landscape.

Further, our analysis confirms that temperature thresholds for in-field development and survival should be re-adjusted with respect to further meteorological (e.g., global radiation) and habitat parameters as conclusions based on laboratory experiments cannot be easily juxtaposed with those based on field studies. While laboratory studies are carried out in a controlled environment, numerous factors are at play in field studies. Next, trap captures do not reflect actual in-field fly densities and we still lack information about realistic in-field population densities since the attractiveness of ACV baited traps varies significantly between habitats and seasons.

A detailed analysis of the effect of landscape on *D. suzukii* captures and determination of weather conditions was beyond the scope of the study. However, it must be noted that the traps were spatially quite clustered and did not map large variations in weather. An analysis of a country-wide monitoring is necessary to validate the results on a bigger scale.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/9/4/125/s1: Figure S1. (a) Example of a new cup, (b) the used stencil for standardized trap construction and (c) the prepared trap. Figure S2. Partial counting of large captures (>5 mL): (a) petri dish (Ø 6 cm) split into six equal sections for catches <15 mL; (b) petri dish (Ø 14 cm) split into eight equal sections for catches >15 mL. The sections were made with wires attached to a plastic ring insert placed inside the petri dish. Figure S3. Captures per day from each trap over the monitoring period (2011–2018) with a smoothing curve (blue), based on which the five seasonal categories were created. The bubble size (small to big) is proportional to the capture size. Note: the y-axis is log-scaled. Figure S4. Box plots of captures/trap/day in each *D. suzukii*-year, grouped by sampled habitat types and *D. suzukii* phenology. The box represents the inter-quartile range (IQR) and the band inside the median. The whiskers represent data that are within 1.5 IQR below or above the first and the third quartiles. The outliers are represented by dots beyond the whiskers. Note: the Y-axis is log-scaled (captures/day/trap + 0.01). Figure S5. Annual sum of *D. suzukii* captures in the *D. suzukii*-years from 2014–2015 until 2017–2018 grouped by *D. suzukii* phenology and site/trap. The size of the blue bubble is proportional to the annual sum. Note: the y-axis scale varies between rows. Table S1. Distances of select traps from the forest border, orchards/vineyards and urban areas, coordinates and altitude. The five traps shown here are located in and around the Forest of Odes and captured significantly higher individuals than the rest of the traps from this study.

Author Contributions: Conceptualization, H.V., F.B., C.H., B.G and A.H. Methodology, F.B., H.V., C.H. and C.E. Formal analysis, A.R.D. and F.B. Data curation, A.R.D. and F.B. Writing, original draft preparation, F.B. and A.R.D. Writing, review and editing, all authors. Supervision, H.V.

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2.2 An invader supported by a parasite: Mistletoe berries as host for food and reproduction of Spotted Wing Drosophila in early spring

Felix Briem, Astrid Eben, Jürgen Gross, Heidrun Vogt

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ORIGINAL PAPER

An invader supported by a parasite: Mistletoe berries as a host for food and reproduction of Spotted Wing Drosophila in early spring

Felix Briem[1] · **Astrid Eben**[1] · **Jürgen Gross**[2] · **Heidrun Vogt**[1]

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Abstract The Spotted Wing Drosophila (SWD), *Drosophila suzukii* Matsumura, an invasive pest species in Europe and the Americas, is able to feed and reproduce on numerous fruit crops and a wide range of wild host plants. SWD is thought to overwinter outside of agricultural fields in forests and hedges. To identify overwintering sites and early spring oviposition hosts, traps were installed in forests. In spring 2015, traps in the canopy of pine trees parasitized by mistletoe, *Viscum album* subsp. *laxum,* captured significantly more SWD than traps in pine trees without mistletoe. We found SWD females with ripe eggs coinciding with ripening and ripe mistletoe berries. We investigated whether mistletoe may serve as a host for SWD. Under laboratory conditions, SWD developed from egg to adult in mistletoe berries. More adults emerged from wounded berries. Females were observed to feed on berries and survived up to eight days without other food. A few adults emerged from wild mistletoe berries. To understand the attraction of SWD to parasitized trees, we analyzed the volatile organic compounds (VOCs) collected from the headspace of mistletoe berries by GC–MS and identified the main components. Thirty-two VOCs were found. Wounded and unwounded berries differed significantly in the quantity of 11 VOCs emitted. The odor spectrum showed many similarities to other typical berry odors. The combination of field surveys and laboratory assays identified a new reproduction host for SWD in spring. This host plant may help SWD to withstand the bottleneck period for survival in winter and spring.

Keywords Alternative host · Overwintering · Invasion biology · Volatile compounds · Reproductive status · Population dynamics

Key message

- Mistletoe berries support SWD nutrition and reproduction.
- SWD abundance in the canopy of pine trees parasitized by *V. album* was higher than in the canopy of *P. sylvestris* without *V. album* or at lower heights in the vegetation.
- The odor spectrum showed many similarities to other typical berry odors.
- Adult SWD emerged from field-collected mistletoe berries indicating that mistletoe is one of the first reproductive hosts for SWD in Central Europe.

Introduction

The invasive Spotted Wing Drosophila (SWD), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), is a worldwide pest species that recently became established in Europe (Cini et al. 2012, 2014). After its discovery in the

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✉ Heidrun Vogt
heidrun.vogt@jki.bund.de

[1] Laboratory of Entomology, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Julius Kühn-Institut (JKI), Schwabenheimer Straße 101, 69221 Dossenheim, Germany

[2] Laboratory of Chemical Ecology, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Julius Kühn-Institut (JKI), Schwabenheimer Straße 101, 69221 Dossenheim, Germany

USA (California) and Southern Europe (Italy & Spain) in 2008, it rapidly dispersed and is now present in many regions of North America and most European countries, and since 2013 in Brazil (Calabria et al. 2012; Deprá et al. 2014; Asplen et al. 2015). In Germany, it was recorded for the first time in 2011 (Heuck 2012; Vogt et al. 2012a, b) and spread rapidly throughout the country. Since the summer 2014, it has been found in all Federal States of Germany (Asplen et al. 2015; Köppler and Vogt 2015; Vogt and Briem 2015).

As a highly polyphagous pest species, SWD infests a broad range of wild hosts and cultivated fruits. Its complete host range has not yet been determined. It infests economically important berry crops (e.g., raspberries and blackberries: *Rubus* spp.), stone fruits (e.g., cherries: *Prunus avium*, *P. cerasus*, plums: *Prunus domestica*), as well as many wild host plants like *Sambucus* sp., *Prunus serotina*, or *Cornus* sp. (Hauser 2011; Cini et al. 2012; Poyet et al. 2014; Lee et al. 2015). The female fly penetrates the skin of ripening and ripe fruits with its sclerotized saw-like ovipositor and lays its eggs underneath the fruit skin (Mitsui et al. 2010; Hauser 2011; Cini et al. 2012; Bellamy et al. 2013). Deposited eggs are detectable by their respiration filaments that protrude from the fruit.

According to current knowledge, SWD is overwintering as adult. It shows phenotypic plasticity with darker and bigger individuals in winter that can survive several months at 1 °C (Shearer PW, personal communication). Winter morphs are the most cold-tolerant life stage, but are chill intolerant. Thus, adults must avoid extreme cold temperatures to survive, e.g., by hiding in sheltered sites (Stephens et al. 2015; Jakobs et al. 2015). In addition, there is no egg production from fall to spring (Mitsui et al. 2010; Gerdeman and Tanigoshi 2012; Zerulla et al. 2015, and own observations, unpublished), suggesting a reproductive diapause. The findings of Ometto et al. (2013) about the low rate of molecular evolution in SWD support this as it could be due to its reduced rate of generations per year compared with its relatives. In temperate climates, SWD is a winter-active species when mean temperatures allow flight activity. Although exact data on temperature-dependent flight are not available, seasonal catches from diverse regions during winter indicate that flight activity seems to be still possible when mean daily temperatures are around 5 °C (Harris et al. 2014; Wiman et al. 2014; Briem et al. 2015; Shearer, pers. communication). During winter, SWD occurs especially in forest edges and interiors, a finding which supports the assumption of fly migration over larger distances away from orchards to suitable overwintering sites (Hamby et al. 2014; Briem et al. 2015). Furthermore, its ability for a fast recovery and survival potential at low temperatures and during short freezing periods by feeding on fruit dropped on the ground or leftover after harvest could enable successful overwintering (Dalton et al. 2011; Jakobs et al. 2015; Stephens et al. 2015).

During the mild winter 2013/2014, SWD adults were caught continuously in traps installed in forests at a standard height of 1.5 m above the ground as well as in the canopy (~18–20 m) of trees (Briem et al. 2015). In 2014, following an extraordinarily warm spring, reproduction of the overwintered population started earlier than in previous years. The shortened generation time due to the high ambient temperature allowed the population to increase rapidly causing high infestation levels in stone and soft fruits early in the season. As population growth in spring depends on winter survival as well as on early available reproduction hosts, identifying wild host plants where SWD can feed and oviposit in early spring might help provide additional knowledge about its population dynamics. Alternative host plants, such as *Lonicera* sp. or mistletoes (*Viscum* sp.), can supply sugar sources to SWD in winter (Lee et al. 2015) and early spring which might increase the survival rate of SWD. Within our landscape monitoring, traps in pine trees infested by mistletoes captured more SWD in spring than traps in pine trees without mistletoes. We hypothesized that mistletoe might serve as an early food source and host for reproduction. Thus, we conducted a study to investigate the suitability of mistletoe berries as food and host for SWD reproduction.

Methods and materials

SWD rearing and colony maintenance

Female SWD used for no-choice assays were obtained from a laboratory colony maintained at the Julius Kühn-Institut (JKI) Dossenheim, Germany since October 2013. The culture was started with offspring of adults that emerged from fruit (blackberry, raspberry, and cherry) sampled at the experimental fields of the JKI Dossenheim. Adult SWD were kept in cages (Bugdorm-1, Megaview, Taiwan) provided with a sugar-water source (5 % sucrose) and dry sugar and brewer's yeast mix (1:1). For rearing offspring JKI standard diet (30 g sugar, 142 g cornmeal, 20 g soy flour (Reformhaus, Germany), 34 g brewer's yeast (Diana, Germany),11.2 g agar and 5 g vitamin mixture (Vanderzant, MP Biomedicals, USA), 9.4 ml propionic acid, 1748 ml water) was used, based on a recipe from Fondazione Edmund Mach, San Michele, Italy (pers. communication G. Anfora). For oviposition, cups (125 ml, Huhtamaki, Finland) filled with JKI standard diet were placed in the cages. After 2–3 days, cups were removed and closed with a perforated lid. These oviposition substrates were stored under the same conditions as above until adults emerged. Then lids were removed and cups were placed in rearing cages. Rearing cages were stored in an environmental chamber at 23 °C, 60 % relative humidity (RH), and a photoperiod of 16 h:8 h (L:D).

Monitoring SWD adults

Transparent plastic cups (Jokey JETB 850, 870 ml, 12 cm high, 10 cm in diameter) closed with a lid and prepared with 20 holes ($d = 2.5$ mm) in the upper half were used as traps for monitoring the abundance of SWD in the field. As bait, 200 ml of naturally clouded apple cider vinegar (K-Classic, Kaufland, Neckarsulm, Germany) was mixed with water (ratio 2:3) and 0,025 % detergent (Ultra Sensitive, dm, Karlsruhe, Germany). The traps were distributed among pine trees in a forest (Dossenwald, N49.418949, E8.564489; 100–114 m a.s.l) between Mannheim and Schwetzingen (Baden-Württemberg, Germany) and in a second forest near Dossenheim (N49.450382, E8.691917; 290–330 a.s.l., Baden-Württemberg, Germany).

The Dossenwald is a nature reserve near Mannheim situated in the plain of the Rhine valley (Breunig and Demuth 2000). It is characterized by a high proportion of Scots pine, *P. sylvestris,* and several other deciduous forest tree species (e.g., *Robinia pseudoacacia, Fagus sylvatica, Prunus avium, P. padus*, *P. serotina, Quercus robur*). The forest near Dossenheim is part of the low mountain range named Odenwald. We chose sites on the western mountain side bordering the Rhine valley. Wild and cultivated host fruits (*Prunus* sp., *Rubus* sp., *Sambucus* sp., *Vitis* sp.) are found along its edges. The main part of the forest is a mixed stand with beech (*F. sylvatica*) and oak trees (*Quercus* sp.). In the sun-exposed parts, *P. sylvestris* are common; moreover, wild cherry trees (*P. avium, P. padus*), maple (*Acer* sp.) and spruce (*Picea abies*) are also present.

At each forest site, traps were installed at a height of about 20 m at the canopy of five *P. sylvestris* (one trap per tree) using a bow and arrow: first a thin string was shot to the treetop, then a thicker string was drawn up with the help of the thin one resulting in an infinite loop. Finally, the trap was fixed to the thick string and pulled to the canopy. For changing the trap, it was lowered with the help of the string loop. In addition, one trap was placed at standard monitoring height (~1.5–1.8 m) in each of the five trees. These trees were distanced by 50–100 m. Throughout the entire year, traps were biweekly exchanged and numbers of male and female SWD captured were determined under a stereo microscope (M3Z, Wild Heerbrugg, Switzerland). Female flies captured between March and May 2015 were stored in 70 % ethanol for further evaluation of ovarian development.

Ovigeny assessment

A total of 522 female individuals caught during March and May 2015 were dissected to evaluate ovarian development. Ovarian development was categorized into five categories (Fig. 1) (King et al. 1956; Zerulla et al. 2015):

1. Indiscernible ovarioles,
2. Unripe ovarioles,
3. Maturing eggs in ovarioles visible,
4. Mature eggs with filaments, and
5. Old eggs.

To determine the developmental stage of the ovaries, the abdomen of each fly was cut with a pair of tweezers and held open between the 3rd and 4th segments with another pair of tweezers. By doing this, the internal organs in the abdomen were exposed and ovarioles could be categorized. Photographs of representative developmental stages of eggs were taken with a photomacroscope (M400, Wild Heerbrugg, Switzerland).

Oviposition preference and development

No-choice trials were conducted to investigate the suitability of mistletoe berries for oviposition and development, and to determine the effect of berry wounding. Berries of *Viscum album* subsp. *laxum* were collected from pine trees in the forest site near Mannheim (N49.418949, E8.564489) at April 14th and 29th 2015. Branches were cut

Fig. 1 Developmental stages of SWD eggs (magnification: ×32). Photographs: J. Just & A. Frank, JKI Dossenheim

from the canopy of *P. sylvestris* at an approximate height of 15–18 m using a manlift (Nifty 150-T, Niftylift LtD, Germany). Fruits were stored at 18 °C until use. Six subsets of 10 berries which were not exposed to female SWD were macerated to determine Brix (% soluble solids) and pH.

The experimental unit was a small cage assembled with a Plexiglas ring ($d = 10$ cm, 3 cm height) and closed at both sides with glass plates (11 × 11 cm). The Plexiglas ring had five ventilation holes ($d = 1.3$ cm) covered with fine gauze and was equipped with a sugar-water source (5 % sucrose). In each of these cages, 10 wounded or 10 unwounded berries, respectively, were offered to ten 11-day-old gravid females. Ten replicates were performed for each treatment. Field-collected berries were checked for intactness by examining each berry for any cracks using a stereomicroscope. Intact berries were used for the treatment "unwounded," whereas for the treatment "wounded," intact berries were slit with a scalpel over a length of ~3–4 mm. The berries were arranged on small plastic lids to ensure that the slit remains on the upper side during the trials. These cages were stored in an environmental chamber at 23 °C, 60 % relative humidity (RH), and a photoperiod of 16:8 (L:D). After 48 h of exposure, females were removed from the cages and the number of eggs laid in berries of *V. album* subsp. *laxum* was counted by checking for egg filaments using a stereo microscope. The berries were then transferred to clear rearing cups (pint-sized Bugdorm 360 ml/12 oz, Megaview, Taiwan) with mesh lids and kept for 18 days in the environmental chamber and emerged adults were counted regularly. The trials were repeated three times, on April 15th, 22nd, and May 4th, 2015.

Survival rate

No-choice trials were conducted to determine the effect of intact or artificially wounded mistletoe berries on the survival rate of female SWD. The experimental unit for the assay was the same as described above, equipped with a water source and a small petri dish ($d = 6$ cm), filled with a moistened cotton pad and water instead of sugar-water. On the cotton pad, as described previously, 10 wounded or 10 unwounded berries per cage were offered to ten 11-day-old gravid females. Ten replicates were conducted per treatment. A third treatment offering only water served as a control. Dead females were counted once per day.

Initial infestation of mistletoe

To determine the infestation rate under natural conditions, a random sample of 100 berries (*V. album* subsp. *laxum* growing on *P. sylvestris)* collected on April 14th in Dossenwald and about 1,000 (*V. album* subsp. *album* growing on *Malus domestica*) on May 19th from apple trees of a meadow orchard near Heddesbach (N49.474216, E8.834986, Odenwald, Germany) were used. The berries were stored in small rearing tents (Bugdorm-2120, Megaview, Taiwan) in the environmental chamber until June 16th and daily checked for emerged adult SWD. As described previously, SWD abundance was assessed in this orchard with one trap in each of four apple trees from May 19th to June 16th 2015.

Sampling of volatile organic compounds (VOCs) emitted by mistletoe berries

To compare the profile of volatile chemical compounds emitted by wounded and unwounded berries of *V. album* subsp. *laxum*, berries (50 g) were put in clean glass petri dishes (without lids) and placed in 0.7-l desiccators continuously flushed with charcoal-filtered air. For each treatment six replicates were sampled. A quantitative 5-channel sampling device according to Rid et al. (2016) was used. Headspace of berries was sampled for 100 min (flow 100 ml/min) in an environmental chamber (18–20 °C). Volatiles were trapped in stainless steel sample tubes (Tenax TA 60/80, PerkinElmer, USA).

GC–MS analysis

Samples were analyzed using a thermal desorber (TurboMatrix ATD 650, PerkinElmer) connected to a gas chromatograph coupled mass spectrometer system (Clarus 680, PerkinElmer). Tubes were desorbed for 10 min at 250 °C. Volatiles were collected in a cold trap (Tenax TA) at −20 °C and transferred to the GC–MS system (99 °C/s to 250 °C, hold 1 min). A nonpolar Rxi-5 ms capillary column (Restek, Germany) was used for volatile separation. Splitless injection was employed using helium as a carrier gas (flow rate 5 ml/min, column head pressure 5 bar). The initial oven temperature of 40 °C was held for 1 min, followed by a gradient (40–180 °C at a rate of 5 °C/min, and a rate of 20 °C/min from 180 to 280 °C, final temperature was held for 6 min). The quadruple mass detector was operated in electron impact (EI) mode at 70 eV. Full-scan mass spectra were collected within the range of 35–350 m/z. Volatile compounds were identified by comparing fragmentation patterns with data from mass spectral libraries (NIST 08 Mass Spectral Library, National Institute of Standards and Technology, Wiley; JKI-OW Library). Peak retention times were compared with standards according to Weintraub and Gross (2013). Relative proportions of selected compounds were calculated from peak areas and the sum of the selected compounds was set at 100 %.

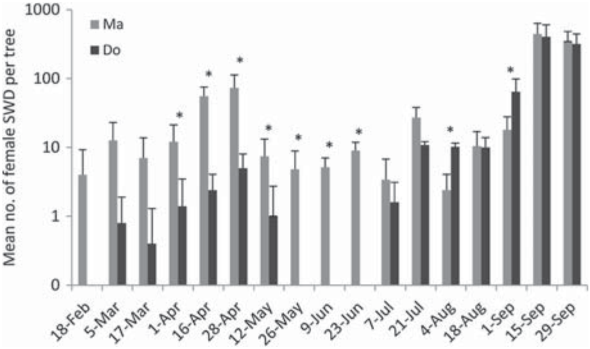

Fig. 2 Flight activity of female SWD monitored in pine trees at Dossenwald (Ma) and Dossenheim (Do) in 2015. *Bars* = mean number of female flies per tree. Log scale with standard deviation (SD) ($n = 5$; 2 traps per tree, i.e., standard height and canopy summarized); statistically significant differences are indicated by * ($p < 0.05$)

Statistical analysis

A linear mixed effect model was fitted (Pinheiro and Bates 2000) to assess the effect of trap height, forest site, and sample date on abundance of female SWD. Using trap ID nested within tree ID as a random factor, this model accounts for non-independent errors that may occur due to repeated sampling of trees. Data were log transformed to achieve normality of residuals. Generalized linear models (GLMs) were used to analyze the effect of treatment and replicate and their interaction on egg number and hatch rate of adults. The GLM was performed using quasi-Poisson family for count data due to the observed overdispersion. Significance of terms was tested using F-test and the function drop1. Both models were simplified by removing nonsignificant interactions ($p > 0.05$) and nonsignificant factors. Factors that figured in significant interactions were kept in the model (Crawley 2002). Post hoc comparisons between treatments and dates were obtained from least-square means and confidence intervals from statistical models using the function lsmeans. *P* values were adjusted using the method of Hochberg (1988). A Mann–Whitney *U* test was performed for the comparison of relative volatile amounts released by unwounded and wounded berries. Significance level was set at $p < 0.05$. All analyses were performed using R (R Development Core Team 2015) with packages lsmeans (Lenth 2015) and nlme (Pinheiro et al. 2015).

Results

Monitoring SWD adults

At the location Dossenheim (Do), no females were caught in February and from mid-May to end of June, while at Dossenwald (Ma) on trees parasitized by *V. album* subsp. *laxum* the first females were trapped in February and occurred continuously throughout the season. Numbers of trapped individuals were significantly greater ($p < 0.05$) at Ma than at Do at most occasions between February and the end of July. Only on August 4th and September 1st numbers were significantly higher ($p < 0.05$) in traps located at Do (Fig. 2).

On several dates from March to May, the abundance of female SWD was higher in traps at both heights in trees with *V. laxum* at Ma when compared with Do where no mistletoe was growing in the canopy, with significant differences observed in April (Fig. 3a, b; $p < 0.05$). More individuals were caught in the canopy than at the standard height at both forest sites, with significant differences on April 28th (Fig. 3c, d; $p < 0.05$).

Ovigeny development

All female SWD captured in Ma and Do from March 18th to April 28th were dissected ($n = 522$). Numbers of females caught in this period were about 10-fold higher in Ma ($n = 480$) than in Do ($n = 42$). Individuals captured before April 1st did not have maturing or mature eggs. Females with mature eggs (9.6 %) were found for the first time in canopy traps from Ma covering the collection period from April 1st to April 16th (Table 1). In the following period, from April 16th to April 28th 71.7 % of the females from the canopy in Ma carried mature eggs, compared with only 26.1 % from traps at the standard height. In Do, 75 % of the captured females in the canopy carried mature eggs. First "old eggs" appeared in females on the April 28th collection date. During the observation period, more individuals with mature eggs were captured in the canopy of Ma ($n = 120$) compared with standard height ($n = 35$) and Do ($n = 18$) (Table 1).

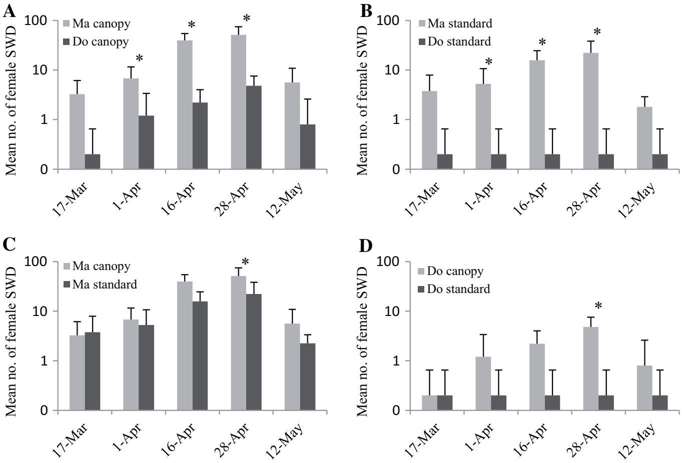

Fig. 3 Female SWD captured at Dossenwald (Ma) and Dossenheim (Do) comparing the sites and the heights (mean of five traps at each height, respectively; log scale, with standard deviation (SD). Statistical differences are indicated by *($p < 0.05$)

Table 1 Status of ovarioles and eggs in females caught in spring 2015 in Dossenwald (Ma) and Dossenheim (Do) in the canopy (C) of pine trees and at standard height (S)

Date	Site	*N*	Indiscernible ovarioles (%)	Unripe ovarioles (%)	Maturing eggs (%)	Mature eggs (%)	Old eggs (%)
01. Apr	Ma-C	17	41.2	58.8	–	–	–
	Ma-S	21	4.8	95.2	–	–	–
	Do-C	6	50	50	–	–	–
	Do-S	–	–	–	–	–	–
16. Apr	Ma-C	115	3.5	61.7	25.2	9.6	–
	Ma-S	41	–	90.2	9.8	–	–
	Do-C	11	–	92.3	7.7	–	–
	Do-S	0	–	–	–	–	–
28. Apr	Ma-C	152	–	12.5	15.1	71.7	0.7
	Ma-S	134	0.7	27.6	45.5	26.1	–
	Do-C	24	8.3	12.5	4.2	75	–
	Do-S	1	–	–	100	–	–

The numbers of dissected females and the percentage per category are given

Performance and survival

In no-choice assays, a higher number of eggs were laid in wounded (17.2 ± 10.7 SD) than in unwounded (6.8 ± 9.9 SD) berries. The model stresses the influence of wounding on egg laying success (Fig. 4a; df = 1; *F* value = 22; $p < 0,05$; $r^2 = 47.4$ %). The number of emerging adults (9–15 days after oviposition) was higher in wounded (8.6 ± 4.4 SD) than in unwounded (2.0 ± 2.7 SD) berries. The model stresses the influence of wounding on successful development into adults (Fig. 4b; df = 1; *F* value = 72; $p < 0.05$, $r^2 = 65.6$ %). Model shows that egg numbers and adult emergence vary among sampling dates (eggs: df = 2, *F* value = 14; $p < 0.05$; hatch: df = 2; $F = 17$; $p < 0.05$). The pH of the berries was 6.35 and degree brix was 20.3°. No female died during the 48-h exposure period. In cages without berries first individuals died at 48 h. All individuals were dead by the fifth day. In cages equipped with unwounded or wounded berries, all individuals survived for 8 days. Flies were

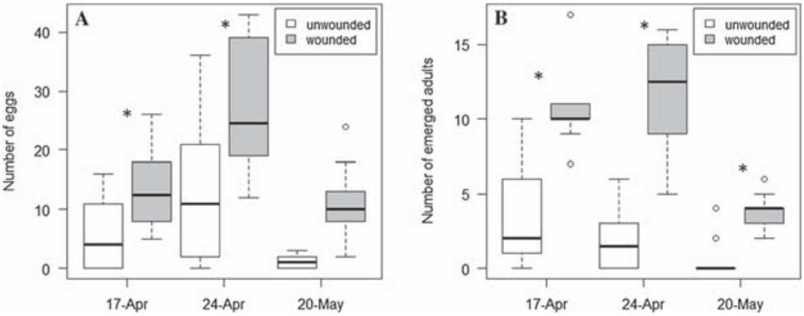

Fig. 4 Boxplots of eggs laid (**a**) and emerged adults (**b**) in no-choice assays. The *bottom* and *top* of the *boxes* represent the lower and upper quartiles, respectively, and the *line* divides the *box* into two parts the median. The ends of the whiskers represent the standard deviation (SD); the dots above the boxplot represent the outliers. In each replicate ($n = 10$), 10 berries of *V. album* subsp. *laxum* were exposed to 10 gravid females for 48 h. Statistical differences are indicated by *($p < 0.05$)

observed feeding on the surface of unwounded and wounded berries.

Field infestation of mistletoes

After four weeks of incubation, no adult SWD emerged out of 100 *V. album* subsp. *laxum* berries collected from Ma (sampling date: April 14th). However, 8 adults (7 ♂, 1 ♀) emerged from the field-collected *V. album* subsp. *album* berries from Heddesbach on May 19th. The monitoring traps in Heddesbach caught 3 male and 2 female SWD in the specified four weeks of trapping.

VOCs emitted by mistletoe berries

In the gas chromatographic analysis of the headspace of both unwounded and wounded berries, 32 peaks of VOCs were detected in the chromatograms (Fig. 5). Twenty-four peaks were selected for a statistical comparison between VOCs emitted by unwounded and wounded berries (Table 2). Of those 19 were identified. While we could not detect any qualitative differences, 11 peaks showed significant differences ($p < 0.05$) in proportional amounts of each compound between unwounded and wounded berries. Out of those benzaldehyde, octanal, methyl salicylate, and farnesene (the isomer could not be precisely determined) could be identified. Seven of the statistically different volatile compounds detected were found in higher amounts in wounded berries (e.g., benzaldehyde and octanal), whereas four volatile compounds were present in higher amounts in unwounded berries (methyl salicylate and farnesene) (Table 2).

Discussion

This study identified mistletoe (*V. album* subsp. a*lbum* and *V. album* subsp. *laxum*) as a new host for SWD in early spring in Central European temperate forests. Adult SWD were successfully reared from *V. album* berries collected from apple trees on a meadow orchard near Heddesbach. Laboratory assays demonstrated that SWD can successfully complete its life cycle in mistletoe berries. SWD laid more eggs in artificially wounded berries compared with undamaged berries. This effect is also known from other fruit crops, e.g., cranberries and grapes (Steffan et al. 2013; Ioriatti et al. 2015). This was more likely due to easier access to the fruit pulp in wounded fruits than to the emitted volatiles, because we could not detect qualitative differences between intact and damaged fruits. The quantitative differences in volatiles measured in our experiments should not have played a role due to the no-choice experimental design. In contrast to cranberries, where SWD can only develop within wounded decaying fruit (Steffan et al. 2013), this study showed for the first time that SWD can develop from egg to adult stage in both unwounded and wounded mistletoe berries.

Furthermore, we showed that adult female SWD were able to subsist on unwounded and wounded berries of mistletoes for at least eight days. As no female died during exposure to berries during the assay, we assume that they could survive even for a longer time on mistletoe as only food source. The flies obviously fed on the surface of berries as they were observed grazing. The microflora on the berry surface seems to offer nutritional components (e.g., microorganisms like yeast, fungi, and bacteria). After

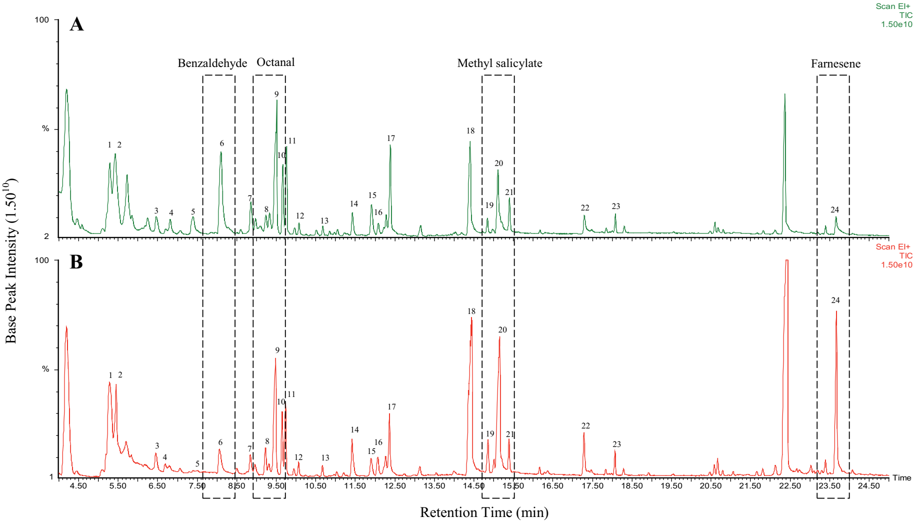

Fig. 5 Total ion current chromatograms obtained from GC–MS analysis of odor spectra of wounded (=A) and unwounded (=B) *V. album* subsp. *laxum* berries. Peaks selected for statistical analysis are indicated by numbers. *Dotted boxes* indicate identified compounds with significant differences ($p < 0.05$)

oviposition, fully intact berries are incised and females can feed on sap or fruit flesh (Walsh et al. 2011; Poyet et al. 2014). Besides, there might be microcracks that can be used for oviposition and feeding as well. A temporal extension of the feeding assay was not possible due to deterioration of the berries and growth of different microorganisms which reduced the differences between wounded and unwounded berries. Further studies could focus on these microorganisms to determine if they have any special attraction to SWD.

SWD has been observed to feed on leftover fruits like overripe and damaged persimmon, figs, and fallen rotting apples during winter (Lee et al. 2015). Additionally, Ioriatti et al. (2015) expected that SWD can utilize grapes wounded by cracking, disease, and/or bird damage in autumn as a nutrient resource, which may result in increased longevity, fecundity, and reproduction. Shearer (pers. communication) reported that winter morphs of SWD were able to survive at 1 °C for several months. We showed that SWD winter survivors can use mistletoe in spring as nutrient and reproduction host. These circumstances support SWD winter and early spring survival success and subsequently its population growth in spring.

The monitoring traps captured greater numbers of SWD in *P. sylvestris* canopies when associated with ripe *V. album* subsp. *laxum* berries. Additionally, traps captured more SWD in the upper canopy of a forest in the presence of mistletoe when compared with traps at standard height. The parasitic mistletoe is always located in the canopy and never at lower heights. Moreover, abundance of SWD captured in traps revealed greater numbers of SWD when *V. album* subsp. *laxum* berries were ripe. These significant differences in the distribution of SWD in forest habitats measured by monitoring traps may be due to a higher attraction of SWD to volatiles produced by mistletoe berries.

Sampling the headspace of mistletoe berries revealed that some volatiles ((E)-2-hexenal and (Z)-3-hexen-1-ol, benzaldehyde, octanal, methyl salicylate, farnesene) were emitted in high amounts. Thus, these chemicals might help SWD moving through the forest canopy to locate potential host fruits. The detected compounds include volatiles typical for ripening fruits (Abraham et al. 2015; Revadi et al. 2015), e.g., benzaldehyde and octanal, and others considered common green leaf volatiles. The volatiles may lure the flies to pine trees parasitized by mistletoe. It was recently shown that SWD is not only more responsive to fruit volatiles than the closely related *D. melanogaster*, but also attracted to green leaf odors (Keesey et al. 2015).

Hexyl acetate and octanal are known from the headspace of ripe strawberries (Keesey et al. 2015). Benzaldehyde is a compound regularly emitted by several berry fruits

Table 2 Volatile organic compounds selected from unwounded and wounded berries of mistletoes

#	RT	Compounds
1	5.28	(E)-2-Hexenal
2	5.45	(Z)-3-Hexen-1-ol
3	6.45	Not ident*
4	6.80	Not ident*
5	7.49	Not ident*
6	8.06	Benzaldehyde*
7	8.85	Not ident*
8	9.31	Octanal*
9	9.48	Cis-3-Hexenyl acetate
10	9.65	Hexyl acetate
11	9.74	Trans-2-Hexenyl acetate
12	10.06	Limonene
13	10.67	Ocimene
14	11.42	Not ident*
15	11.90	Not ident*
16	12.07	Not ident*
17	12.36	Pelargonaldehyde
18	14.44	Ethyl benzoate
19	14.86	Cis-3-Hexenyl butyrate
20	15.15	Methyl salicylate*
21	15.40	Decanal
22	17.29	Ethyl salicylate
23	18.07	Tridecane
24	23.63	Farnesene*

Peak number

Statistical differences are indicated by * based on Mann–Whitney *U* tests at $p < 0.05$

RT Retention time (Rxi-5 ms capillary)

(Abraham et al. 2015), and a common constituent of leaf odors of many green plants (Steck et al. 2012). (E, E)-α-farnesene is the main constituent from flower volatiles of several subspecies of *V. album* (Bungert et al. 2002). Interestingly, another isomer β-farnesene has only been reported from *V. album* a parasite exclusive to *P. sylvestris* (Bungert et al. 2002). With our analytic equipment, it was not possible to distinguish between the different isomers of farnesene. Thus, further analysis is necessary to identify the isomer of farnesene produced by mistletoe berries. Methyl salicylate was emitted in significantly lower amounts by artificially wounded berries than by intact fruits. This organic ester is derived from the shikimic acid pathway (Dicke et al. 2009) and increases in the headspace of plants after attack by herbivores with chewing feeding behavior (Heil 2007; Van Poecke et al. 2001), and in plants infested by pathogens (Mann et al. 2012; Rid et al. 2016). However, methyl salicylate is naturally produced by many species of plants and has been reported from numerous floral scents (Knudsen et al. 1993). It can also be found in the headspace of fruits from several *Rubus* sp. and cherries (Keesey et al. 2015). It was recently shown that methyl salicylate can be detected by olfactory sensory neurons of SWD (Keesey et al. 2015).

Brix (20.6°) and pH (6.35) levels of berries used in our study are comparable to other susceptible hosts, e.g., grapes (Ioriatti et al. 2015). It was shown that higher pH and Brix levels increased the number of eggs laid from which a higher percentage of adults emerged (Lee et al. 2011). In contrast, Lee et al. (2015) could not identify any trend in wild and non-crop ornamental fruits in relations of pH and Brix suggesting that other fruit characteristics are affecting susceptibility of SWD, e.g., fruit firmness and fruit skin characteristics. Additionally, we assume that wounded berries (e.g., by birds or severe storms) increased the susceptibility of mistletoe to SWD.

No infestation by SWD of field-collected berries at Ma from April 14th was observed. Dissections of females captured at this time revealed few or no mature eggs. Unfortunately, it was not possible to sample further mistletoe berries at Ma. For this reason, we collected further berries (*Viscum album* subs. *album*) in May at Heddesbach from scattered apple trees. After four weeks, seven males and one female emerged out of several hundred berries. SWD was the only species that emerged from these fruits. Thus, we showed for the first time that SWD is able to lay eggs and complete its development entirely within mistletoe berries at sites where berries and gravid females occur at the same time.

The newly identified wild host, *V. album,* may increase reproductive success when ripening and ripe berries coincide with gravid females bearing mature eggs. We observed such a scenario during April 2015. The dissection of field-captured SWD females showed that the majority of the females had ripe eggs in their ovaries in the second half of April. At that time the mistletoe berries were fully ripe. No-choice assays of unwounded and wounded berries showed that both are accepted by SWD for oviposition. However, more eggs were laid in wounded berries yielding a higher percentage of emerged adults. Thus, susceptibility of *V. album* to attack by SWD may increase due to severe storms, feeding birds, and rain cracking of berries. This study indicates that SWD can feed, oviposit, and fully develop to adults on mistletoe berries in early spring. Further studies on this newly reported host should provide more information on initial infestation at different sites and different years. Besides fruit availability, other aspects like microclimate or light conditions may explain why SWD is more abundant in the canopy of *P. sylvestris* when compared with SWD abundance at the standard height. One next step is to investigate the abundance of SWD in other tree species compared. More knowledge about wild hosts is needed in order to better understand the population dynamics of SWD, especially with regard to the starting point for seasonal built-up of populations. Such information can be used to develop forecasting models to estimate the potential threat to fruit production.

Author contribution

FB, JG, and HV conceived and designed the study relating to monitoring, survival, and reproduction; FB and JG conducted headspace sampling and GC–MS analyses of mistletoe volatiles. FB and AE conducted the oviposition experiments. FB, JG, and HV analyzed the data. HV supervised the study. FB wrote the first draft of the manuscript, which was supplemented by all authors. All authors approved the final version of the manuscript.

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2.3 Response of *Drosophila suzukii* (Diptera: Drosophilidae) to extreme heat

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Response of *Drosophila suzukii* (Diptera: Drosophilidae) to extreme heat and dryness

Astrid Eben[*], Maria Reifenrath[†], Felix Briem[*], Sebastian Pink[‡] and Heidrun Vogt[*]

[*]*Julius Kühn – Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Strasse 101, 69221 Dossenheim, Germany,* [†]*Faculty of Biosciences, Heidelberg University, Im Neuenheimer Feld 234, 69120 Heidelberg, Germany and* [‡]*Centre for European Social Research, Mannheim University A5, 6 68131 Mannheim, Germany*

Abstract

1 *Drosophila suzukii* Matsumura (Diptera) is a polyphagous herbivore native to East Asia that develops in cultivated and wild fruits. In 2011, it appeared in Germany. In 2012, economic damage was recorded and, in 2014, the harvest of stone and soft fruits was lost in some regions. By contrast, during 2015, populations remained lower. Record temperatures and dryness might have impeded population growth during that year.

2 To test this hypothesis, flies were exposed to a 4-day simulation. We evaluated the effect of fluctuating temperature and humidity on mortality and reproduction of *D. suzukii* from three age classes (average age: class 1: 3 days; class 2: 11 days; class 3: 20 days). Maximum temperatures were 27, 33 and 39 °C. Relative humidity oscillated between 18% and 85%.

3 Fly mortality through heat stress ranged from 50% to 80%. Higher rates died of oldest flies and females. Offspring per female did not differ between heat stressed and control groups.

4 Flies of both sexes were not sterilized through heat and dryness. Prior acclimation reduced any negative effects.

5 We concluded that heat waves and extreme dryness during 2015 were one cause of the low population densities of *D. suzukii* observed under field conditions.

Keywords Age classes, fluctuating temperatures, heat record, mortality, reproduction, spotted wing *Drosophila*.

Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae) or spotted wing drosophila is native to East Asia and was first described in Japan in 1931 by Matsumura (Kanzawa, 1939). In 2008, it invaded the continental U.S.A. (Hauser, 2011) and southern Europe, where it was found simultaneously in Spain and Italy (Calabria *et al.*, 2012; Cini *et al.*, 2012, 2014). In subsequent years, it invaded further European countries and was detected for the first time in Germany in 2011 (Heuck, 2012; Vogt *et al.*, 2012). To date, it is widely distributed in Europe (Asplen *et al.*, 2015). It is highly polyphagous and damages a broad spectrum of cultivated host plants (Beers *et al.*, 2011; Goodhue *et al.*, 2011; Lee *et al.*, 2011; Walsh *et al.*, 2011; De Ros *et al.*, 2015), as well as many wild fruits from over 20 plant families (Mitsui *et al.*, 2010; Poyet *et al.*, 2015; Briem *et al.*, 2016; Kenis *et al.*, 2016). Females use their species-specific, serrated ovipositor to cut the skin of undamaged, ripe fruits and deposit their eggs in the fruit pulp underneath. Infested fruits quickly collapse and become unmarketable. The development time from egg to adult depends mainly on temperature and ranges from 9 to 79 days (Kanzawa, 1939; Lee *et al.*, 2011; Tochen *et al.*, 2014; Ryan *et al.*, 2016). The highest number of generations per year was described for Japan, with up to 13 being reported (Kanzawa, 1939). Based on laboratory experiments and field observations, temperature-dependent survival and reproduction are the main factors influencing these population dynamics (Wiman *et al.*, 2014, 2016). *Drosophila suzukii* is active at temperatures between 10 and 30 °C (Kanzawa, 1939; Hamby *et al.*, 2013) and the most favourable temperature range for development is 20–25 °C. It has a lower thermal threshold of 5 °C and its reproduction is assumed to cease at 31 °C (Kinjo *et al.*, 2014; Asplen *et al.*, 2015). Under optimal weather conditions, its short

Correspondence: Astrid Eben. Tel.: +49 6221 86 805 28; fax: +49 6221 86 805 15; e-mail: astrid.eben@julius-kuehn.de

generation time combined with a high reproductive potential can lead to rapid population growth.

Within less than 5 years after its introduction, *D. suzukii* became a serious threat for cultivated fruits, with high financial losses in many of the newly invaded areas (Asplen *et al.*, 2015; De Ros *et al.*, 2015). As a result of the broad host range of this species, host fruits are colonized in a temporal sequence of ripening fruits in orchards and natural habitats. Nevertheless, numbers of flies caught in field traps show large local and seasonal variations (Harris *et al.*, 2014; Briem *et al.*, 2015; Mazzetto *et al.*, 2015).

After its first report in Germany, the numbers of *D. suzukii* have increased steadily. In September 2014, hundreds and occasionally more than thousand of flies were trapped per week and site (Vogt & Köppler, 2014). During 2015, fly abundance remained low in most fruit growing regions of Germany. Moreover, monitoring data revealed that the seasonal peak in fly abundance was reached later in 2015 compared with the previous year (Wissensportal JKI, 2016; http://drosophila.julius-kuehn.de). It can be assumed that the 2-week period in February 2015 with continuous temperatures below 5 °C, followed by a prolonged cold spell in spring, and later by a very hot and dry summer, may have caused the slow population increase. Total precipitation recorded for July and August was only 58 mm in 2015, whereas it was 154 mm in 2014 (Deutscher Wetterdienst, 2015). In these particular months, there were some days with extremely high temperatures. During the first heat wave in July 2015, the heat record for Germany was broken, with a temperature of 40.3 °C (Kitzingen, Bavaria, Germany) (Deutscher Wetterdienst, 2015). Both summer months are particularly important for fruit cultivation because ripening and harvest of most commercial sweet and sour cherry varieties, as well as berry fruits, takes place during this time of the year. The present study aimed to evaluate the potential impact of such short periods of extreme heat on age-dependent mortality and reproduction of *D. suzukii*.

Materials and methods

Insects

Adult *D. suzukii* were obtained from a laboratory colony. Flies were reared under an LD 16 : 8 h photocycle at 23 °C and 60% relative humidity (RH) in a climate chamber at the Julius Kühn – Institut (JKI), Dossenheim (Heraeus Vötsch Industrietechnik GmbH, Germany). A mix of dried brewer's yeast (Diana, Germany) with sucrose 1 : 1 and a 5% sucrose solution served as a food supply for the adult flies. As substrate for oviposition, the JKI standard diet [30 g of sugar, 142 g of cornmeal, 20 g of soy flour (Reformhaus, Germany), 34 g of brewer's yeast, 11.2 g of agar, 5 g of vitamin mixture (Vanderzant; MP Biomedicals, Santa Ana, California), 9.4 mL of propionic acid, 1748 mL of water, based on a recipe from Fondazione Edmund Mach, San Michele, Italy] was offered in 50-mL cups (Huhtamaki, Finland). For standard rearing, this substrate is replaced every 48 h and kept in the rearing chamber until adult eclosure.

Three age classes were evaluated in each experiment. All ages given in days reflect the age of the insects at the onset of each experimental replication. After a pre-oviposition period of <4 days, the oviposition rate of newly eclosed *D. suzukii* increases until it reaches a peak at the age of 20 days (Asplen *et al.*, 2015). Based on this pattern, we defined the age classes: age class 1 included males and females with an age (mean ± SD) of 2.7 ± 1.1 days, age class 2 comprised flies aged 10.8 ± 0.9 days and age class 3 comprised flies aged 20 ± 1 days.

For the experiments, the same cages as for standard fly rearing were used (BugDorm-insect rearing cage, 30 × 30 × 30 cm; MegaView, Taiwan). Each cage contained two plastic cups (7 × 4 cm; neoLab, Germany) with a lid modified to hold a sponge and filled with 80 mL of a 5% sucrose solution. Insect diet medium was offered as food source and oviposition substrate in accordance with the standard rearing procedure.

Heat wave simulation

Temperature and humidity values are continuously recorded at the meteorological station of the JKI in Dossenheim (AME 1650; Hoffmann, Germany). Based on these data, a 4-day program for the heat simulation experiment was developed. For the program, actual temperatures of July and August 2015 were used to create fluctuating profiles for the present study. These profiles were programmed and recorded using SIMPATI, version 4.0 (Heraeus Vötsch Industrietechnik GmbH) and the simulation was performed in a climate chamber (Heraeus Vötsch Industrietechnik). In the chamber, RH could not be regulated. Therefore, a defined volume of fresh air (10 – 15 m^3/h) was constantly introduced with a ventilator. This ensured that RH inside the chamber fluctuated during the simulation experiment in correspondence with temperature (Fig. 1). The values reached for RH showed a variation of less than 10% among the nine replications at any given time point. During the entire experiment, temperature and RH were recorded automatically every 10 min using SIMPATI, version 4.0 and, additionally, with a datalogger (Testo 175H2; Testo AG, Germany) placed inside the experimental climate chamber.

We did not expose the same individual flies to subsequent simulations because, in summer 2015, a period of more than 4 weeks occurred between both heat waves and probably at least one additional generation of *D. suzukii* had emerged in that time. Since the main goal of the present study was the examination of a potential effect of the extreme temperatures recorded in Germany on 5 July 2015 (Deutscher Wetterdienst, 2015), the experimental conditions for humidity and temperature for the third experimental day resembled that day. To permit acclimation of the flies to high temperatures as it is possible for them under natural conditions, the first experimental day simulated an average summer day with a peak temperature of 27 °C (Table 1). The 4-day program was run in a 3-day-rotation because the first and fourth day had the same temperature profile (Fig. 1 and Table 1). This program was repeated nine times to run nine replicates for each treatment and age class.

Impact of heat stress on adult survival and reproduction: 4-day simulation

At the beginning of the first experimental day, 50 individuals of either sex were released into each test cage in the simulation

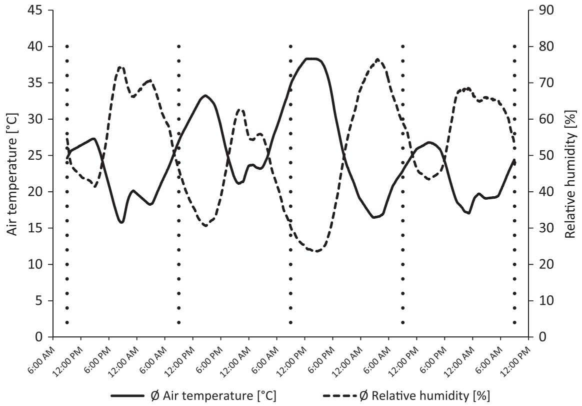

Figure 1 Mean temperature and relative humidity (RH) profiles from nine replications of the 4-day program run in the climate chamber. Vertical lines indicate the 4-day program defined as lasting 24 h (from 09.00 to 9.00 h).

Table 1 Daily minimum and maximum value for temperature and relative humidity (RH) and daily duration (h) of heat levels that are particularly important for *Drosophila suzukii* biology during the simulation experiments

Parameters	Day 1	Day 2	Day 3	Day 4
Minimum temperature (°C)	15.4	18	18.1	15.2
Maximum temperature (°C)	27.4	33.5	38.9	27.4
h > 25 °C	6	12	16	6
h > 30 °C	—	7	12	—
h > 33 °C	—	3	9	—
h > 35 °C	—	—	7	—
Minimum RH%	46.5	29.8	18.1	26.2
Maximum RH%	85.2	72.6	76.9	66.9

chamber and likewise into control cages kept in the rearing chamber ($n = 100$ flies per cage). To prevent additional stress for the test insects and to avoid a potential influence on mortality rate, all experiments were started at 09.00 h (MEZ) when the conditions in both the rearing chamber and the simulation-chamber were the same (LD 16 : 8 h, 23 °C, 60% RH, 10 000 lux). In the rearing chamber, environmental conditions were maintained at a constant throughout the experiments. A separate cage in each chamber was set up for each age class ($n = 600$ flies per replication). Dead males and females were counted and removed from the cages every 24 h. Oviposition substrates in each cage were exposed to *D. suzukii* for 24 h and replaced the next day at 09.00 h. Removed Petri dishes were covered with a perforated lid and kept in the rearing chamber. To allow undisturbed larval development and pupation, pupae were counted after 8–10 days (M3Z; Wild Heerbrugg, Switzerland). Adults hatched after a pupal period of 16–18 days and were transferred into 70% ethanol after anaesthesia with CO_2. These numbers of male and female offspring were recorded for each age class, treatment and replication.

At the end of the 4-day simulation experiments, surviving males and females were counted and cages were transferred to the rearing chamber. Those flies were then kept for another 7 days under optimal conditions to assess survival. Moreover, for the evaluation, if the surviving adults remained fertile after the heat stress treatments, they were also offered oviposition media. These media were monitored until the eclosure of live adults.

Effect of extreme temperature without prior acclimation: 1-day simulation

The effect of extreme temperatures on *D. suzukii* without prior acclimation was examined in a 1-day temperature stress test. The experimental set-up was the same as in the 4-day experiment, although the test insects of each of the three age classes were exposed only to the 24-h program of the extreme temperature day (day 3). The flies spent the extreme heat day with a peak of 39 °C in the simulation chamber and were returned to the rearing chamber afterwards. Mortality was assessed and survivors were kept for another 7 days under optimal conditions. The number of dead flies was recorded daily.

To determine whether egg maturation took place during the temperature simulations, dead females from the heat stress experiments with and without prior acclimation were dissected. The presence of ripe eggs in the ovaries was recorded. Ten females were used from each age group and treatment.

Effect of extreme temperatures on male fertility: 6-day mating experiment

For the evaluation of a potential effect of temperatures above 30 °C on male fertility, males from age class 2 were subjected to high temperature treatments. Fifty males and 50 females were released into each of two cages set up in the simulation chamber ($n = 100$ flies per cage). Water and oviposition media were offered as food source. One cage was removed after day 2 with a maximum temperature of 33 °C, whereas the second cage was exposed to both hot days: to a temperature of 33 °C (day 2) and to the temperature maximum of 39 °C (day 3). Twenty surviving males from the respective cages were individually transferred to test containers. Cups (1000 mL) containing two dental wicks soaked with 8 mL of sucrose solution (5%) and 20 mL of oviposition medium in a 50-mL cup were used in this assay. All other surviving males and all live females were frozen. One recently eclosed, unmated female fly from the laboratory colony was added to each of the test containers containing a male. All females used in the tests were transferred to these test containers within a maximum of 3 h after eclosure to assure no prior mating. The control consisted of 20 males kept in the rearing chamber under optimal conditions prior to the addition of unmated females. Furthermore, 20 newly emerged females were maintained individually without males to control for oviposition of unfertilized eggs.

Test containers containing a fly couple were kept in the rearing chamber. All flies were removed from the containers after 6 days. Presence of eggs or larvae was verified for each oviposition cup with the aid of a stereomicroscope. Larvae were allowed to pupate, and number and sex of the offspring of each couple was recorded after 14 days.

Statistical analysis

The experimental set-up followed the tested fly groups over a course of 4 days measuring surviving proportions of the same cohorts after each day. Mortality as a result of fluctuating temperature and humidity during each day of the 4-day simulation experiments was evaluated by means of survival analysis (Kalbfleisch & Prentice, 2002; Ma & Bechinski, 2008; Scherber *et al.*, 2013). We estimated different exponentially distributed hazard rates (i.e. four time dummies) for different groups of flies (i.e. sex, age) and tested them daywise for statistical differences. The model treated age and sex as fixed effects. The interpretation is such that the higher the hazard rate, the higher the mortality observed after each treatment day. The dataset for survivorship contained 19 102 data points for a total of 5400 flies. Survivorship status and day were used to calculate the hazard rate of mortality using the streg function of STATA, version 14 (Stata Corp., College Station, Texas).

The effect of the 4-day simulation treatment on offspring number, the impact of previous acclimation on mortality and fecundity, and the effect of heat stress on male fertility were analyzed by analysis of variance, and means were separated by Tukey's honestly significant difference after a significant F-test at $P < 0.05$ (JMP, version 12; SAS Institute Inc., Cary, North Carolina).

Results

Temperature and RH profile of the heat wave simulation

The program run in the climate chamber for the 4-day temperature simulation experiments was automatically controlled to confirm that the desired temperature and humidity profiles were maintained for all nine replications. Humidity during the 4-day experiments fluctuated in accordance with the daily increase and decrease of temperature (Fig. 1 and Table 1).

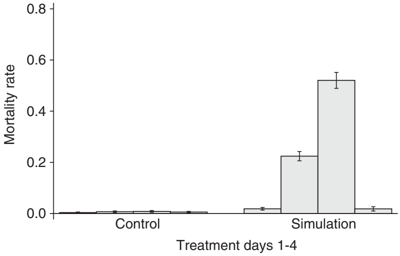

Figure 2 Mortality rates of the tested flies for each day of the 4-day temperature simulation in the climate chamber. Bars represent values of the mean ± SE. Each experiment started with 50 males and 50 females ($n = 100$ per cage) from each age class ($n = 900$ for nine replications) in the simulation treatment and control ($n = 5400$ flies). Heat stressed flies in the simulation experiment died in significantly higher numbers than control flies on all 4 days.

Impact of heat stress on adult survival: 4-day simulation

An increased daily temperature led to a higher mortality for insects from all age classes compared with the control groups. In the control groups under optimal conditions, no significant difference in mortality was detected between age classes and among consecutive days. Overall mortality was low and ranged between 0.1% (age classes 1 and 2) and 2.4% (age class 3).

Varying percentages of *D. suzukii* died on each day of the four climate simulation days. We compared the magnitude of the estimated hazard rates for statistical differences based on the linear combinations of effects from which these hazard rates were calculated. Mortality was significantly higher in heat stressed flies than in control flies after all four experimental days ($P < 0.001$). Insect mortality differed significantly ($P < 0.05$) when comparing consecutive days of the simulation experiment (Fig. 2).

Within all age classes, we observed a significant difference ($P < 0.05$) in the mortality of the test flies among consecutive days of the 4-day simulation. The pattern was similar for all age classes ($n = 900$ per age class) (Fig. 3), although the total mortality after the 4-day simulation differed significantly between age classes (number of dead flies per age class: class 1 = 52.4 ± 7.4 flies; class 2 = 60.9 ± 8.9 flies; class 3 = 80 ± 8.6 flies). Older flies in age class 3 died in significantly higher numbers than younger flies in age class 1 and 2 during all 4 days of the experiment ($P < 0.05$) (Fig. 3). Youngest flies in age class 1 differed in their susceptibility to fluctuating temperatures only on day 2 compared with medium-aged flies in age class 2 ($P < 0.05$) (Fig. 3). On day 2 with a maximum temperature of 33 °C, more than twice the number of flies died in age class 3 (40.7 ± 8.6%) compared with age class 2 (17.9 ± 3.9%) and four times more flies died in age class 3 compared with age class 1 (9.1 ± 4.1%). During day 3 with a maximum temperature of 39 °C, mortality rate was highest with 46.8 ± 6.1%, 51.5 ± 10.8% and 61.6 ± 13.7% dead *D. suzukii*, respectively, for the three age classes. Low numbers of flies in all age classes died during day 1 (1–3%) and day 4 (0.9–6.3%) with a maximum temperature of 27 °C.

Female flies were more susceptible than male flies and died in significantly higher numbers on day 2 ($P < 0.0001$), day 3

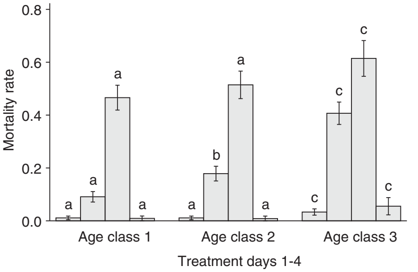

Figure 3 Differences in mortality rates are depicted for flies from different age classes that experienced the 4-day simulation experiments. Age classes were defined as age class 1 = 2.7 ± 1.1 days, age class 2 = 10.8 ± 0.9 days and age class 3 = 20 ± 1 days**.** Bars represent the mean ± SE of nine replications ($n = 900$ flies). Different letters indicate significant differences in mortality rates between age classes when comparing the same experimental day ($P < 0.05$). The age-dependent mortality pattern was similar for male and female flies; thus, sexes were combined for analysis.

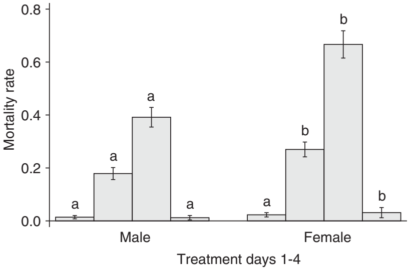

Figure 4 Mortality rates for female and male flies during each day of the 4-day temperature simulation experiments. Bars represent the mean ± SE of nine replications ($n = 900$ flies). Different letters indicate significant differences in mortality rates between male and female insects when comparing the same experimental day ($P < 0.05$). The pattern was the same for all three age classes; therefore, age classes were combined in this analysis.

($P < 0.0001$) and day 4 ($P = 0.045$) of the simulation experiments (Fig. 4). The difference between female and male mortality was significant within all age classes ($P < 0.05$). During the 4-day simulation, fly mortality differed significantly between consecutive days ($P < 0.05$) in males and females alike.

Impact of heat stress on adult reproduction: 4-day simulation

Egg counts under a dissection microscope revealed no reliable relationship between visually detectable eggs in the oviposition media and the number of pupae found later on, whereas the number of pupae counted and the number of emerged adult flies was the same. Therefore, statistical analyses compared pupal numbers between treatments. During our simulation experiments in the climate chamber, the temperature of the oviposition media reached a maximum of 34 °C during the extreme heat days. Temperature was measured on the surface and <0.5 cm below the surface of the media with a digital thermometer (Testo 108; Testo AG). High temperatures did not lead to sterilization of male or female flies. Total number of pupae from each female was not significantly different after days 1, 2 and 4 between heat stressed females and control groups for all age classes ($F = 2.7203$, d.f. = 5, $P = 0.2873$). Offspring number was significantly reduced during day 3 for all three age classes ($P < 0.05$). Within both, the heat stressed and the control insects the number of pupae per female was significantly lower in age class 1 compared with age class 2 and 3 ($P = 0.0018$).

For heat stressed females of age class 1, the highest fertility was reached on day 2 (number of pupae per female: 0.4 ± 0.6) and the lowest on day 3 (number of pupae: 0.04 ± 0.06). The fertility of females of age class 2 was highest on day 2 (0.9 ± 0.5 pupae per female) and lowest on day 3 (0.2 ± 0.1 pupae per female) of the four temperature simulation days. The number of pupae per female of age class 3 was reduced by seven-fold on day 3 (0.1 ± 0.2) compared with day 2 (0.7 ± 1.0). On day 4, the highest number of pupae per female (2.9 ± 5.8) was obtained. In control females, the number of pupae per female and day increased during the course of the experiment (Fig. 5).

Effect of extreme temperature without prior acclimation: 1-day simulation

Extreme heat and low humidity of experimental day 3 caused a significantly higher mortality of not acclimated flies in all age classes compared with acclimated groups ($F = 195.59$, d.f. = 1, $P < 0.0001$) (Fig. 6). The interaction of age by treatment ($F = 3.97$, d.f. = 2, $P = 0.022$) was significant. More not acclimated females than males died in all age classes ($F = 74.89$, d.f. = 1, $P < 0.0001$). Age classes within treatments did not differ in their mortality rates.

No significant difference in offspring number between acclimated and not acclimated flies was found after experiencing the day with maximum temperature of 39 °C ($F = 4.22$, d.f. = 5, $P = 0.83$). Reproduction of not acclimated *D. suzukii* females of age class 1 was significantly lower compared with not acclimated flies of age classes 2 and 3 ($F = 6.05$, d.f. = 2, $P = 0.005$) (Fig. 7). No difference was found in number of offspring between the age classes in acclimated flies.

The evaluation of ovarian development in females that experienced extreme conditions showed that, independently of their age, all female *D. suzukii* had either immature eggs in her ovaries or underdeveloped ovaries. By contrast, eight out of 10 unstressed control females had mature eggs present in their ovaries.

Effect of extreme temperatures on male fertility: 6-day mating experiment

All male flies that survived the conditions of the 4-day simulation remained alive during the course of the 6-day mating experiment.

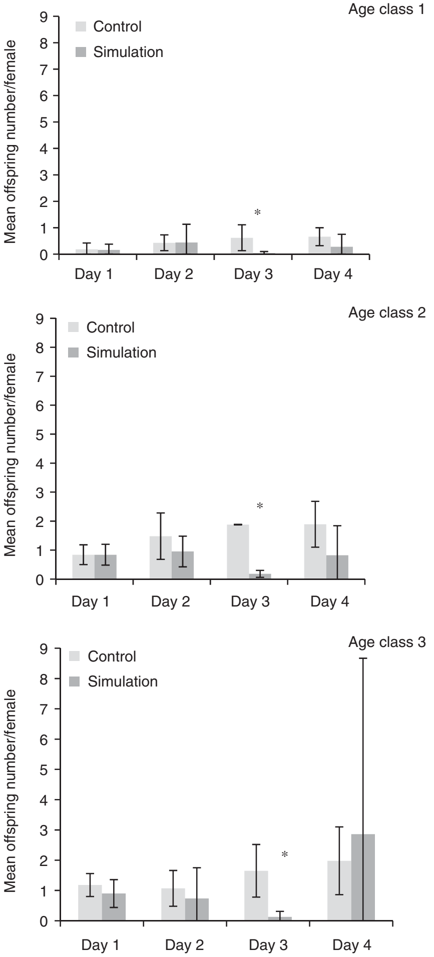

Figure 5 Mean number of adult offspring produced by each female of the P-generation ($n = 50$ male and 50 female flies per replication) in each age class over the 4-day simulation experiments. Bars represent the mean ± SD of seven replications. The control shows the mean number of offspring produced by unstressed females of the same age classes. Significant differences in offspring numbers are indicated by an asterisk (${}^*P < 0.05$) (F-test, Tukey's honestly significant difference).

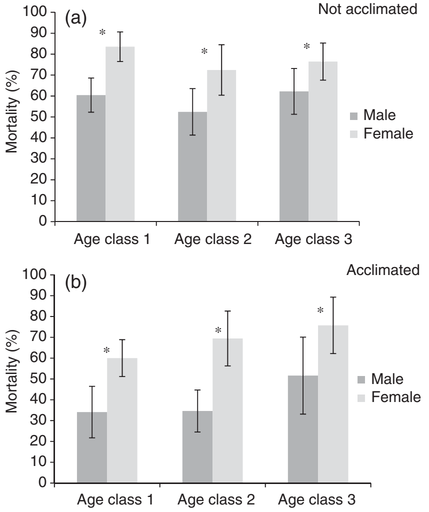

Figure 6 Bars represent the mean ± SD of dead *Drosphila suzukii* after 24 h of extreme heat stress (peak temperature 39 °C for 3 h) ($n = 50$ males and 50 females per replication, $n = 900$). (a, b) Not acclimated flies of all age classes died in higher proportions than acclimatized flies ($P < 0.05$). Statistically significant differences between sexes are indicated by an asterisk (${}^*P < 0.05$) (F-test, Tukey's honestly significant difference).

Offspring number was not different between males that survived heat stress at 33 or 39 °C and males from control groups ($F = 0.31$, d.f. $= 2$, $P = 0.74$). There was no interaction between sex of offspring and treatment ($F = 0.08$, d.f. $= 2$, $P = 0.09$), although the overall number of female offspring was greater than the number of male offspring ($F = 6.71$, d.f. $= 1$, $P = 0.01$). Females kept without mating partner did not lay eggs.

Discussion

To our knowledge, this is the first experimental test of the effect of temperature and humidity on *D. suzukii* that evaluated naturally fluctuating profiles. This is also the first report of an age-related difference in heat susceptibility of adult flies. Similar studies did either not control for age or tested *Drosophila* spp. from only one age class (Bubliy *et al.*, 2013; Kinjo *et al.*, 2014; Tochen *et al.*, 2014, 2015; Ryan *et al.*, 2016; Enriquez & Colinet, 2017).

Under controlled conditions in a climate chamber, fluctuating high temperatures and corresponding low humidity significantly reduced adult survival by 52–80%, depending on age class. Consequently, this resulted in a lower number of total offspring. Regardless of age, females were more susceptible than males to extreme heat and low humidity. The number of viable offspring produced per female during the day with extreme heat and low humidity was significantly lower in heat stressed than in unstressed control groups. The dissection of tested females revealed that heat stress interrupted egg production. Nevertheless, this was reversible because survivors continued to lay fertile eggs. The age-related curve for the oviposition rate

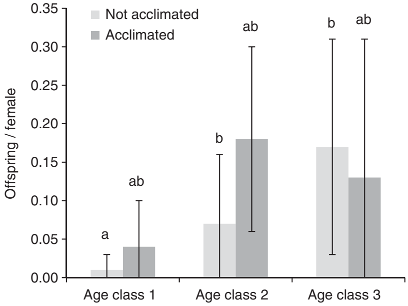

Figure 7 Comparison of offspring number per female that survived day 3 with a maximum temperature of 39 °C. Bars represent the mean ± SD of seven replications. Results are shown for females of each age class with (acclimated) and without (not acclimated) prior acclimation to the day with 39 °C temperature maximum (day 3). Different lowercase letters indicate significant differences ($P < 0.05$) (*F*-test, Tukey's honestly significant difference).

of *D. suzukii* described in previous studies (Kinjo *et al.*, 2014; Asplen *et al.*, 2015) could clearly be observed in our experiments for both the control and the treatment groups.

In our simulations, flies were exposed to temperatures above their optimum of 25 °C (Kinjo *et al.*, 2014; Tochen *et al.*, 2014) and the corresponding low RH for a period of 6–16 h according to each particular day of the 4-day experiments. Extreme temperatures above 33 °C were held for 3 h on day 2 and for 9 h on day 3, similar to data recorded under natural conditions. Temperature and humidity fluctuated broadly similar to natural conditions in our 4-day simulations. Therefore, the number of eggs laid during 24 h, pupation rate and adult mortality rate per experimental day were not correlated with a particular temperature. We observed that *D. suzukii* continued to lay viable eggs even during the day with maximum temperatures of 39 °C and low humidity. By contrast, under conditions of constant, low humidity (20–33%), female flies survived for only 3 days on average (Tochen *et al.*, 2015). Likewise, under 60% RH and a constant temperature of 31 °C for a period of 4 days during mating and oviposition, no eggs hatched and, under a constant temperature of 33 °C for 24 h, all larvae died prior to pupation. Four days of exposure of adults to 25 and 31 °C (LD 12 : 12 h) or to 25 and 33 °C (LD 16 : 8 h) resulted in reduced egg laying and egg hatch for the latter combination (Kinjo *et al.*, 2014).

Because *D. suzukii* has an activity peak for mating in the morning and a peak for oviposition in the late evening (Lin *et al.*, 2014; Hamby *et al.*, 2016; Evans *et al.*, 2017), our experiments ran during the entire 24 h of each experimental day. Therefore, peak activity periods of the flies were included during times with a much lower than maximum temperature and higher humidity levels.

Low humidity in combination with heat was reported as a limiting factor for the reproduction (Calabria *et al.*, 2012; Tochen *et al.*, 2015) and survival (Enriquez & Colinet, 2017) of *D. suzukii*. Under field conditions, Mitsui *et al.* (2010) observed little reproduction of *D. suzukii* during midsummer at low altitudes in central Japan and Kinjo *et al.* (2014) noted that *D. suzukii* were not found during August 2010 in the field when temperatures reached a maximum of 33 °C. That temperature, however, was considerably lower than the German heat record for 2015.

The results of our 4-day simulation experiments support the hypothesis that the comparatively slow population build-up of *D. suzukii* observed during the 2015 season was caused by record heat waves and low humidity. The negative effects might vary locally because adult flies can move to a shadier and moister habitat and thus escape extreme radiation and dryness during the hottest time of the day. On the other hand, eggs or larvae inside fruits may suffer from high temperatures through sun exposure.

Heat stress experiments without prior acclimation showed that up to 40% of the tested adult *D. suzukii* were able to withstand adverse climatic conditions even without any previous adaptation period. The surviving individuals were fully capable of producing viable offspring. During the course of a field season, natural populations might be repeatedly subjected to selection for heat resistance through such short heat waves. A phenotypic plasticity to withstand adverse conditions is often found in successful invasive species (Chown *et al.*, 2007; Davidson *et al.*, 2011). Prior acclimation reduced the mortality of adult *D. suzukii* by 15–30% compared with not-acclimated flies. Similar effects were reported for *D. melanogaster* (Krebs & Loeschcke, 1994). In our heat wave simulations, female *D. suzukii* suffered more from extreme heat and desiccation than males. Similarly, Tochen *et al.* (2014) reported that more males developed into adulthood at 26–28 °C compared with higher numbers of females completing development at lower temperatures from 18 to 22 °C.

The number of eggs deposited under heat stress (i.e pupae obtained) per surviving female during each experimental day was not significantly lower in heat stressed flies compared with control groups. Oviposition cups were transferred after each experimental day to the rearing chamber and kept there under constant optimal conditions. This means that, in contrast to the thresholds described previously (Tochen *et al.*, 2014; Kinjo *et al.*, 2014), it can be assumed that, under field conditions with fluctuating heat and humidity, the negative impact of adverse climatic conditions on the local population dynamic of *D. suzukii* is difficult to predict. Interestingly, studies on the impact of heat stress on pest insects are scarce. Published research testing the response of insects to extreme heat kept the experimental temperature or humidity constant with a variable duration or repeated the identical peak pattern several times (Kimura, 2004; Davis *et al.*, 2006; Piyaphongkul *et al.*, 2012; Chen *et al.*, 2013; Sentis *et al.*, 2013; Chiu *et al.*, 2015).

The negative effects of heat and dryness on fly survival observed in the climate chamber were more pronounced for older flies. This was similar to findings of a study by Stratman and Markow (1998) who reported less heat tolerance for older individuals of several desert-inhabiting *Drosophila* spp. They assumed this was a result of senescence because young flies might still carry a certain concentration of heat shock proteins that can help to withstand extreme heat. In *D. buzzatii*, eggs and young larvae were found to be less susceptible to heat than older larvae and adult flies, and pupae were the least heat susceptible life stage (Krebs & Loeschcke, 1995). These observations are of crucial importance as a result of the overlap of all developmental stages and several generations of *D. suzukii* throughout the growing season under natural conditions.

The physiological difference of resistance to high temperatures versus resistance to desiccation in *Drosophila* spp. during temperature stress experiments was discussed by Stratman and Markow (1998). To partially avoid desiccation, we provided adult *D. suzukii* with a water source and observed a large proportion of flies gathering on the water filled sponges in each cage during the hottest and driest time of all experimental days. To date, the mechanism of thermal tolerance in *D. suzukii* is not yet well understood. Further research is necessary, especially on the susceptibility of egg and larval stages of *D. suzukii* to extreme temperatures, to evaluate its potential for physiological and behavioural adaptations (Kinjo *et al.*, 2014; Wiman *et al.*, 2014).

Kellermann *et al.* (2012) reconstructed the phylogenetic relationships of *Drosophila* species and compared their climate-related distribution pattern. The phylogeny obtained showed that the evolution of desiccation resistance differs between the two subgenera *Drosophila* and *Sophophora*, with *Sophophora* being less tolerant to dryness. The invasive species *D. suzukii* was recovered within this latter subgenus. The pattern so far observed for the invasion of new habitats by *D. suzukii* over three different continents (Europe, North and South America) is in agreement with this finding (Asplen *et al.*, 2015).

In conclusion, *D. suzukii* is a very recent invasive pest species in Germany and detailed knowledge on its reproduction, development, migratory behaviour, host plants and physiology is still lacking. Collection of additional field and laboratory data is needed to understand and predict its locally variable population dynamic, as well as the subsequent infestation threat to cultivated fruits.

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2.4 Identifying plant DNA in the sponging-feeding insect pest *Drosophila suzukii*

Felix Briem,Christiane Zeisler, Yasemin Guenay, Karin Staudacher, Heidrun Vogt, Michael Traugott

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ORIGINAL PAPER

Identifying plant DNA in the sponging–feeding insect pest *Drosophila suzukii*

Felix Briem[1] · Christiane Zeisler[2] · Yasemin Guenay[2,3] · Karin Staudacher[2] · Heidrun Vogt[1] · Michael Traugott[2]

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Abstract

Drosophila suzukii (Diptera: Drosophilidae) is a highly polyphagous invasive pest threatening fruit production in the Americas and Europe. The current knowledge of its host plants is mainly based on oviposition and larval development in fruits, while little is known on the diet of the adult flies. This information is important for developing effective control strategies. Here, we examine DNA-based techniques to determine food plants of *D. suzukii*. Adult flies were fed with raspberries (*Rubus idaeus*) and allowed to digest up to 72 h after feeding. Raspberry DNA was detected by diagnostic PCR for up to 48 h post-feeding with a significant negative effect of time on DNA detection success but no significant differences between male and female flies in detection probabilities. As *D. suzukii* walks on plants, its body surface can get contaminated with DNA. With a bleaching experiment, we succeeded to remove contaminating external plant DNA, while the DNA in the gut content stayed unaffected. Finally, field-collected flies were subjected to a next-generation sequencing approach, demonstrating that plant DNA from different host plants can be efficiently detected in both bleached and non-bleached specimens. In order to safeguard against erroneous host plant detections, we recommend bleaching flies before they are subjected to DNA extraction. The current findings encourage the use of DNA-based gut content analysis in *D. suzukii* to obtain a better understanding of its feeding ecology which is a prerequisite for developing successful control strategies.

Keywords Chloroplast DNA · Diet metabarcoding · Feeding experiment · Molecular gut content analysis · Spotted Wing Drosophila · High-throughput sequencing · NGS

Key message

- We developed and evaluated PCR-based methods to detect plant DNA ingested by *D. suzukii*.
- DNA of ingested plants could be identified for up to 48 h post-feeding with no significant differences between males and females.
- Flies were successfully decontaminated from external eDNA by a newly adapted bleaching protocol.
- Host plant DNA could be identified within field-collected specimens using a next-generation sequencing approach.
- DNA-based gut content analysis provides a promising approach to unravel *D. suzukii* feeding ecology.

Introduction

The invasive and highly polyphagous frugivore pest species *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) is threatening soft and thin-skinned fruit crops in the Americas, Europe and its native Sino-Japanese realm, causing severe economic impacts (Asplen et al. 2015; Farnsworth et al. 2017; Mazzi et al. 2017). Unlike native Drosophilidae that infest rotten fruits, female *D. suzukii* own a serrated

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✉ Felix Briem
Felix.Briem@julius-kuehn.de

[1] Laboratory of Entomology, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Julius Kühn-Institut (JKI), Schwabenheimer Straße 101, 69221 Dossenheim, Germany

[2] Mountain Agriculture Research Unit, Institute of Ecology, University of Innsbruck, Technikerstraße 25, 6020 Innsbruck, Austria

[3] Institute of Interdisciplinary Mountain Research, IGF, Austrian Academy of Sciences, Technikerstraße 21a, 6020 Innsbruck, Austria

ovipositor enabling them to oviposit directly into ripening and ripe fruits (Atallah et al. 2014). Infested fruits quickly collapse and become unmarketable.

Since its first observation in Southern Europe (Italy and Spain) and North America (California) in 2008 (Calabria et al. 2012; Cini et al. 2012; Hauser 2011), several studies focused on the host plants of this pest (Kenis et al. 2016; Poyet et al. 2015). Accordingly, a wide range of cultivated, ornamental and wild fruits has been examined and more than 80 host plants are described for Europe (Briem et al. 2016; Kenis et al. 2016; Poyet et al. 2015). This knowledge is mainly based on studies dealing with field-collected fruit samples, laboratory feeding and oviposition trials, development of larvae and the emergence of adult flies (Hamby et al. 2016; Mazzetto et al. 2015; Poyet et al. 2014; Tochen et al. 2016). However, studies on infield food choices are rare. This is because identifying food plants is usually difficult: direct observations of feeding activity in the field are problematic in (1) habitats with limited access (e.g. forest canopy) and (2) due to correct identification of small insects such as *D. suzukii*. However, during laboratory feeding trials insects often feed on and/or oviposit into host plants which are not typically used infield (Garcia-Robledo et al. 2013). As a consequence, the diet range may be overestimated by the above-mentioned approaches and we lack information about the plants utilized as food source by *D. suzukii* under natural conditions. This knowledge, however, is crucial for developing more effective biotechnological control strategies such as “attract & kill” or “push & pull” (Alnajjar et al. 2017; Renkema et al. 2016). For these strategies, either the food plants itself or the volatile organic compounds emitted by such plants could be used. In the latter, further research is needed to identify such compounds.

Sponging–feeding insects such as *D. suzukii* are well adapted to feed on fruit or tree sap, extrafloral and floral nectar using a haustellum (Kanzawa 1939; Tochen et al. 2016). Therefore, studying its diet by dissecting the flies is impossible as no microscopically identifiable food remains can be found within the gut content. Several studies showed that DNA-based techniques provide a tool to obtain detailed information on the gut content in such situations, including the detection of DNA from ingested plants (Avanesyan and Culley 2015; De la Cadena et al. 2017; Staudacher et al. 2013; Wallinger et al. 2015). Junnila et al. (2011) and Lima et al. (2016) already showed that plant DNA is identifiable in the guts of blood feeding sand flies (Diptera: Nematocera) which have sucking mouthparts. In Drosophilidae (Diptera: Brachycera) which are sponging feeders that soak up small amounts of food in a liquid form, this has not yet been shown. Here, we test how well the molecular approach is suited to identify plant DNA ingested by *D. suzukii* using a series of laboratory experiments employing diagnostic PCR and next-generation sequencing (NGS). Besides assessing post-feeding food DNA detection intervals for *D. suzukii*, we also considered sex-specific effects on plant food detection probabilities in our experiments.

Field-collected insects can be externally contaminated with DNA (so-called environmental DNA: eDNA) which stems from the environment they live in (Greenstone et al. 2012). This might be especially relevant for species such as *D. suzukii* which walk on plants and fruits they feed on with their typical foraging behavior (Kanzawa 1939; Poyet et al. 2015). Extracting the DNA from whole bodies of those individuals includes the risk of also amplifying eDNA, leading to false-positive assignments of food sources (Greenstone et al. 2012; Wallinger et al. 2013). To check whether such eDNA is a potential source of error and if so, how it can be removed from the flies before DNA extraction, the bleaching method (Greenstone et al. 2012; Wallinger et al. 2013) was adapted and modified for *D. suzukii*. Experiments were conducted where fed flies were externally contaminated with plant DNA and subjected to a bleach cleaning treatment.

Finally, as *D. suzukii* is highly polyphagous an NGS approach was applied to a set of field-collected adult flies to check how well this technique is suited to identify DNA of consumed plants. The NGS approach includes general plant primers which amplify DNA from virtually all plant species which might be utilized by *D. suzukii*, while a diagnostic approach employing species- or genus-specific primers would only allow assessing the consumption of these particular plant taxa. For example, the NGS approach should be especially useful to unravel the food resources utilized by this pest in winter and spring, which is a bottleneck period for the species. Furthermore, we examined with these field-collected specimens how important eDNA might be as a source of contamination when using NGS to identify food plants under natural conditions.

According to the knowledge gaps identified above, our study had three aims: (1) to develop new protocols to identify plant DNA ingested by *D. suzukii* via diagnostic PCR to determine for how long post-feeding plant DNA can be detected in its gut content and whether post-feeding detection intervals differ between males and females, (2) to develop a bleaching protocol to decontaminate *D. suzukii*’s outer body surface from eDNA without affecting ingested plant DNA and (3) to test how well plant DNA can be identified in field-caught individuals using a NGS-based approach of diet analysis.

Materials and methods

Colony maintenance

Individuals of *D. suzukii* used in this study were obtained from the laboratory colony maintained at the Julius

Kühn-Institut (JKI) in Dossenheim (Germany) since October 2013. The culture has been started with adults that emerged from different fruits (i.e. blackberry, cherry and raspberry) sampled at the experimental fields of the JKI Dossenheim and is yearly refreshed. Flies were kept in an environmental chamber (Vötsch Industrietechnik GmbH, Germany) at constant conditions with 23 °C, 60% RH and a photoperiod of 16L:8D. As nutrition, a mixture of dried brewer's yeast (Diana, Germany) with sucrose (1:1) and a 5% sucrose–water solution was offered. For oviposition, plastic cups (125 ml, Huhtamaki, Finland) filled with 75 ml JKI standard diet (Briem et al. 2016) were placed in the cages for 2–3 days. These oviposition substrates were stored under the same conditions as the colony in a second environmental chamber until adult emergence. All individuals emerged within 8–10 days before the experiments started.

Feeding experiment

Adult *D. suzukii* were starved for at least 24 h to adjust them to the same starving level. Then, mashed defrosted raspberries (*Rubus idaeus*, Luxfrost Sárl, Luxemburg) were offered as experimental food to the flies. After 1 h, the food was removed and individuals were checked for feeding activity, indicated by a red abdomen (Fig. 1). Feeding active individuals ($n = 150$ males and $n = 150$ females) were separated by sex and transferred in batches of 10 individuals per sex to starving containers (2 L, Rotilabo®, Carl Roth + Co. KG, Germany) for each post-feeding time point (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 32, 48 and 72 h). Those containers were prepared with gauze (mesh size 500 µm) for ventilation, and a small petri dish (∅ 35 mm, Greiner Bio-One International GmbH, Austria) at the bottom was filled with a moistened cotton pad. To avoid stress due to starvation every 12 h post-feeding, the JKI standard diet was offered for 1 h. Nevertheless, only 3 males survived for 72 h. After reaching the desired post-feeding time point, individuals were separately stored in 2-ml safe seal reaction tubes (Sarstedt AG & Co., Germany) at − 28 °C until DNA extraction.

Fig. 1 Female *D. suzukii* fed with raspberry for 1 h indicated by the red abdomen (left) and a starved female with a clear abdomen (right); photographs: A. Frank, F. Briem, J. Just; JKI Dossenheim. (Color figure online)

eDNA decontamination of the flies' body surface

A new protocol to remove eDNA contamination sticking on the fly's outer body surface based on previously published studies using similar methods was developed (Greenstone et al. 2012; Remen et al. 2010; Wallinger et al. 2013). To establish this method, 20 *R. idaeus*-fed females were artificially contaminated with mistletoe (*Viscum album*), sampled at the Dossenwald near Mannheim, Germany (Briem et al. 2016). Females were dipped softly with a tibia or wingtip into the mashed *V. album* berries to simulate an "authentic" scenario. A "worst-case" scenario was simulated by dipping another 10 females with a tibia or wingtip AND their abdomen into mashed *V. album* berries. Ten females from the "authentic" scenario served as control ("no-bleach"). For easier handling and to avoid unpredictable fly movement, the females of both scenarios were CO_2 anesthetized before dipping them into the mashed mistletoe berries. In both scenarios, we used sodium hypochlorite ("bleach," Sigma-Aldrich, St. Louis, Missouri, USA) with a concentration of 1–1.5% (incl. 0.02% Tween® 20, Sigma-Aldrich) as bleaching agent for 30 s based on the outcomes of testing varying concentrations of sodium hypochlorite and incubation durations. After bleaching, all individuals were washed with molecular grade water.

Primer design and screening for plant DNA

For primer design, several *trn*L sequences of *R. idaeus* and *V. album* which included the binding site for the universal primer *c* (5′-CGAAATCGGTAGACGCTACG-3′) (Taberlet et al. 1991) were downloaded from Genbank. Sequences were assembled in BioEdit Sequence Alignment Editor v7.1.9 (Hall 1999), and the alignment was extended with further plant sequences such as maize and soy which are components as corn and soy flour of the JKI standard diet for *D. suzukii*. Primer Premier 5 (PREMIER Biosoft International, Palo Alto, USA) was used to design specific reverse primers targeting *R. idaeus* and *V. album*, respectively. We chose primers that can be combined with the universal forward primer *c* to amplify a 245-bp fragment for *R idaeus* (Rub-ida-A575; 5′-GGAAGGATTCCTTTACGAACAC-3′) and a 170-bp fragment for *V. album* (Vis-alb-A576; 5′-TATTGTTGTTTGCTTGGATAAGCT-3′). The primers' specificity and sensitivity were evaluated in vitro and a final singleplex PCR protocol established: 15 µl PCRs contained 4 µl DNA extract, 7.5 µl 2× Type-it Mutation Detect PCR Kit (Qiagen, Hilden, Germany), each primer at 0.5 µM, 0.5 µg BSA (Sigma-Aldrich) and PCR grade water to adjust the volume. Amplifications were carried out under the following thermocycling conditions: 5 min at 95 °C, 35 cycles of 20 s at 92 °C, 90 s at 60 °C and 30 s at 70 °C and finally 5 min at 70 °C.

DNA was extracted from whole *D. suzukii* specimens. Each individual was submerged in 200 µl TES buffer (0.1 M TRIS, 10 mM EDTA, 2% SDS, pH 8) and 5 µl proteinase K (20 mg/ml). Then, they were homogenized with three to four 3-mm glass beads in a Precellys® 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) for 120 s at 5000 rpm. The samples were incubated overnight at 56 °C. DNA was extracted with the Biosprint 96 DNA blood kit on a Biosprint 96 extraction robotic platform (both: Qiagen) following the manufacturer's instructions except for the modification of the lysis described above and elution in 200 µl 1× TE buffer. Each 96-well plate contained four extraction negative controls. All extractions were done in a separate pre-PCR laboratory using an UVC-equipped laminar flow hood.

PCRs were run with two positive (raspberry and mistletoe DNA, respectively) and two negative (molecular grade water) controls per 96-well. No cross-sample contamination was detected by testing the extraction and the PCR negative controls using the diagnostic assays described above.

All PCR products were visualized using the automated capillary electrophoresis system QIAxcel (Qiagen), and the results were scored with BioCalculator "Fast Analysis software version 3.0" (Qiagen). Samples showing the expected fragment length with a signal above 0.1 relative fluorescent units (RFUs) were assigned as positive. It is important to note that the diagnostic assays developed here were not tested for their specificity beyond the species involved in this feeding experiment, also including ingredients of the JKI standard diet and that further specificity testing would be required if these assays are to be applied in systems which contain other (plant) species.

Field sampling and sample preparation for NGS

For NGS analysis, 40 (20 male and 20 female) *D. suzukii* were collected from the raspberry plantation (variety: Himbotop) of the JKI Dossenheim and checked for feeding activity (Fig. 1). Half of the individuals were washed with bleach as described above (eDNA decontamination) prior to DNA extraction. The remaining 20 flies were processed without this cleaning step, and all 40 flies were DNA-extracted as described above. Additionally to these field-collected flies, those 5 samples of the eDNA decontamination experiment ("worst-case" contamination scenario) that were still positive for DNA of *V. album* were also processed for NGS to test whether sequences of the contaminating *V. album* DNA can be obtained.

To analyze a broad spectrum of ingested plant species from the whole-body extracts of *D. suzukii* without a priori decision on focal groups, we used the universal primers B49317 and B49466 (Taberlet et al. 1991, 2007) that amplify a fragment of the *trn*L intron and modified them in terms of adding the binding region for the Illumina Nextera adaptors. The PCR was performed with the Type-it Mutation Detect PCR Kit (Qiagen), using additional BSA (Sigma-Aldrich) under the following PCR conditions: 5 min at 95 °C followed by 40 cycles of 20 s at 92 °C, 90 s at 55 °C, 60 s at 70 °C and 5 min at 70 °C once. Positive and negative controls (as described above) were included in these PCRs as well. The resulting PCR products were checked with a QIAxcel as described above for appropriate size and purified using the magnetic bead capture kit SPRIselect (left side selection) (Beckman Coulter, Inc., Brea, USA) as recommended by the manufacturer.

NGS of field-collected individuals

The NGS was conducted at the Department of Genomic and Applied Microbiology (University of Göttingen, Germany). Purified PCR products were used to attach indices and Illumina sequencing adapters using the Nextera XT Index kit (Illumina, San Diego, USA). Index PCR was performed using 5 µl of template PCR product, 2.5 µl of each index primer, 12.5 µl of 2× KAPA HiFi HotStart ReadyMix and 2.5 µl of molecular grade water. Thermal cycling scheme was as follows: 95 °C for 3 min, 8 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C and a final extension at 72 °C for 5 min. Quantification of the products was performed using the Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Invitrogen GmbH, Karlsruhe, Germany) following the manufacturer's instructions. MagSi-NGSPREP Plus Magnetic beads (Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany) were used for purification of the indexed products as recommended by the manufacturer, and normalization was performed using the Janus Automated Workstation (Perkin-Elmer, Waltham Massachusetts, USA). Sequencing was conducted at an Illumina MiSeq platform using dual indexing and MiSeq reagent kit v3 (600 cycles) according to the manufacturer's protocol.

NGS data processing

Quality control checks of the paired-end sequencing raw data files were run with the software FastQC-0.11.4 (Andrews 2010). Due to detected Nextera transposase sequences in each R1 (1.7–3.4%) and R2 file (11.5–20. 9%) of the 45 samples, reads were 3′ end trimmed using cutadapt-1.14 (Martin 2011) and afterward merged with PEAR-0.9.10 (Zhang et al. 2014). As universal chloroplast *trn*L primers for targeted sequencing were utilized, these primer sequences were removed with cutadapt before doing local BLAST search. Therefore, all BLAST+ (Camacho et al. 2009) searches on the downloaded nt database (ftp://ncbi.nlm.nih.gov/blast/db/, status: 25/7/2017) were performed on the high-performance compute cluster LEO3e of the University of Innsbruck. Each sample file was split into file pieces containing 4000 or 8000 sequences using Linux split command and then executed separately, and for evaluation their output files joined together per sample with the Linux cat command.

Statistical analysis

Generalized linear models (GLMs) were used to analyze the influence of ingested *R. idaeus* DNA detection success over time using the package "MASS" (Venables and Ripley 2002), "VGAM" (Yee 2010) and "car" (Fox and Weisberg 2011). The GLM was performed using binomial family due to the binary type of the data (detection/no detection). Significance of terms was tested using Chi-square test and the function drop1. Models were simplified by removing nonsignificant interactions ($P > 0.05$) and nonsignificant factors. Factors that figured in significant interactions were kept in the model (Crawley 2002). The time point for food DNA detection probability of 25, 50 and 75% and their 95% confidence intervals were calculated using the package "drc" (Ritz et al. 2015). *T* tests were performed to test for significant differences in RFUs measured. Significance level was set at $P < 0.05$. All analyses were performed using R (R Development Core Team 2016).

For the analysis of the NGS sequence data, *t* tests for independent samples were performed using SPSS 24.0 to test for significant differences in the mean number of generated sequences per sample between bleached and non-bleached individuals. This comparison was conducted because the bleach treatment might negatively affect the DNA quality and/or the subsequent molecular downstream analysis.

Results

Detection of ingested plant DNA by diagnostic PCR

DNA of ingested *R. idaeus* was detected by diagnostic PCR in the guts of males and females for up to 24 and 48 h, respectively (Fig. 2). Over all time points tested in our experiment, *R. idaeus* DNA was detected in 38.2% of all analyzed specimens (males 39.9%; females 36.7%). The detection success of plant DNA was negatively related to digestion time (pseudo-$R^2 = 43.184$, $SE = 0.143$, $P < 0.005$) (Fig. 2). No significant differences were present in *R. idaeus* detection rates between males and females. Directly after the feeding period, the RFUs measured for ingested plant DNA, providing a proxy for the food plant DNA concentrations in the samples, showed no significant differences between males (3.3 ± 0.85 SD) and females (3.9 ± 1.11 SD) ($t = 1.4658$, $df = 16.835$, $P = 0.1611$).

Removal of eDNA via bleaching

All individuals were decontaminated successfully of eDNA (*V. album*) within the "authentic" scenario, whereas in 25% of females (5 out of 20 samples) tested in the "worst-case"

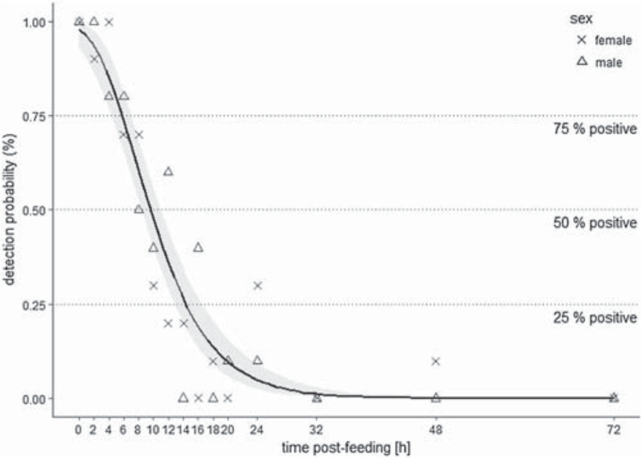

Fig. 2 Plant DNA detection success in whole-body extracts of female and male *D. suzukii* fed with raspberry (*R. idaeus*) at different time points ranging from 0 to 72 h post-feeding. Detection rates measured are provided as Δ = males and *x* = females and a fitted CLOGLOG model including the lower and upper 95% confidence intervals. Time points where the detection probability equals 25, 50 and 75% are given as dotted lines. Ten individuals were tested at each time point for each sex, except for 72 h (only 3 males survived)

scenario eDNA was still amplified. In the control groups (“no-bleach”), 79 and 100% of the females were tested positive for eDNA of *V. album* in the “authentic” and the “worst-case” scenario, respectively. Ingested DNA of *R. idaeus* stayed unaffected in both scenarios (Fig. 3). In four out of the five individuals which tested positive for mistletoe DNA, sequences of *V. album* were generated with NGS accounting for 2.3–37.2% of all sequences obtained from these samples.

Detection of ingested plant DNA in field-collected *D. suzukii* using NGS

In total, 6,282,930 sequences for 45 *D. suzukii* samples were generated, with a mean sequence number of 139,620 sequences per sample (range 39,264–396,573). After read trimming and merging, still 99.25% of the sequence data were appropriate for BLAST search.

All sequences with the minimum number of 1% of all sequences found within a specific sample were taken into account. Thus, we identified three plant species in field-collected *D. suzukii*: *R. idaeus*, *Urtica dioica* and *Polygonum humifusum*. Additionally, BLAST hits for *Prunus* spp., *Pinus* spp. or Rosaceae species other than *R. idaeus* were found but could not be identified at species level due to identical sequences for *trn*L amplicons within each genus (26 different *Prunus* and 69 *Pinus* species) or family (*Comarum salesovianum*, 2 *Sibbaldianthe*, 5 *Rosa*, 9 *Potentilla*, 11 *Fragaria* and 33 *Rubus* species with the same sequence).

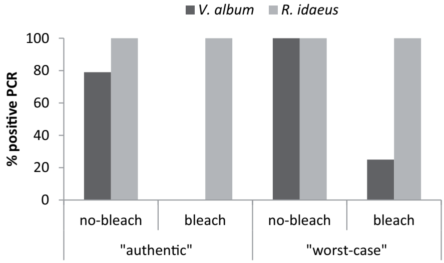

Fig. 3 Plant DNA detection rates (%) of mistletoe (*V. album*), sticking on the outer body surface and raspberry (*R. idaeus)* in the gut content of *D. suzukii* in the “authentic” and the “worst-case” scenario. For each scenario, the percentage of positive non-decontaminated (no-bleach) and decontaminated (bleach) individuals is shown

In all except one of the field-collected individuals, the proportion of *R. idaeus* (or other unidentified Rosaceae) sequences made up for more than 90% of the reads obtained. DNA of *R. idaeus* was the most frequently found type of DNA as in 85% of the field-collected individuals only sequences of this species were identified. While in one sample the unidentified Rosaceae made up > 85% of all sequences, the other two samples where this taxon was detected yielded 7.4 and 1.4% of the total sequences per sample. Sequences from *Pinus* spp. were detected in one sample accounting for 14.2% of the reads, while the single sample detections of *Prunus* spp., *U. dioica* and *P. humifusum* stayed under 2% of the sequences generated (Table 1).

Assuming that the unknown Rosaceae sequences are likely to be *R. idaeus,* only raspberry sequences were found in bleached specimens, while in non-bleached flies three out of 20 flies provided sequences of four additional plant taxa. Also regarding the total number of sequences generated per sample showed no significant difference between bleached (149,348.9 ± 87,680.48 SD) and non-bleached (123,055 ± 80,294.03 SD) individuals ($t = 0.989$, $P = 0.329$).

Discussion

In this study, we developed a PCR-based method to detect plant DNA ingested by *D. suzukii*. The current results demonstrate that ingested plant DNA is amplifiable with both diagnostic PCR and NGS within whole-body extracts of *D. suzukii.* Additionally, eDNA was successfully removed from the flies’ outer body surface using the developed bleaching protocol.

Post-feeding plant DNA detection in *D. suzukii* was possible for a maximum of 48 h post-feeding which is longer than in mirids such as *Apolygus lucorum* (~ 16–20 h) (Wang et al. 2017), but shorter than in caterpillars of *Helicoverpa armigera* or *Tuta absoluta* (50% detection after 24 h) (Pumarino

Table 1 Percentages of total reads per sample from gut content analysis of field-collected individuals of *D. suzukii* that showed more than one plant species in the NGS run. Provided are the sample name (#), the bleach treatment (bleach) followed by the plant taxa identified using BLAST

#	Bleach	*Rubus idaeus*	*Rosaceae*	*Prunus* spp.	*Pinus* spp.	*Urtica dioica*	*Polygonum humifusum*
a14	Yes	14.14	85.12				
a26	Yes	92.40	7.39				
a22	No	98.48	1.40				
a23	No	84.86			14.22		
a39	No	95.71				1.09	1.92
a41	No	96.63		1.29			

et al. 2011) and soil-living *Agriotes* larvae (> 72 h) (Staudacher et al. 2011). *Agriotes* larvae, *T. absoluta* and *H. armigera* are chewing plant tissue, whereas *A. lucorum* and *M. pigmaeus* have sucking mouthparts to take up plant sap, while *D. suzukii* is a sponging–feeding insect ingesting plant fluids. Considering the difference between chewing and liquid feeding insects, plant fluids are probably broken down faster by enzymatic processes than chewed plant tissue (Pumarino et al. 2011; Staudacher et al. 2011), explaining the relatively shorter post-feeding detection intervals found for *D. suzukii*. Besides the feeding mode, the ambient temperature might also affect food DNA detection success: this is because digestion, as any other enzymatic processes, is temperature dependent and the detection interval of food DNA is generally shortened by higher temperatures (von Berg et al. 2008; Hoogendoorn and Heimpel 2001; Hosseini et al. 2008). The extended detection rates post-feeding in *Agriotes* larvae may therefore also be ascribed to a lower ambient temperature of 16 °C during the experiments compared to 25 °C used for *A. lucosum* and *M. pigmaeus* (Pumarino et al. 2011; Wang et al. 2017). The latter are more similar to our study (23 °C) and thus might also contribute to the more similar post-feeding plant DNA detection intervals.

The sex of the flies might have possibly affected food DNA detection periods as, for example, in insects females are often bigger than conspecific males and thus have larger meal sizes (Hosseini et al. 2008). However, Sheppard et al. (2005), Hosseini et al. (2008) and Hoogendoorn and Heimpel (2001) did not find any influence by sex, weight or even the developmental stage on post-feeding food DNA detection periods, corroborating the current findings. Still, in our study, ingested DNA in females could be detected for longer post-feeding periods than in males. Directly after feeding, we found no significantly higher RFUs in females than in males; however, a tendency toward more food intake (higher RFUs) of females was identifiable, perhaps contributing to the longer post-feeding detection intervals in females compared to males.

Comparable to previous studies on herbivorous insects (Jurado-Rivera et al. 2009; Pumarino et al. 2011; Staudacher et al. 2011; Wallinger et al. 2013), we used whole-body DNA extracts to detect ingested plant DNA. To remove unwanted DNA which might be present on the body surface of the flies, we tested several bleach protocols and chose the best one to apply to the flies of our "authentic" and the "worst-case" contamination scenario. None of the individuals of the "authentic" scenario showed any contaminating DNA of *V. album* as indicated by the negative results obtained by diagnostic PCR. Thus, the newly adopted bleaching protocol for *D. suzukii* seems to be feasible for removing potential eDNA contamination without destroying the DNA of the ingested plants. Moreover, we showed that the detection of ingested plant DNA via NGS in whole-body extracts and the reads generated per sample are not negatively affected by the bleach treatment. In 25% of the individuals of the "worst-case" scenario, however, we were still able to amplify DNA of *V. album* after bleaching, indicating that the DNA in heavily contaminated specimens might not always be removed successfully. In all but one of these *V. album*-positive flies eDNA of mistletoe was also obtained by subjecting these specimens to NGS, highlighting the importance of the bleach cleaning step when analyzing food plant DNA via high-throughput sequencing.

Diagnostic PCR employing species-specific primers represents an efficient approach to test for a limited number of specific DNA types such as consumers from feeding experiments to determine post-feeding detection intervals. However, this approach becomes impractical in situations when many different food sources need to be identified. Here, NGS-based techniques provide a much better way to examine the broad spectrum of host plants which might be consumed by herbivores under field conditions (Valentini et al. 2009). In contrast to NGS assays with universal plant primers, a limited range of plant species is selected a priori when species- or genus-specific primers are used (Wallinger et al. 2012). However, this selection implies two disadvantages: (1) it is not adequate to detect unknown food sources and (2) it reflects only a small range of the plants that can be used as food source by polyphagous herbivores. Besides the *trn*L locus, the two other alternatives would be the chloroplast *rbc*L and *mat*K loci (Hollingsworth et al. 2016). We are not aware of a locus which would allow identifying all species from all plant families. Based on the description of the wide range of several plant families by Taberlet et al. (1991, 2007), the universal plant primer with the locus *trn*L was chosen in this study. Further studies have to consider that one particular DNA locus does most likely not enable an unambiguous identification of all plant species. Thus, when the identification on species level is required, especially without a detailed record of the vegetation, several universal plant primers might be employed.

By testing field-collected *D. suzukii* samples with the NGS approach, DNA of *R. idaeus* was detected in all individuals. Further, we identified additional plant species and genera in 6 of the 40 individuals tested. The BLAST hits for the family Rosaceae or *Prunus* spp. resulted in multiple species hits. *Prunus* spp. is known as host/food plant (Kanzawa 1939; Poyet et al. 2014; Uchino 2005), whereas this is not the case for *U. dioica*, *P. humifusum* and *Pinus* species. It is also important to note that the presented BLAST results only illustrate which of the DNA sequences is closest related to one species within the NCBI nt database. *Polygonum humifusum* is an Asian and North American species and to our knowledge not present in Europe. However, a related European species, *P. aviculare,* is very common. Since Tochen et al. (2016) found a higher survivorship of *D. suzukii* provided with flowers, we assume that the collected individuals might have fed on nectar or pollen of flowering *P. aviculare* and *U. dioica* and/or on pollen of these plants which was wind-drifted to the raspberry bushes where the flies were collected from. *Pinus sylvestris* is cultivated at the JKI in Dossenheim and is also growing in the surroundings of the experimental fields. As *P. sylvestris* was not flowering during the sampling period, but made up a big proportion in the sequences of the respective sample, we suggest that this particular individual of *D. suzukii* may have fed on *Pinus* spp. tree sap as described by Kanzawa (1939) for *Quercus* spp. Another possibility explaining these finding might be the consumption of honeydew which has been mentioned as possible food source for *D. suzukii* (Lee et al. 2015; Walsh et al. 2011). As we do not know for field-collected specimens when and how much they fed, we may find host plant DNA in many different digestion stages. To minimize the influence on the variability on plant DNA detection success between individual samples, it is recommended to analyze a large number of samples. Moreover, there are studies which showed species-specific differences in the detection probabilities of plant DNA within the gut content of plant-eating insects (Wallinger et al. 2013), which suggests that such feeding experiments could be employed for important host plants to allow for a better interpretation of the field-derived molecular data.

Nevertheless, the detection of these other plant taxa might not depict feeding but external contamination with eDNA. This highlights the need to apply bleach cleaning procedures before DNA extraction. Moreover, the sampling in this study was conducted only in raspberries, and as the levels of eDNA contamination might vary between locations and habitats, bleaching field-collected specimens is strongly recommended. Furthermore, with increasing sequencing depth, minor amounts of contaminating eDNA might become visible (Oskar Rennstam Rubbmark personal communication).

In conclusion, our study demonstrates that the DNA of ingested plants can be identified from the gut contents of the polyphagous pest *D. suzukii* for several hours after the feeding event, allowing for a meaningful assessment of the diet of this important pest using DNA-based techniques. Moreover, it is recommended to decontaminate flies before they are subjected to DNA extraction to safeguard against erroneous host plant detections due to external eDNA. As such, our findings encourage the use of DNA-based gut content analysis in *D. suzukii* and other sponging–feeding dipterans to obtain a better understanding of their feeding ecology under natural conditions.

Author contribution statement

FB, KS, HV and MT conceived and designed the study. KS designed the primers. KS, CZ and FB established the molecular assays and performed the laboratory work. Flies were starved, fed and field-caught by FB at the JKI in Dossenheim, Germany. YG performed the bioinformatic analyses. CZ, YG and MT interpreted the NGS output and BLAST results. FB analyzed the data and compiled tables and figures. FB wrote the manuscript. CZ, YG, KS, MT and HV revised and improved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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3 GENERAL DISCUSSION

The results of this thesis expand the understanding of *D. suzukii*'s spatio-temporal dispersal. The focus of the present thesis was to acquire a greater understanding of the biology of *D. suzukii* in the Upper Rhine Valley characterized by small-structured landscape use with numerous small orchards, vineyards and the hilly landscape along the mountain chains in the west, the Palatinate forest and the Forest of Odes in the east. The subject was split into gaining knowledge on the spatial-temporal dispersal and the survival capacity under challenging weather conditions. Information on the feeding ecology of *D. suzukii* is lacking, therefore a molecular method to eliminate this knowledge gap was developed.

The population size of *D. suzukii* depends a lot on the combination of weather conditions and the availability of nutritional sources throughout the whole season. The Rhine Valley in Southwest Germany with its temperate oceanic climate (Cfb on the Köppen & Geiger climate classification) is well suited for *D. suzukii*. The small-structured landscape provides a variety of abiotic and biotic conditions and a vast number of cultivated, wild and ornamental fruits, bordering the hilly landscape in the east (e.g. Black Forest, Forest of Odes). A large number of studies already focused on exploring the spatio-temporal dispersal of *D. suzukii* using monitoring traps (e.g. Kremmer et al. 2017; Renkema et al. 2018; Seebacher et al. 2017; Thistlewood et al. 2018). In this thesis, the abundance and dispersal of *D. suzukii* was examined through a seven-year period (chapter 2.1, Briem et al. 2018a). The monitoring data analysed in correlation with weather and landscape indices showed that semi-natural habitats such as orchards or vineyards are poor overwintering habitats for *D. suzukii*. Forested areas and hedges revealed to be more suitable, even in the course of the year. Thus, the landscape composition influences abiotic or biotic conditions and might provide overwintering habitats better suited for *D. suzukii* (Wallingford et al. 2018). Forested areas can mitigate harsh weather conditions, as they are protected from wind and are often milder and moister than semi-natural landscapes (Tougeron et al. 2016). Further, forested areas bordering cultivated areas provide a nearby shelter and numerous potential overwintering food sources (Wallingford et al. 2018). The presence of berries in these areas at various stages of ripeness partly all throughout the year (Briem et al. 2015; Poyet et al. 2014; Poyet et al. 2015) is an important aspect why forested areas and hedges always recorded very high capture rates. *D. suzukii* is known to feed on dropped fruit, tree sap, nectar or left overs (e.g. grapes for ice-wine) to sustain itself energetically during periods when no host fruits are available (Bal et al. 2017; Kimura 2004; Mitsui et al. 2010; Tochen et al. 2016). Beside ripening and ripe cultivated, ornamental or wild fruits, microorganisms growing on the surface of evergreen trees or tree sap provide nutritional sources supporting *D. suzukii*'s survival (Fountain et al. 2018; Kanzawa 1939). Hence, several sources provide nutrition for *D. suzukii* to withstand severe conditions.

Trap captures indicated that *D. suzukii* is active on mild days during winter, probably searching for the above-mentioned nutrition (Wallingford et al. 2018). A wide spectrum of more than 80 host plants both indigenous and exotic is described, solely for Europe (Kenis et al. 2016; Lee et al. 2015b; Poyet et al. 2015). Most of those host plants are fruiting during the warm seasons and are often used as food plants as well. Knowledge on nutritional sources and first reproduction hosts in spring during the severe winter and spring months are still scarce. This thesis showed in chapter 2.2 (Briem et al. 2016) the utilization and importance of *V. album* for *D. suzukii* for early season survival and as first reproduction host with both laboratory-based assays and with monitoring traps in the canopy of *P. sylvestris* parasitized with *V. album*. The dissection of females revealed mature ovaries from mid-April onwards coinciding with ripe berries of *V. album* assuming that those berries might be an adequate protein source. Recently it was also mentioned that mushrooms or bird feces might be an adequate protein source as well (Wallingford et al. 2018), available all year round. Consequently, *D. suzukii* can exploit several sources during the cold season not only for nutritional reasons, but also for first reproduction in the early season in temperate forests.

The above determined flight activity during mild winter conditions was proven by trap captures in chapter 2.1 (Briem et al. 2018a). Frost events can certainly reduce winter survival dramatically. Consequently, the first early season population build-up can be slowed down with the result that infestation in early season cherries or strawberries might be reduced or even prevented. Winter active specimens might further be attracted to the canopies by the green leaf odours of host plants (Keesey et al. 2015) or microorganisms in order to find nutritional sources. Besides the potential availability of nutrition, further aspects, such as a better suited microclimate (Tanabe 2002) and thus shelter, may explain the higher abundance of *D. suzukii* in the canopy of trees (Briem et al. 2015; Tanabe 2002). It was further assumed that *D. suzukii* might move between different habitats and elevations, depending on the season, in search for more suitable conditions (Santoiemma et al. 2019; Santoiemma et al. 2018; Tait et al. 2018). These movements might proceed from semi-natural habitats (e.g. orchards or vineyards) towards forested areas in autumn and vice versa when blossoms and green leaves appear in cultivated fruit crops in spring, the latest when fruits are available. With five traps of the 7-year monitoring located between the Forest of Odes and the plains in the Rhine Valley, this circumstance was proven impressively for Southwest Germany. Thus, five to ten times more individuals were captured in a trap located prominently on a small mountain ledge at the forest border when compared with traps in the interior of the forest. This is consistent with Toda (1992) who described a higher abundance of individuals at the forest border than in the interior. Additionally, he determined a higher abundance of Drosophilidae in or above the canopy of the forest. However, the observed incident of this study mainly occurred during the first week of November, when temperatures dropped for the first time below 8 °C and the orchards were completely defoliated. *D. suzukii* is a highly visually orientated insect, like many other Brachycera. Without enough sheltered habitats or nutrition left in cultivated fruit crops, specimens of *D. suzukii* from the Rhine valley might use the silhouette of the Forest of Odes on the mountains to orientate and move there in order to survive upcoming severe winter conditions. Further, shelter and nutrition in the canopy of indeciduous trees might play an important role (Briem et al. 2018a; Briem et al. 2016).

The detected decline in trap captures due to hot temperatures in chapter 2.1 potentially explains the decreasing trap captures as shown in chapter 2.3 (Eben et al. 2018) where heat stressed females tend to oviposit less. The hypothesis of chapter 2.3 that the observed slow population build-up in 2015 was caused by extreme hot and dry weather conditions was proven by the analysis of the monitoring data in chapter 2.1 (Briem et al. 2018a). Negative effects might vary locally, and adult individuals could move to shadier and moister habitats avoiding extreme weather conditions, whereas eggs or larvae inside fruits cannot move to sheltered sites. Thus, fruit temperatures during summer when temperatures exceed 30 °C reduce or even inhibit larval and pupal development (Evans et al. 2018). The hot summer of 2015 when fruits reached extreme temperatures (Briem, unpublished) while exposed to the sun (blackberries, max. 50.1 °C; raspberries, max. 42.6 °C) and even in the shadow (blackberries, max. 39.5 °C and raspberries, max. 37.4 °C) contributed to a reduced abundance and, as a consequence, to less infestation and/or larval development in that year in Southwest Germany. In addition, the analysis of the seven year monitoring highlighted that a high number of days exceeding 30 °C in late spring (April–June) coincided with less precipitation after a severe winter. As a possible consequence, the reduced population of *D. suzukii* was not able to recover and build up the population as fast as in other years. In the laboratory assays of chapter 2.3 (Eben et al. 2018), males suffered more from extreme summer temperatures as already mentioned by Plantamp et al. (2016). In the analysis of the monitoring data, it was vice versa. More males were captured in summer and autumn, by tendency, assuming that females suffer more from hot and dry weather conditions. These inconclusive results confirm that laboratory experiments cannot be easily juxtaposed with those based on field studies. Laboratory studies are usually carried out in a controlled environment, whereas different factors influence field studies. Consequently, reduced capture rates of females during the warm seasons and males during the cold seasons may be based on reduced flight activity and not on reduced survival. The reduced capture rates of females could further be a function of the attractant in the traps and females might prefer to search out oviposition sites instead of food sources.

The molecular gut content analysis developed in this thesis (chapter 2.4, Briem et al. 2018c) revealed to be a feasible option for assessing the feeding behaviour of *D. suzukii*. The study was successfully developed by focusing on a single food plant fed and another single host plant sticking on the outer body surface. Later it was successfully applied to a batch of field collected individuals with DNA of several crop plants inside and outside the specimens. The method can prospectively help to understand the nutritional resources usage and the movements between focal crops and the surroundings. In contrast to classical methods used for tracking the movement of insects (e.g. mark-recapture), the gut content analysis might provide knowledge on both the feeding behaviour and the movements of the insect (Diepenbrock et al. 2018). It is important to mention that results from the gut content analysis of *D. suzukii* with the NGS approach have to be verified prior classifying detected plant DNA as host plant. In the case of the field collected specimens where hitherto unknown host plants such as *Urtica dioica* or *Polygonum* spp. were identified, it is more likely that *D. suzukii* fed on e.g. ripe berries of *Rubus idaeus*, where accidentally some pollen has landed on. This circumstance depicts impressively the importance to question the reliability of the results and the need to combine laboratory results with the knowledge on the biology of the insect examined (Briem et al 2018c). Further, identifying the proportion of flies that fed upon defined crop hosts both within and outside of a field or the distance a fly moves from one host plant to another could contribute to understand the host usage and the movements of the pest (Diepenbrock et al. 2018). As the knowledge on the influence of *D. suzukii* populations in wild host plants bordering cultivated fruit crops is still not clarified, the molecular gut content analysis could contribute to gain better knowledge on the (feeding) behaviour within or outside plantations. In addition, it can contribute to the knowledge about nutritional sources used, beside microorganisms (Fountain et al. 2018), when no fruits are available, especially during winter and spring. Since the dispersal of *D. suzukii* is not only based on weather conditions, the successfully adapted molecular gut content analysis might prospectively unravel the whole feeding ecology of *D. suzukii* and thus support a better understanding of the spatio-temporal behaviour of the pest. This knowledge might help to modify field management or to manage those ideal microhabitats and refuges to reduce *D. suzukii* populations before they move into cultivated fruits.

3.1 Conclusion and future prospects

The analysis of the long-time monitoring revealed that temperature thresholds for in-field development and survival should be re-adjusted with respect to further meteorological (e.g., global radiation) and habitat parameters, as conclusions based on laboratory experiments cannot be easily juxtaposed with those based on field studies. It has to be considered that ripening and ripe fruits are more attractive to *D. suzukii* than traps e.g. baited with ACV-mixtures and trap captures do not reflect the in-field fly abundance adequately. Furthermore, information about realistic population densities is still lacking since the attractiveness of traps varies largely between habitats and seasons. Moreover, once attracted to VOCs emitted by the host plant, *D. suzukii* individuals orientate visually, like many other Brachycera, to the round, reddish and ripe fruits (pers. communicated T. Dekker).

Consequently, trap captures are not representative for the in-field abundance of *D. suzukii*. Thus, in periods when no fruits are available, they might overestimate the abundance, and it is still unknown whether higher trap captures in forests are a result of higher *D. suzukii* abundance or a higher attraction of traps due to less competition by fruits. Despite the great number of traps and baits for field captures of *D. suzukii,* traps solely indicate their presence and flight activity. Trap captures should be interpreted with caution as the attraction of the traps is strongly influenced by the ambient habitat, the phenology and the availability of nourishment and shelter. The above-mentioned circumstances depict the complexity of this issue.

In cases when the feeding ecology is still unknown, the capability of the molecular gut content analysis (MGCA) could help to determine the feeding activity as it provides a powerful tool to study the feeding ecology of pest insects in general. Moreover, the relationship of an insect with a specific host/food plant or a group of host/food plants could be identified too. Moreover, the findings of chapter 2.4 (Briem et al. 2018c) encourage the use of MGCA with *D. suzukii* to obtain a better understanding of its feeding behaviour and movements under natural conditions. It should be applied on field trapped individuals all year round from different sites. In winter and spring, it could provide better knowledge on overwintering nutrition and thus overwintering sites as well as on the early season feeding behaviour. This could offer the opportunity to prevent or reduce infestation in early varieties of e.g. strawberries or cherries.

Since food plant DNA might be found in many different digestion stages in the flies, it is recommended to analyse a large number of individuals to minimize the influence on the DNA detection success (Briem et al 2018c). Furthermore, more studies are necessary to determine the digestion rates of field captured specimens of winter and summer morphs at different weather conditions in the course of the year. Still, a well performing trap to ensure host plant DNA in the gut content of trapped specimens needs to be developed. Trapping the individuals alive might not be efficient enough as metabolism can be quite fast, depending on the ambient temperature. However, a system that stops metabolism at the same moment the flies are captured is necessary. It could simplify the sampling of larger numbers of individuals and a higher number of trapped specimens could contain amplifiable plant DNA. In times when increasing numbers of invasive pest insects spread through the continents, the method can expedite the understanding of its ecology and support the development of sustainable management strategies (Diepenbrock et al. 2018).

Especially early ripening fruit varieties might be protected better by developing strategies of population dynamics management. However, more precise predictions on infestation of other crops could be made along the course of the year. Another major and important point in the control of *D. suzukii* will be the characterization and identification of overwintering sites. This may help to facilitate the development of targeted control strategies to delay population dynamics in the following season. For this purpose, further research has to focus on the development of environmentally friendly control methods in order to control *D. suzukii* in the overwintering sites during winter or before they spread into cropped fruits.

Although the research results presented in this thesis have provided important insights into the spatio-temporal behaviour of *D. suzukii*, many questions remain unanswered and further research is necessary with respect to the invasion of *D. suzukii* all over the world. The still ongoing climate change can facilitate *D. suzukii* to spread even further into hitherto unsuited regions. The world map of climates preferred and tolerated by *D. suzukii* (Figure 2) will change prospectively. As seen in the long-time monitoring analysis, *D. suzukii* is capable of withstanding severe weather conditions, even extreme heat or dryness. As an r-strategist with up to 8 generations per year, with their number depending on the ambient conditions, *D. suzukii* may even adapt to these changing conditions much faster than many other animals. These adaptations should be addressed in future projects in order enhance our understanding of *D. suzukii*.

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APPENDIX

Supplemental Material: Explorative Data Analysis of *Drosophila suzukii* Trap Catches from a Seven-Year Monitoring Program in Southwest Germany

Felix Briem, Anto Raja Dominic, Burkhard Golla, christoph Hoffmann, Camilla Englert, Annette Herz and Heidrun Vogt

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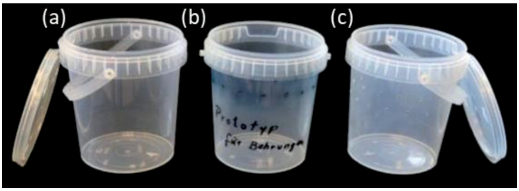

Figure S1. (a) Example of a new cup, (b) the used stencil for standardized trap construction and (c) the prepared trap.

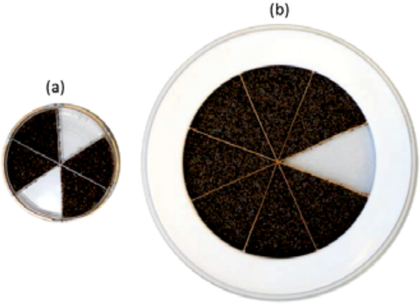

Figure S2. Partial counting of large captures (> 5 ml): (a) petri dish (Ø 6 cm) split into six equal sections for catches < 15 ml; (b) petri dish (Ø 14 cm) split into eight equal sections for catches >15 ml. The sections were made with wires attached to a plastic ring insert placed inside the petri dish.

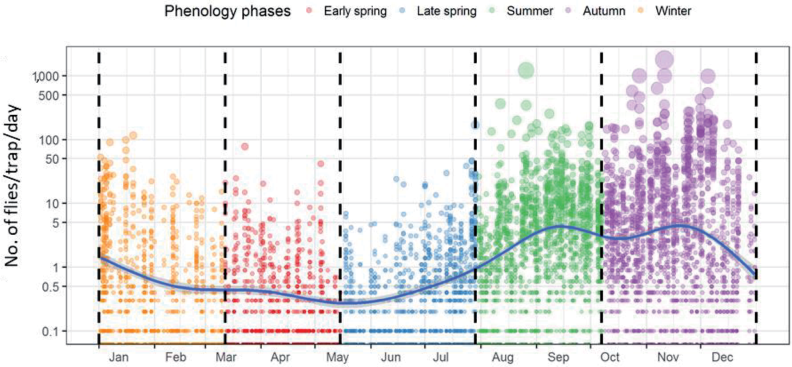

Figure S3. Captures per day from each trap over the monitoring period (2011 – 2018) with a smoothing curve (blue), based on which the five seasonal categories were created. The bubble size (small to big) is proportional to the capture size. Note: the y-axis is log-scaled.

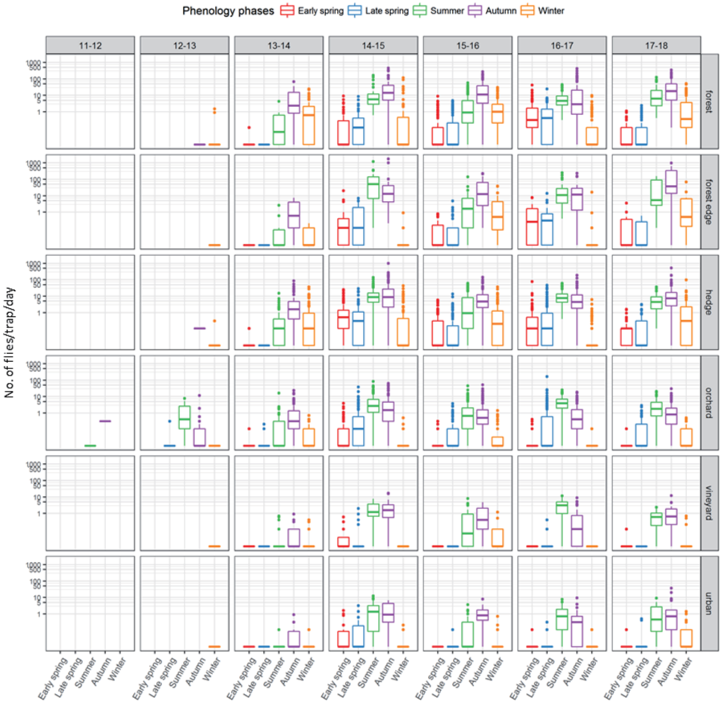

Figure S4. Box plots of captures/trap/day in each *D. suzukii* - year, grouped by sampled habitat types and *D. suzukii* phenology. The box represents the inter-quartile range (IQR) and the band inside, the median. The whiskers represent data that are within 1.5 IQR below or above the 1st and the 3rd quartiles. The outliers are represented by dots beyond the whiskers. Note.: Y-axis is log-scaled (captures/day/trap + 0.01).

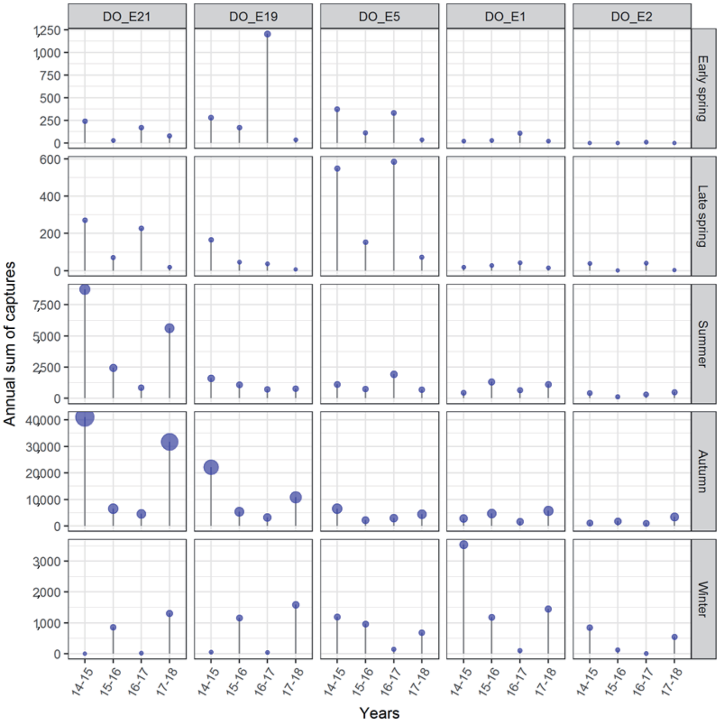

Figure S5. Annual sum of *D. suzukii* captures in the *D. suzukii* -years from 2014-2015 until 2017-2018 grouped by *D. suzukii* -phenology and site/trap. The size of the blue bubble is proportional to the annual sum; Note: y-axis scale varies between rows.

Table S1. Distances of select traps from the forest border, orchards/vineyards and urban areas, coordinates and altitude. The five traps shown here are located in and around the Forest of Odes and captured significantly higher individuals than the rest of the traps from this study.

Trap name	Trap location	Distance to forest border (m)	Distance to orchard/vineyard (m)	Distance to residential area (m)	Coordinates	Altitude asl (m)
DO_E1	On the forest border	0	300	110	E8.68300703 N49.44914396	236
DO_E2	In the forest	700	900	165	E8.69164199 N49.45003001	285
DO_E5	Near the forest border	150	1	390	E8.67215700 N49.46331901	167
DO_E19	On the forest border	17	5	170	E8.67126801 N9.47380100	237
DO_E21	On the forest border	13	10	100	E 8.67096903 N49.47397602	231

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DANKE!

CURRICULUM VITAE

Personal Information

Name:	Felix Briem
Date of birth:	07.06.1984
Place of birth	Freiburg/Brsg.
Nationality:	German

Work Experience

since 10/2013	Research assistant at Julius Kühn-Institut (JKI) , Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Dossenheim, Germany Topic: *“Behavior and phenology of the invasive pest insect Drosophila suzukii”*
03/2012 – 09/2013	Research assistant, State Institute for Viticulture and Enology, Freiburg, Germany
12/2010 – 08/2011	Student assistant, German Environmental Specimen Bank (ESB), Trier University, Germany
06/2010 – 08/2010	Student assistant, Alfred Wegener Institute, Sylt, Germany
11/2009 – 04/2010	Student assistant, German Environmental Specimen Bank (ESB), Trier University, Germany

Education

Since 02/2016	PhD student at the Martin-Luther-University, Halle-Wittenberg, Faculty I of Natural Science - Biological Science
2005 – 2011	Studies in “Applied Biogeography”, University of Trier, Germany Diploma thesis: “*Effects of the severe winter 2009/2010 on alien species around the island of Sylt*“ Diplom-Biogeographer
1996 – 2004	Abitur (A-level) at the Martin-Schongauer-Gymnasium, Breisach, Germany

PUBLICATIONS AND PROCEEDINGS

Publications – peer-reviewed

Briem F, Dominic AR, Golla B, Hoffmann C, Englert C, Herz A, Vogt H (2018) Explorative Data Analysis of *Drosophila suzukii* Trap Catches from a Seven-Year Monitoring Program in Southwest Germany. Insects 9,125.

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Conference Contributions: Talks

Briem F, Dominic AR, Golla B, Hoffmann C, Englert E, Herz A, Vogt H (2019) Explorative data analysis of a 7-year *Drosophila suzukii* monitoring program in Southwest Germany- patterns and relationships to weather conditions, PheroFIP 19 (IOBC), 20. – 25.01.2019, Lissabon, Portugal

Briem F, Dominic AR, Golla B, Hoffmann C, Englert C, Herz A, Vogt H (2018) Explorative Datenanalyse zum Auftreten der Kirschessigfliege, *Drosophila suzukii*, anhand von Monitoringdaten aus DrosoMon, 61. Deutsche Pflanzenschutztagung, 11. – 14.09.2018, Hohenheim.

Briem F, Eben A, Boehnke B, Golla B, Hoffmann C, Herz A, Englert C, Kleepsies R, Biganski S, Pinggera J (2017) Herausforderung Kirschessigfliege: Aktuelles aus der Forschung und zur Bekämpfung, 72. ALVA-Tagung, Zukunft Obstbau, 22. – 23.05.2017, Waldkirchen am Wesen, Österreich.

Briem F, Zeisler C, Staudacher K, Traugott M, Vogt H (2017) Developing a new molecular approach to analyze the diet of *Drosophila suzukii*, Integrated Management of *Drosophila suzukii*, Deutsch-Chinesischer Workshop, 26.06. – 2.07.2017, Darmstadt.

Briem F, Zeisler C, Traugott M, Vogt H (2017) Habitat use, digestion time and diurnal activity of *Drosophila suzukii*, Tagung der Deutschen Gesellschaft für allgemeine und angewandte Entomologie (DGaaE), 13.-16.03.2017, Freising.

Briem F, Vogt H (2016) Managment strategies and case studies for the invasive pest *Drosophila suzukii* in Germany, Journées *Drosophila suzukii*, 01. – 02.12.2016, Centre Ctifl de Balandran, Bellegarde, Frankreich.

Briem F, Vogt H (2016): Landscape-level movements and a molecular approach to analyze the diet of *Drosophila suzukii*. 9th Young Scientists Meeting, 9. – 11.11.2016, Quedlinburg.

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Briem F, Staudacher K, Zeisler C, Porsche F, Eben A, Traugott M, Gross J, Vogt H (2016) Habitat use and a molecular approach to analyze the diet of *Drosophila suzukii*. 9th international Conference on Integrated Fruit Production, 04. – 08.09.2016, Thessaloniki, Griechenland. – Abstract awarded with the student paper award.

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Briem F, Eben A, Gross J, Vogt H (2015) Neues zum Populationsaufbau im Frühjahr, AG Kirschessigfliege, 08.-09.12.2015, Erfurt.

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Briem F, Vogt H (2015) Die Kirschessigfliege – neueste Erfahrungen und Erkenntnisse, 24. Thüringer Obstbautag, 10.02.2015, Erfurt.

Briem F, Vogt H (2015) Occurrence and infestation potential of the recently introduced *Drosophila suzukii*. Tagung der Deutschen Gesellschaft für allgemeine und angewandte Entomologie (DGaaE), 20.-25.03.2015, Frankfurt/M.

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Briem F, Breuer M, Köppler K, Vogt H (2014) Die Kirschessigfliege in Deutschland: quo vadis? 59. Deutsche Pflanzenschutztagung, 23-26.9.2014, Freiburg.

Briem F, Breuer M, Köppler K, Vogt H (2014) Phenology and occurrence of Spotted Wing Drosophila in Germany and case studies for its control in berry crops. Seminar im Julius Kühn Institut, Juli 2014, Dossenheim.

Briem F, Vogt H (2014) Auftreten und Populationsentwicklung der Kirschessigfliege in Deutschland, Arbeitssitzung PSD, März 2014.

Briem F, Vogt H (2014) *Drosophila suzukii* – quo vadis? 7th Young Scientists Meeting, 26. – 28.11.2014, Julius Kühn-Insitut, Quedlinburg.

Briem F, Vogt H (2014) Auftreten der Kirschessigfliege im Jahresverlauf in Obstkulturen und auf Landschaftsebene; AG Kirschessigfliege, 9. – 10.12.2014, Freiburg.

Conference Contributions: Poster

Briem F, Neukampf R, Vogt H (2018) Einsatz einer halbautomatischen Falle zur Erfassung der Kirschessigfliege im Tages- und Jahresverlauf. 61. Deutsche Pflanzenschutztagung, 11. – 14.09.2018, Hohenheim.

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EIDESSTATTLICHE ERKLÄRUNG / DECLARATION UNTER OATH

Ich erkläre an Eides statt, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word and content.

Roxheim, den 20.05.2019

Felix Briem

JKI

Julius Kühn-Institut

Bundesforschungsinstitut für Kulturpflanzen