Evaluation of Natural Antagonists for Biological Control of Drosophila suzukii

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Evaluation of Natural Antagonists for Biological Control of *Drosophila suzukii*

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Unless you try to do something beyond what you have already mastered,
You will never grow.

-Ralph Waldo Emerson/Ronald E. Osborne

In loving memories of my parents
For the faithful companions on my way

CONTENT

LIST OF ABBREVIATIONS

%	percent
× *g*	multiple of gravitation speed
°C	degree Celsius
a.i.	active ingredient
B.t.i.	*Bacillus thuringiensis* servovar. *israelensis*
B.t.k.	*Bacillus thuringiensis* servovar. *kurstaki*
B.t.t.	*Bacillus thuringiensis* servovar. *thuringiensis*
BC	biological control
BCA	biological control agent
Bio	Biomükk, *B.t.i.* product
BLAST	Basic Length Alignment Search Tool
bp	base pairs
C	control
cm	centimeter
CRISPR/Cas	Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR associated genes
CTAB	cetyltrimethylammonium bromide
DiNV	Drosophila innublia nudivirus
DM	*Drosophila melanogaster*
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOM	*Drosophila* oviposition medium
dpi	days post inoculation
DS	*Drosophila suzukii*
dsDNA	double-stranded DNA
DW	*Drosophila willistoni*
e. g.	*exempli gratia*, for example
Ed./Eds.	editor/editors
EF	elongation factor
EPF	entomopathogenic fungi
EPN	entomopathogenic nematodes
ERH	enemy release hypothesis

et al.	*et alia*, and other
etc.	*et cetera*
EU	European Union
F	forward oligonucleotide primer
g	gram
GMO	genetically modified organism
IAS	invasive alien species
IPM	integrated pest management
IPTG	isopropyl-β-D-1-thiogalactopyranoside
ITS	internal transcribed spacer
JKI	Julius Kühn-Institut
kbp	kilobase pairs
KV	Kallithea virus
l	liter
L1, L2, L3	first, second, third instar larva
LB	Lysogeny Broth medium
LC_{50}	median lethal concentration
LSU	large ribosomal subunit
mg	milligram
mHz	mega Hertz
min	minutes
ml	milliliter
ML	Maximum Likelihood method
mM	milimolar
MP	Maximum Parsimony method
N, N^{o}	number of tested samples/individuals
n.d.	not determined
Neu	Stechmückenfrei (Neudomück), *B.t.i.* product
ng	nanogram
NGS	next generation sequencing
NJ	Neighbor-Joining method
nm	nanometer
Ø	diameter

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	*potentia hydrogenii*, measure of acidity or alkalinity of aqueous solution
R	reverse oligonucleotide primer
r.H.	relative humidity
R/N	number of replicates/number of samples
RC	recommended concentration
rDNA	ribosomal deoxyribonucleic acid
RNAi	ribonucleic acid interference
RPB1	largest subunit of RNA polymerase II
rpm	rounds per minute
RT-qPCR	real-time quantitative PCR
SAM	*Drosophila suzukii* American population
SD	standard deviation
SE	standard error
sec	seconds
SIT	sterile insect technique
Sol	Solbac, *B.t.i.* product
sp. nov.	*species nova*, new species
sp./spp.	*species*/*species pluralis*, species/multiple species
SSU	small ribosomal subunit
SWD	spotted wing drosophila
T	treatment, application of test substances
TAE	tris base, acetate and EDTA buffer solution
Taq	*Thermus aquaticus*
TSA	Tryptic Soy Agar
USA	United States of America
V	Volt
*w*Suz	*Wolbachia* sp. from *D. suzukii*
XGAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	microgram
µl	microliter
µm	micrometer

SUMMARY

The polyphagous insect *Drosophila suzukii* (eponymous: spotted wing drosophila, SWD) is indigenous to Southeast Asia and has been spreading rapidly in North American and European countries since 2008. It became primarily a pest insect of berries, grapes and stone fruits as it lays eggs in ripening fruits leading to rapid fruit collapse and causing high economic losses. Because of its life cycle, chemical control of SWD is very complicated and often impossible. Hence, the evaluation and development of biological control measures are of great importance for conventional and organic fruit growers.

As a first approach (Chapter II), biological control agents (BCA) that are already available for Diptera have been tested for their efficacy against SWD. For this purpose, various products of the gram-positive subspecies *Bacillus thuringiensis* serovar. *israelensis* (*B.t.i.*), which is specific for the Diptera, such as mosquitos and blackflies, were applied to SWD larvae and adults. It could be demonstrated in laboratory experiments, that the examined products showed neither an increased mortality after exposure to SWD larvae or adults nor a repellent effect on the oviposition behaviour after application to host fruits. Thus, *B.t.i.* products could be excluded as possible candidates for SWD control.

As shown in Chapter III, Neem oil, an extract from the seeds of the Neem tree *Azadirachta indica*, was also inefficient against SWD. The extract is mainly applied to larvae of leaf-sucking insects, on which the active ingredient Azadirachtin A has a lethal effect and inhibits ecdysis (moulting). These effects also appeared on SWD larvae, but only at concentrations ten times higher than suggested for target organisms. In addition, no repellent effect to SWD was noted.

Another approach to find host-specific antagonists suitable as BCA is to examine natural populations for pathogens that are already associated to the respective species. In Chapter IV, such a pathogen belonging to the phylum Microsporidia is described. Based on comprehensive light and electron microscopic studies as well as molecular analysis of rDNA sequences and phylogenetic studies revealed a new microsporidian species, which was eventually named *Tubulinosema suzukii* sp. nov.

T. suzukii was further tested for its competence as an antagonist of SWD (Chapter V). The median lethal spore concentration (LC_{50}: 6900 spores/µl) and concentration-dependent mortality after exposure of larvae showed moderate to high virulence of *T. suzukii* to early developmental stages of SWD. Molecular examination of the infection process via RT-qPCR showed that replication of *T. suzukii* increased especially during the larval and pupal stages of SWD. This resulted in restricted or delayed development of adult SWD. In addition, population-reducing effects were evaluated by experiments on survival rates and on the

ability to lay eggs (oviposition). After inoculation of SWD larvae with *T. suzukii*, hatching rates were significantly reduced (over 70%), and the survival rates of hatched adult flies as well as their reproductive ability (up to 70% less progeny) were severely impaired. These effects were less pronounced when adult SWD were exposed to *T. suzukii*. Considering the clear effects on viability and egg deposition as well as generally chronic and sublethal impact of microsporidia on its infected hosts, a long-term effect of *T. suzukii* infection affecting SWD populations seems to be likely.

The results of this thesis indicate that application of *B.t.i.* products or Neem oil do not offer sufficient control options for SWD since they are either ineffective or would require an excessive and therefore uneconomical application rate. On the other hand, a new microsporidian species, *T. suzukii*, was isolated and characterized. It showed highly promising effects on larval stages of SWD and its finding encourages further evaluations in semi-field trials.

ZUSAMMENFASSUNG

Das polyphage Insekt *Drosophila suzukii* stammt aus Südostasien und hat sich seit 2008 rasant in Nordamerika und Europa verbreitet. Es ist insbesondere ein Schadinsekt bei Beeren, Reben und Steinfrüchten. Das Ablegen seiner Eier in reifende Früchte führt zu deren Kollabieren und hohen ökonomischen Schäden. Aufgrund seines Lebenszyklus ist es sehr schwierig, *D. suzukii* chemisch zu bekämpfen. Daher besteht die Notwendigkeit, biologische Bekämpfungsverfahren, die sowohl im konventionellen wie im ökologischen Obstbau eingesetzt werden können, zu evaluieren und zu entwickeln.

In einem ersten Ansatz (Kapitel II) wurde die Wirkung biologischer Pflanzenschutzmittel, die zur Bekämpfung von zweiflügligen Schadinsekten bereits verfügbar sind, auf *D. suzukii* getestet. Hierzu wurden Larven und adulte Fliegen von *D. suzukii* mit verschiedenen Produkten der gram-positiven Unterart *Bacillus thuringiensis*, welche spezifisch auf Dipteren (z.B. Stechmücken und Kriebelmücken) wirkt, behandelt. In Laborversuchen konnte gezeigt werden, dass die getesteten Produkte weder eine erhöhte Mortalität behandelter Larven oder Adulten von *D. suzukii* hervorriefen, noch eine repellente Wirkung auf das Ovipositionsverhalten nach Applikation auf Wirtsfrüchte entfalteten. Somit konnten auf *B.t.i.* basierende Produkte als potentielle Kandidaten zur Bekämpfung von *D. suzukii* ausgeschlossen werden.

Wie in Kapitel III gezeigt, war auch Neemöl, ein Extrakt aus den Samen des Neembaums, gegenüber *D. suzukii* uneffektiv. Der Extrakt wird vornehmlich zur Bekämpfung von Larven blattsaugender Insekten, auf welche der Wirkstoff Azadirachtin A des Neemöls einen häutungsinhibierenden und letalen Effekt hat, verwendet. Zwar konnten diese Effekte auch bei Larven von *D. suzukii* beobachtet werden, allerdings erst bei einer zehnfach höheren Konzentration als für Zielorganismen vorgeschlagen. Zudem konnte kein repellenter Effekt auf *D. suzukii* festgestellt werden.

Ein anderer Ansatz, wirtsspezifische Antagonisten zu finden, welche sich als mikrobiologische Bekämpfungsmittel nutzen lassen, ist die Untersuchung von natürlichen Populationen auf bereits assoziierte Pathogene. Im Kapitel IV wird ein solches Pathogen des Phylums Microsporidia beschrieben. Basierend auf umfänglichen licht- und elektronenmikroskopischen Untersuchungen, molekulargenetischen Analysen von rDNA-Sequenzen und phylogenetischen Studien konnte eine neue Mikrosporidien-Art, *Tubulinosema suzukii* sp. nov. identifiziert und charakterisiert werden

Des Weiteren wurde *T. suzukii* auf seine Kompetenz als Antagonist gegen *D. suzukii* getestet (Kapitel V). Die mittlere letale Sporenkonzentration (LC_{50}: 6900 Sporen/µl) und die konzentrationsabhängige Mortalität nach Exposition von Larven zeigten eine moderate bis

hohe Virulenz von *T. suzukii* gegenüber frühen Entwicklungsstadien von *D. suzukii*. Durch die Verfolgung des Infektionsverlaufs mittels RT-qPCR konnte gezeigt werden, dass die Replikation von *T. suzukii* besonders innerhalb der Larval- und Pupalphase von *D. suzukii* ansteigt, was zu einer eingeschränkten oder verzögerten Entwicklung von adulten *D. suzukii* führt. Außerdem wurden populationsreduzierende Effekte durch Experimente zu Überlebensraten und Eiablagefähigkeit evaluiert. Es zeigte sich, dass nach Inokulation von *D. suzukii*-Larven mit *T. suzukii* die Schlupfraten signifikant reduziert (über 70%) waren; ebenso waren auch die Überlebensraten geschlüpfter adulter *D. suzukii* und deren Reproduktionsfähigkeit (bis zu 70% weniger Nachkommen) stark beeinträchtigt. Diese Effekte waren weniger stark ausgeprägt, wenn adulte *D. suzukii* den *T. suzukii*-Sporen exponiert waren. In Anbetracht der deutlichen Effekte von *T. suzukii* auf die Überlebensfähigkeit und die Eiablage von *D. suzukii* und die generellen chronischen, subletale Auswirkungen von Mikrosporidien-Infektionen auf ihre Wirte, ist ein populationsreduzierender Langzeiteffekt einer *T. suzukii*-Infektion auf *D. suzukii* möglich.

Die Ergebnisse dieser Arbeit zeigen, dass die Anwendung von *B.t.i.*-Produkten oder Neemöl keine ausreichenden Bekämpfungsoptionen von *D. suzukii* darstellen, da sie entweder unwirksam sind oder eine zu hohe und damit unwirtschaftliche Aufwandmenge benötigen würden. Andererseits konnte ein neues Mikrosporidium aus einer *D. suzukii*-Laborpopulation isoliert und charakterisiert werden. Es zeigte durchaus vielversprechende Wirkungen auf Larvenstadien von *D. suzukii*. Diese Ergebnisse sind eine Ermutigung für weiterführende Wirkungsstudien in Halbfreilandversuchen.

CHAPTER I: GENERAL INTRODUCTION

DROSOPHILA SUZUKII: BIOLOGY, DISTRIBUTION AND IMPORTANCE AS INVASIVE INSECT PEST

Biology and ecology of *D. suzukii*

The insect species *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) was recorded for the first time in 1916, initially in Japan, Korea, Thailand, India, Myanmar and China (Matsumura, 1916; Hauser, 2011). The small fruit fly has a pale brown or yellowish-brown-banded thorax and abdomen and red eyes. The morphology of the fruit fly shows a sexual dimorphism. Male flies (2.8-3.1 mm, Figure 1-1 a) are 10-20% smaller than female flies (3.0-3.4 mm, Figure 1-1 b) (Walsh et al., 2011; Cini et al., 2012). Male flies have one black spot on each wing tip (Figure 1-1 a) that is eponymous for the English trivial name and synonym "spotted wing drosophila" (SWD). Generally, these spots appear one to two days after hatching (Hauser, 2011). Moreover, the first and second tarsal segments on the forelegs have small crests, also called "sex-combs", which are important for mating (Figure 1-2 a) (Vlach, 2010). The female fly has a unique serrated ovipositor allowing penetration of intact thin-skinned fruits (Figure 1-2 b). One female can lay 300-400 eggs in a lifetime, most directly under the fruit skin of preferably intact, unwounded fruits (Figure 1-3 a) (Mitsui et al., 2006; Cini et al., 2012). Generally, the two respiratory filaments of the whitish eggs are protruding from the fruit skin. Depending on temperature, the first instar (L1) larvae are hatching within 2 to 5 days having a transparent-whitish and cylindrical body with black mouthparts (Figure 1-3 b) (Walsh et al., 2011; Emiljanowicz et al., 2014). Larval development occurs inside fruits. During development to second (L2) and third instar (L3) the larval body turns white by expansion of adipose tissue making internal organs invisible (Figure 1-3 b) (Walsh et al., 2011). Pupation needs up to two weeks and can occur inside and outside fruits but also at the ground (Figure 1-3 c) (Cini et al., 2012; Woltz and Lee, 2017).

Figure 1-1: SWD sexual dimorphism.
(a) SWD male and (b) female adult fly. Images from Felix Briem, Julius Kühn-Institut.

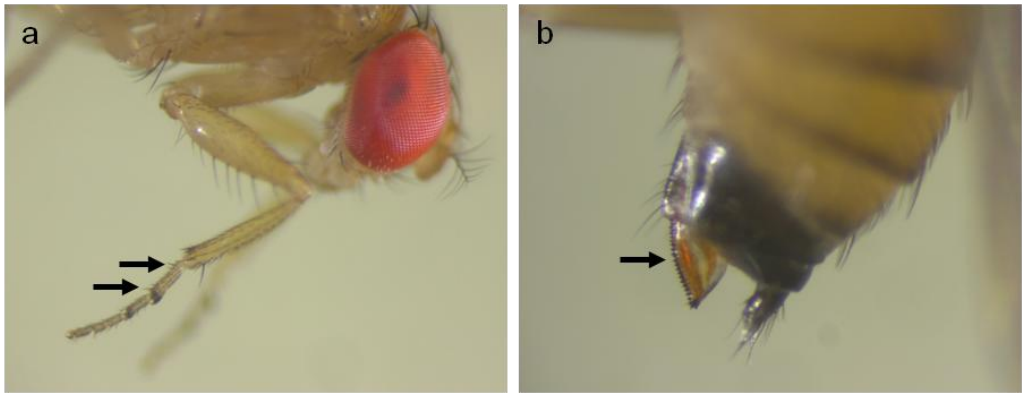

Figure 1-2: SWD morphological characteristics.
(a) Male SWD with species-specific characteristic crests (arrows) on the last tarsal element. (b) The serrated ovipositor of the female fly (arrow). Images from Felix Briem, Julius Kühn-Institut.

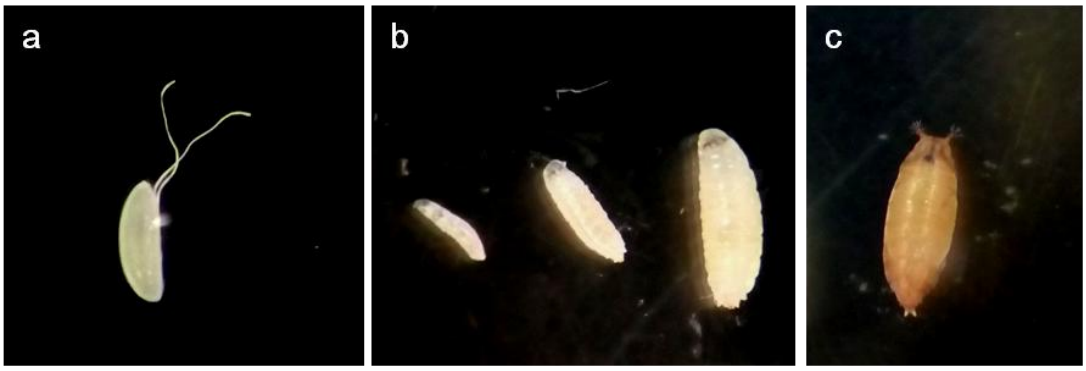

Figure 1-3: Developmental stages of SWD.
(a) Egg with respiratory filaments. (b) From left to right: L1, L2 and L3 larval stage. (c) SWD pupa.

Seasonal conditions induce phenotypic dichotomy of adult flies leading to two adult morphotypes, namely winter and summer morphs (Kanzawa, 1936; Shearer et al., 2016). Both are particularly characterized by different body size and colour whereby summer morphs are much smaller but brighter than winter morphs (Shearer et al., 2016). The larger body size possibly enables winter morphs to store more energy by increasing the proportion of adipose tissue. For that reasons female winter morphs may enhance their cold tolerance during overwintering (Zerulla et al., 2015; Shearer et al., 2016).

SWD overwinters as adult fly (Mitsui et al., 2010; Asplen et al., 2015). Mated females survive up to six months at cold temperature and initiate and establish the first progeny generation in the following year (Kanzawa, 1939; Mitsui et al., 2010; Walsh et al., 2011; Cini et al., 2012). On days with less than 10 °C, flies are staying cold-protected under leaf litter with

warmer microclimate conditions, whereas temperatures below 3 °C cause high mortality (Dalton et al., 2011; Tochen et al., 2014; Wallingford et al., 2018; Stockton et al., 2019). In mild winters and early spring, flies actively look for alternative food sources such as berries of mistletoe and ivy (Poyet et al., 2015; Briem et al., 2016). Seasonal population models show two increasing peaks in spring and autumn, when host plants and fruits are available, but a decrease in summer when temperatures raise over 30 °C leading to high mortality rates (Kanzawa, 1939; Lee et al., 2011b; Tochen et al., 2014; Wang et al., 2016). SWD is polyphagous; preferred host plants are nearly all kind of soft small fruits (raspberry, blueberries, strawberry, elderberry), and thin-skinned stone fruits (cherry, apricots, peaches) (Figure 1-4 a-c) (Walsh et al., 2011; Cini et al., 2012). Infestation of grapevine occurs rarely and can be assumed as a secondary infestation following previous fruit damage (Figure 1-4 d) (Entling and Hoffmann, 2020). In Europe and the USA, also non-fruit plants, ornamental flowers and wild fruits were identified as host plants (Kenis et al., 2016).

Figure 1-4: Pattern of damage for fruits infested by SWD.
(a) SWD pattern of damage on cherries. (b) Infested raspberry with larvae. (c) Elderberries with alimentary filaments (arrow) of a SWD egg. (d) Primary infection of grapevine with a fungus, secondary infestation of SWD egg (arrow). Images: (a) Heidrun Vogt, Julius Kühn-Institut Dossenheim; (b) Jürgen Just, Julius Kühn-Institut Dossenheim; (c, d) Christoph Hoffmann, Julius Kühn-Institut Siebeldingen.

Geographical distribution and importance of *D. suzukii* as invasive insect pest

SWD is endemic to East and Southeast Asian countries (Hauser, 2011). In 1934, first damage was recorded in Japanese cherry orchards (Kanzawa, 1934; Kanzawa, 1939). During the 1980s, the fly was frequently found on the Hawaiian Islands and from there it probably dispersed to the mainland of the USA (O'Grady et al., 2002; Hauser, 2011) but also to South America, including Brazil, Peru and Argentina (Santadino et al., 2015; Andreazza et al., 2017). Grassi et al. (2011) and Calabria et al. (2012) reported its presence in Europe from 2009 with first occurrence in Spain and later in Italy. Shortly thereafter, *D. suzukii* dispersed to Northern Europe, arriving Germany in 2011 and the United Kingdom in 2012, even though species distribution models predicted much larger geographic expansion with global distribution in areas of Mediterranean and temperate climate conditions (Vogt et al., 2012; Harris and Shaw, 2014; Dos Santos et al., 2017; Ørsted and Ørsted, 2019). The current worldwide distribution of *D. suzukii* is shown in Figure 1-5. It has been found almost all over the USA and Europe.

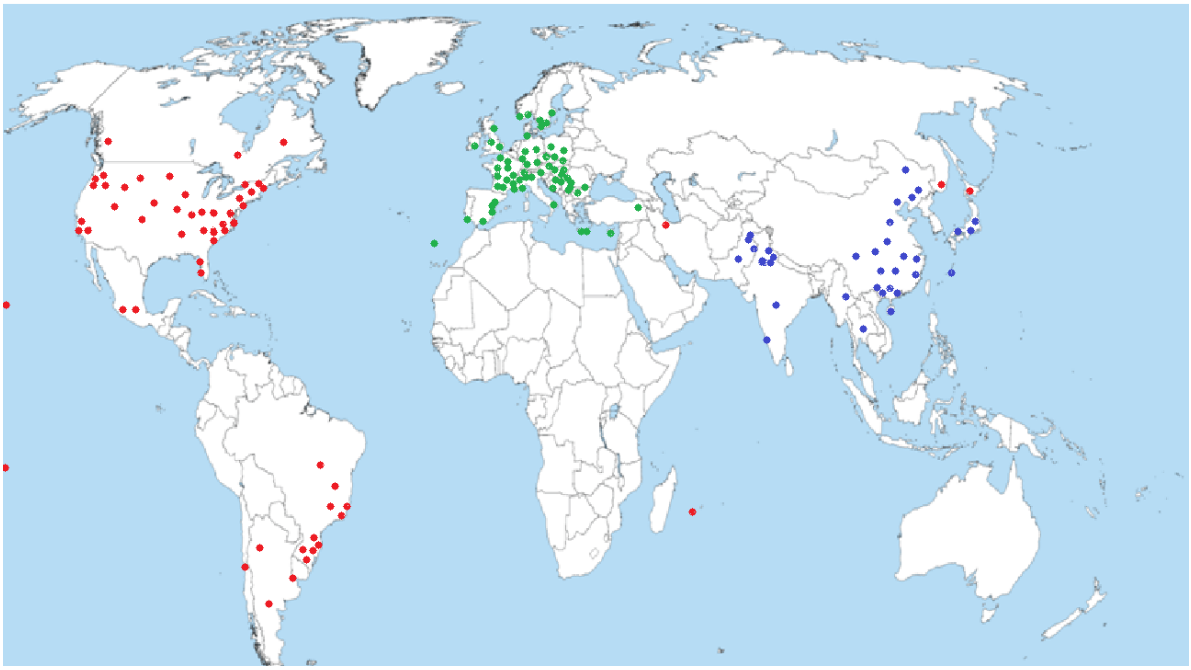

Figure 1-5: Global distribution of *D. suzukii* populations in 2019 modified from Ørsted and Ørsted (2019) and Asplen et al. (2015).

Blue dots represent *D. suzukii* population occurrence in native countries (from Japan to Pakistan), green dots mark the distribution of European population and red dots show distribution of the American population.

Invasive alien species (IAS) are non-indigenous species which are introduced either deliberately (like ornamental plants) or unintentionally (like exotic insect eggs/larvae via contaminated fruits/plants) to new habitats and mainly by human activities like travelling or global trade (reviewed by Paini et al., 2016). Biological invasion frequently results in significant negative consequences for the native ecosystems as shown for numerous examples of exotic plant and animal species (DAISIE, 2009). New habitats are occupied and indigenous species may be repressed by competition for resources (Kenis et al., 2009; Roques et al., 2009). Commonly, no natural antagonists occur in invaded habitats making a rapid occupation possible. If considerable economic damage is caused by the IAS, it is called an invasive pest (Mooney and Hobbs, 2000; Mooney et. al, 2005). SWD is an invasive insect pest that attacks ripening and overripe fruits making it a serious agricultural pest (Lee et al., 2011b). Whereas most drosophilids are attracted by fermentation volatiles of fruits and lay their eggs on rotten fruits, only *D. suzukii*, *Drosophila pulchrella* and *Drosophila subpulchrella* prefer ripening and ripe fruits for oviposition using visual stimuli of fruit colour (Kanzawa, 1934; Cha et al., 2012; Hamby et al., 2012; Landolt et al., 2012a; Landolt et al., 2012b). Blue and red colours were noted to be most attractive to *D. suzukii*, representing the spectrum of many ripening small berry fruits (Lee et al., 2011a). Mostly preferred are virtually all cherry and berry cultivars but also thin-skinned stone fruits and grapevine berries (Cini et al., 2012). In a global view, SWD has enormous damage potential on fruit industry because key economical fruits with high value like sweet cherry, strawberries and blueberry are affected. From 2008, the US American and European fruit growers reported crop losses in these cultivars of more than 30-80% (Lee et al., 2011b; Tochen et al., 2014). In 2008/2009, *D. suzukii* was first recorded at the Pacific West Coast of the USA (California, Oregon), whereby estimated yield losses in economically important small fruit production were up to 500 million US dollar per year (Goodhue et al., 2011). In 2010, the fly was also observed in Eastern USA but without enormous crop losses as reported for the West Coast. In Europe, considerable damage appeared in Italy with up to 3.3 million Euro estimated annual crop losses in small fruit production (De Ros et al., 2013). Potential host fruit preferences are yearly up-scaled as new cultivars and non-crop fruits were detected to be infested by the fly.

CONTROL STRATEGIES: INTEGRATED PEST MANAGEMENT OF *D. SUZUKII*

Within the past ten years, progress in *D. suzukii* control has been achieved with application of chemical pesticides such as spinosyns, organophosphates and pyrethroids (Bruck et al., 2011; Van Timmeren and Isaacs, 2013; Pavlova et al., 2017). Control is needed just before harvest, which is in conflict with preharvest intervals of many chemical pesticides. In case of SWD, some tested chemicals are acting as broad-spectrum insecticides; neonicotinoids for example have high toxicity on larval SWD but were not satisfactory when used to control

SWD adults in sweet cherry cultivars and had a high detrimental impact on beneficials, leaving them not recommendable for *D. suzukii* control (Beers et al., 2011; Bruck et al., 2011).

Integrated pest management (IPM) focuses on the preferential use of environmentally friendly, non-chemical strategies. Successful control of SWD was achieved with construction of physical barriers with exclusion nets or foil tunnels covering fruit cultivars but they are very expensive to install and labour-intensive during growth and harvest (Kawase et al., 2008; Leach et al., 2016; Rogers et al., 2016). Furthermore, sanitary measures like removal of infested, dropped or leftover fruits and defoliation are sustainable methods, which prevent premature introduction of *D. suzukii* by avoiding to give them an early refuge (Walsh et al., 2011; Cini et al., 2012). Also, solarisation or cold treatment of already infested fruits can help to reduce upcoming population (Lee et al., 2011b). Mass-trapping by attract-and-kill strategies are a further consideration. However, luring *D. suzukii* by bait traps using synthetically produced pheromones or special coloured traps focussing on visual cues seems to act insufficiently compared to natural attractants like host fruits (Beers et al., 2011; Landolt et al., 2012a; Grassi et al., 2015; Rice et al., 2017). Application of sterile insect technique (SIT) is a valuable strategy for control of many insect pests including those acting as vectors for diseases infectious for human and livestock (Krafsur, 1998; Benedict and Robinson, 2003). The method is based on population reduction by release of gamma-irradiated, sterile males and their ability to mate with wild-type females resulting in unfertilized eggs producing only female offspring or no progeny at all. Although few studies reported successful laboratory trials, others were questioning an insufficient mating of released males with wild-type females, but also upcoming high costs since fast generation time of *D. suzukii* would end up in mass rearing and numerous releases per year (Lanouette et al., 2017; Schetelig et al., 2018). Considerations have been also given to CRISPR/Cas and RNAi techniques (Li and Scott, 2016; Taning et al., 2016). Both based on biotechnology and genetic engineering using transgenic, genetically modified organisms (GMO) which renders these techniques challenging for application due to current legislation in Europe and other countries (Schetelig et al., 2018).

BIOLOGICAL CONTROL: ECO-FRIENDLY MEASURES WITH MICRO- AND MACROORGANISMS

Biological control (BC) basically addresses control strategies with living organisms (Eilenberg et al., 2001; Eilenberg, 2006). It gains more and more importance due to increasing environment and health awareness of consumers, growers, and politicians (Torjusen et al., 2001; Sanders et al., 2019). Some plant extracts contain secondary metabolites and essential oils which have repellent or growth-inhibiting effects on insect pests feeding on leaves or fruits. For example, Neem oil, an extract from leaves and seeds of the Neem tree

(*Azadirachta indica*), is widely used against lepidopteran and dipteran pests (Schmutterer, 1990; Sharma et al., 1993). On the other hand, macroorganisms like entomopathogenic nematodes, parasitic wasps (parasitoids) and predators but also naturally occurring microorganisms like entomopathogenic bacteria, viruses and fungi are important BCA (Eilenberg et al., 2001; Eilenberg, 2006). Thus, invasive insect pests and their invaded habitat can be screened for naturally occurring enemies and pathogens.

According to the enemy release hypothesis (ERH), invasive species benefit from escaping their natural enemies in the native habitat and the absence of new enemies in invaded areas (Williamson, 1996; Keane and Crawley, 2002). Thus, searching for effective antagonists is most promising in the countries of origin of an IAS but needs previous risk assessment before introducing the exotic antagonist (Van Lenteren et al., 2006). Moreover, IAS can also act as a "vector" for invasive parasites and pathogens. Some cases suggest a pathogen spill over from invasive to indigenous species hitchhiked with the invasive insects and were more virulent to native host species, predators and parasitoids acting then as "biological weapons" (Vilcinskas, 2019). This phenomenon was shown for some protozoan parasites, viruses, fungi but also microsporidia (Strauss et al., 2012; Arbetman et al., 2013; Rode et al., 2013; Lymbery et al., 2014). Due to that issue, evaluation of potential spill over to indigenous host and non-host species is needed preliminary to introduction of non-indigenous microbial antagonists.

On the other hand, already available entomopathogens and beneficials, which are known to be effective against related insect pest species may be a source of natural control agents.

Nematodes, predators and parasitic wasps

In recent years, the application of antagonistic macroorganisms like entomopathogenic nematodes (EPN), predators and parasitic wasps gained more and more attention for BC. For *D. suzukii* control, three *Steinernema* species (Rhabditida: Steinernematidae) but also *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) nematodes were tested and showed suppression of larval, pupal and adult SWD development (Woltz et al., 2015; Cuthbertson and Audsley, 2016; Hübner et al., 2017). Common cosmopolitan predators, such as *Orius majusculus*, *Chrysoperla carnea* and *Forficula auricularia* accepted SWD eggs, larvae and pupae as prey, but were not successfully attacking adult flies (Cuthbertson et al., 2014a; Woltz et al., 2015; Englert and Herz, 2019).

Moreover, these species had some difficulties to detect hidden developmental stages inside fruits. For SWD control, pupal parasitic wasps (parasitoids), such as *Pachycrepoideus* spp. (Hymenoptera: Pteromalidae) may promise more success than larval parasitoids, to which

D. suzukii shows tolerance by melanisation of parasitoid eggs inside larval hemolymph (Woltz et al., 2015). The immune response induces an increased hemocyte production in the host larva resulting in successful defence against the wasp egg (Kacsoh and Schlenke, 2012). Especially *Pachycrepoideus vindemiae* (Figure 1-6 a) was found to naturally parasitize SWD pupae but parasitization rates were low (Rossi-Stacconi et al., 2013). In contrast, laboratory trials with *Trichopria drosophilae* (Hymenoptera: Diapriidae) (Figure 1-6 b) were highly promising, encouraging field trials with augmented release (Rossi-Stacconi et al., 2015; Rossi-Stacconi et al., 2019). *Spalangia erythromera* (Figure 1-6 c) were found naturally parasitizing SWD and utilizing SWD pupae as host but offspring rates were lower compared to *T. drosophilae* and *P. vindemia*e (Mazzetto et al., 2016b; Knoll et al., 2017; Collatz et al., 2018).

Figure 1-6: Parasitic wasps with the ability for parasitizing *D. suzukii*.
(a) *Pachycrepoideus vindemiae*, (b) *Trichopria drosophilae*, and (c) *Spalangia erythromera* parasitizing SWD pupae. Images (b) and (c) from Lara Winterwerber/Camilla Englert, Julius Kühn-Institut.

Bacteria

Bacillus thuringiensis

The bacterium *Bacillus thuringiensis* (Firmicutes: Bacillaceae) was once isolated from the lepidopteran storage pest, *Ephestia kuehniella* BERLINER (Lepidoptera: Pyralidae) (Berliner, 1915). Since then, hundreds of new isolates and strains of the ubiquitous occurring *B. thuringiensis* effective against Lepidoptera, Coleoptera and Diptera were found (Norris, 1964; De Barjac and Bonnefoi, 1968). *B. thuringiensis* strains express host specific crystal proteins named δ-endotoxins during sporulation and VIP proteins during vegetative growth, which both are toxic for insect larvae (Bulla et al., 1980; Schnepf et al., 1998; Melo et al., 2016). Since the 1960s, numerous strains have been developed as commercial products (Melo et al., 2016). Studies from Khyami-Horani (2002) showed *Bacillus thuringiensis* serovar. *israelensis*, a strain toxic to mosquitoes, showed some efficacy against *D. melanogaster* larvae. Hence, Cossentine et al. (2016b), Cahenzli et al. (2018) and Babin et al. (2019) tested the biological activity and repellent effect of various *B.t.* strains on

drosophilids, including SWD, showing some inconsistent results on larval and adult mortality and repellence.

Wolbachia

Wolbachia spp. (Alphaproteobacteria: Rickettsiaceae) are endosymbiontic bacteria associated with most arthropods including many insect orders (Werren et al., 1995; Shoemaker et al., 2002). These mainly maternally transmitted bacteria gained scientific interest due to their ability to cause reproductive manipulation of infected hosts to increase *Wolbachia* transmission (O'Neill et al., 1992; Charlat et al., 2003). Four manipulation strategies are known: (i) cytoplasmic incompatibility, where *Wolbachia*-infected females mating with uninfected males (or vice versa, both is incompatible) leads to increased mortality of progeny (Turelli and Hoffmann, 1995), (ii) male killing by infected females produce female-biased progeny (Hurst and Jiggins, 2000), (iii) feminization of genetic males (Rousset et al., 1992), and (iv) parthenogenesis induction by asexual production of progeny of only one sex (Rousset et al., 1992). In *D. suzukii*, a *Wolbachia* strain was discovered named *w*Suz showing widespread occurrence of 7-20% in US American populations and up to 46% in European populations (Hamm et al., 2014; Cattel et al., 2016a; Fountain et al., 2018). Whereas Mazzetto et al. (2015) recorded a fecundity reduction of 30-50% when females were infected, other studies showed that *w*Suz had no overall progeny-reducing effect but instead induced increased fitness by antiviral intracellular activities (Cattel et al., 2016b; Rainey et al., 2016).

Viruses

Entomopathogenic viruses are widely used in BC. The most successful example is the use of baculoviruses with more than 600 representatives isolated from Lepidoptera, Diptera, and Hymenoptera (Martignoni and Iwai, 1981). These double stranded DNA viruses were initially isolated from caterpillars of lepidopteran insect pests and have three main biological characteristics making them highly attractive for insect control: (i) narrow host range, (ii) biological and environmental safety, (iii) high virulence to hosts and fast-killing mode (Fuxa, 1991; Eberle et al., 2012a). Today, they are globally used for control of diverse range of insect pests (Maramorosch and Sherman, 1985; Lacey et al., 2015). After identification of DNA viruses naturally occurring in *D. melanogaster*, the viriom of *D. suzukii* was recently published showing some interesting DNA viruses associated with the invasive fly (Unckless, 2011; Medd et al., 2017). Especially Drosophila innublia nudivirus (DiNV, *Nudiviridae*) and Kallithea virus (*Nudiviridae*) caught the attention for further *in vivo* bioassays (Palmer et al.,

2018). Moreover, RNA viruses such as Drosophila A virus (DAV, *Permutoretroviridae*), Drosophila C virus (DCV, *Sigmaviridae*) and LaJolla virus (LJV, *Iflaviridiae*) were identified from SWD and tested on fitness-reducing effects showing a significant decline in survival of intrathoracically injected flies (Lee and Vilcinskas, 2017; Carrau et al., 2018).

Fungi

Entomopathogenic fungi (EPF) of the genus *Beauveria*, *Metarhizium* and *Isaria* have been intensively studied for their potential as BCA (Lacey et al., 2015). Today they are used to control soil-borne caterpillars but also locusts, aphids, scarab beetles, and a wide range of lepidopteran pests (Shah and Pell, 2003). Having different modes of action, fungal spores generally attach to the host´s cuticle, germinate and grow into the insect body and circulatory system (hemocoel and tracheae); infection eventually results in death by starvation or by toxic fungal metabolites (Roberts, 1981; Shah and Pell, 2003).

Several studies evaluating EPF for SWD control showed highly varying results on efficacy: in addition to *Beauveria bassiana* and *Metarhizium anisopliae* with 30-60% mortality on pupa and adults, also *Isaria fumosorosea* was shown to cause 30-85% mortality (Cuthbertson et al., 2014b; Naranjo-Lazaro et al., 2014; Woltz et al., 2015; Cossentine et al., 2016a; Cuthbertson and Audsley, 2016). Authors stated that results need to be confirmed in field trials but it seems that the sole application of EPF is not efficient enough, whereas rotation programs with insecticides and/or nematodes could be a possible way for SWD population reduction (Cuthbertson et al., 2014b; Naranjo-Lazaro et al., 2014; Cuthbertson and Audsley, 2016; Rhodes et al., 2018).

Microsporidia

Historical review

Microsporidia are single-celled, obligate intracellular eukaryotic parasites. The Microsporidia phylum is nowadays classified as Fungi, despite a completely divergent life cycle (Corradi and Keeling, 2009; Capella-Gutiérrez et al., 2012; Keeling, 2014; Xiang et al., 2014). The diverse phylum consists of about 200 genera with more than 1300 species (Sprague and Becnel, 1999; Becnel et al., 2014; Wadi and Reinke, 2020). Microsporidia can be found in invertebrates and vertebrates and some species are known to be harmful to economically and agricultural important hosts (Sprague, 1977b; Sprague and Becnel, 1999). In 1857, the first microsporidium caused disease was discovered by Nägeli in silkworm (*Bombyx mori* LINNAEUS*,* Lepidoptera: Bombycidiae), named “Pebrine disease” (Nägeli, 1857). In the early

19th century, French silk production was affected by this epidemic to such extent that European silk production almost collapsed (reviewed by Franzen, 2008). Louis Pasteur discovered the protozoan parasite as causative agent of the disease which was later determined as *Nosema bombycis* by Balbiani (1882) leading to black spotted larvae unable to produce a silk cocoon. Aside from that disease, the honeybee-infecting *Nosema apis* ZANDER (1911) was the second microsporidian species catching attention as pollination and honey production was heavily affected (Zander, 1911; Kudo, 1920). In the 1960s, another economically important microsporidian, *Loma salmonae*, was discovered causing the "Microsporidia Gill Disease of Salmon" with high mortality rates in salmon hatcheries (Putz et al., 1965; Kent and Speare, 2005).

Microsporidia from agriculturally important hosts

There are numerous examples of microsporidia discovered from agricultural pest insects. *Nosema pyrausta*, isolated from the European corn borer, *Ostrinia nubilalis* HÜBNER (Lepidoptera: Crambidae), was shown to have population-regulating effects on the pest which has caused severe damage in the US American corn production since early 20th century (Lewis et al., 2006; Lewis et al., 2009; Zimmermann et al., 2016).

After identification of a new microsporidium, *Nosema* (=*Paranosema*, =*Antonospora*) *locustae*, from locusts and grasshoppers in the 1950s, *Locusta migratoria* LINNAEUS (Orthoptera: Acrididae) have been actively sprayed with the pathogen in Africa during the 1970-80s when locust swarms infested farmland leading to massive crop losses (Canning, 1953; Henry and Oma, 1981; Lockwood et al., 1999).

Furthermore, the Eurasian gypsy moth, *Lymantria dispar* LINNAEUS (Lepidoptera: Noctuidae), is a major concern in natural forest ecosystems and forestry in North America and Canada as outbreaks cause severe defoliation (Bjørnson and Oi, 2014). In 1986, the introduction of *Nosema* spp. and *Vavraia* sp. in the population resulted in recovery of natural forests but nowadays also Lymantria dispar nucleopolyhedrovirus and *Bacillus thuringiensis* are applied for BC (Weiser and Novotný, 1987; Jeffords et al., 1989; Maddox et al., 1999; Bjørnson and Oi, 2014).

Classification and taxonomy

Several attempts for classification and coherent taxonomy of microsporidia have been made using characteristics of morphology of developmental stages, ultrastructure, chromosome number, and later genetic markers (Kudo, 1924; Sprague, 1977a; Larsson, 1986; Sprague et

al., 1992; Vossbrinck et al., 1993, 2014). Also, polar filament arrangement, coil turns and fine structure are widely used characterization criteria for discrimination of microsporidia on genus and species level (Jensen and Wellings, 1970; Burges et al., 1974; Vávra, 1976; Vávra and Larsson, 2014). Recently, a new classification system based on correlation of host habitat and taxonomic grouping and phylogeny was established, which is still updated due to the isolation of new species and inclusion of more molecular data (Vossbrinck and Debrunner-Vossbrinck, 2005; Vossbrinck et al., 2014). This system classifies microsporidia into five clades (Figure 1-7): (i) clade I and III comprise microsporidia with aquatic freshwater hosts (Aquasporidia); clade III includes some exceptional species rapidly switching the host and habitat. (ii) The clades II and IV include the Terresporidia from terrestrial hosts like insect and mammals, whereby many insect species switch their habitat from freshwater to terrestrial. (iii) Clade V are Marinosporidia which are found in hosts with marine origin, e. g. fish and crustaceans, although habitat switching also occurs for several species like salmon (Vossbrinck and Debrunner-Vossbrinck, 2005; Vossbrinck et al., 2014). The probably most studied arthropod-infecting group of microsporidia is the *Nosema-Vairimorpha* clade belonging to the clade IV Terresporidia that consists of more than 150 species infecting at least 12 insect orders (Becnel and Andreadis, 2014; Becnel et al., 2014; Tokarev et al., 2020). During the last decades, many of these taxa were undergoing several genus reassignments depending on different underlying characterisation methods (Tokarev et al., 2020). The outstanding morphological characteristics of the genus *Nosema* are diplokaryotic or binucleate developmental stages, a direct host cell interaction of meronts and/or sporonts and the disporoblastic sporogony. Commonly, two types of spores are formed: a binucleate, thin-walled premature spore and a binucleate mature spore with thick-walled endospore (for spore ultrastructure see Figure 1-8 a). For some microsporidia, once determined as *Nosema kingi*, *Nosema acridophagus* and *Nosema maroccanus* due to congruent morphological characteristics, some additional features, small microtubules on the membrane surface of merogonial stages being in direct contact with host cell cytoplasm, were shown (Kramer, 1964; Krilova and Nurzhanov, 1987; Streett and Henry, 1993; Franzen et al., 2006; Issi et al., 2008). These new ultrastructural details as well as their phylogenetic close relatedness motivated Franzen et al. (2005b) to establish a new family, Tubulinosematidae, which nowadays consists of 10 species found in Hymenoptera, Lepidoptera, Orthoptera and Diptera. Two *Tubulinosema* species (*Tubulinosema ratisbonensis*, *Tubulinosema kingi*) infect drosophilids (Kramer 1964; Franzen et al., 2005b, 2006). They cause profound detrimental effects on fitness, survival and longevity which are important population growth parameters (Armstrong, 1976; Taylor, 1980; Armstrong and Bass, 1989; Franzen et al., 2005b). Considering the wide spread of microsporidia in all insect orders as well as in closely related drosophilid species, their occurrence in *D. suzukii* cannot be excluded.

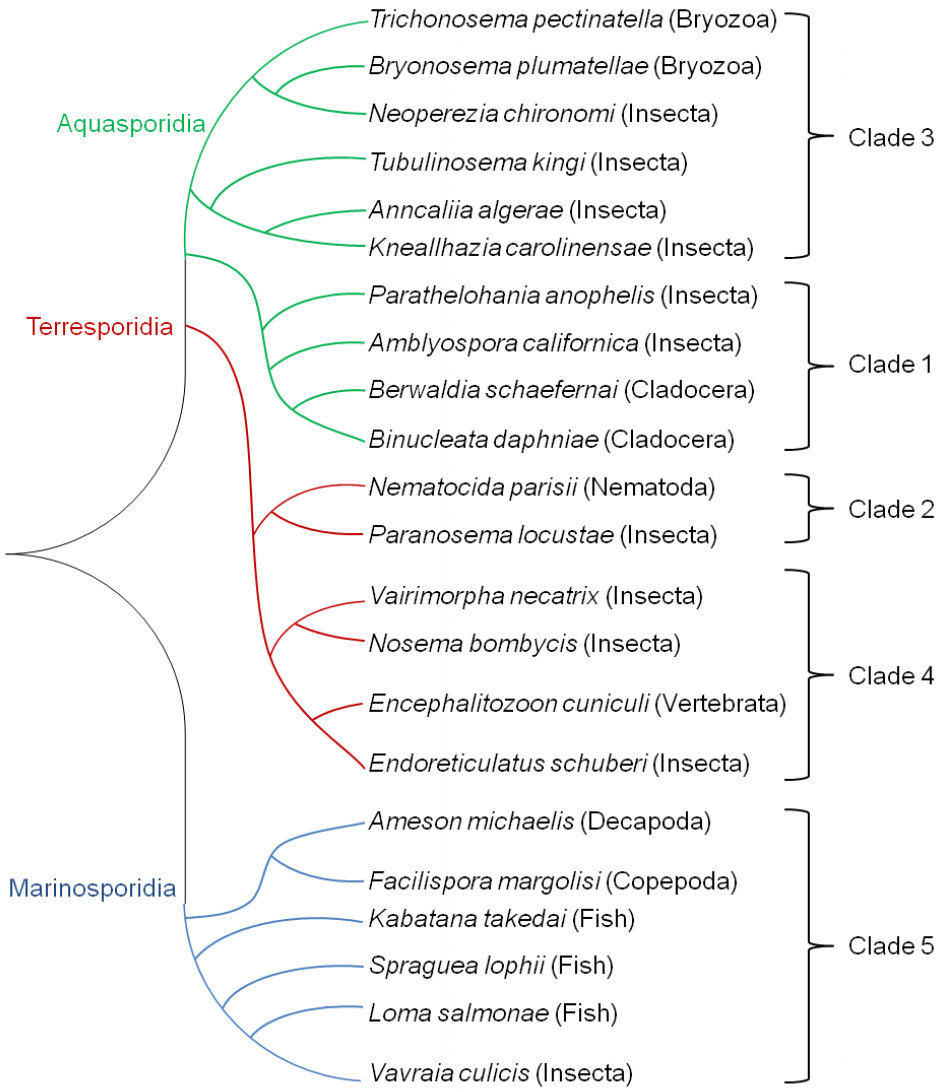

Figure 1-7: Microsporidia clades indicating the environmental habitats of hosts (origin).
Shortened most parsimonious tree of 22 microsporidia representatives with assignment to five clades, host, and host origin (Aqua-, Terre-, Marinosporidia), modified from Vossbrinck and Debrunner-Vossbrinck (2005) and Vossbrinck et al. (2014).

Mode of action: life cycle

Most microsporidia cause slow-acting, chronic infections with progressive seriousness in hosts, whereas acute and fast-acting infections are rarely described (Becnel and Andreadis, 2014). Spores (Figure 1-8 a-c) are the environmental resistant developmental stages which are ingested orally by hosts, transmitted horizontally via cadaver or contaminated faeces, and/or vertically via gonads, eggs or sperms (Becnel and Andreadis, 2014).

Intracellular developmental stages are meronts, sporonts and sporoblasts. The infection route of microsporidia is schematically illustrated in Figure 1-9, whereby three different

phases can be distinguished: infective or environmental phase (Figure 1-9 a, b), the merogonial or proliferative phase where meronts and sporonts replicate and divide (Figure 1-9 c, d) and the sporogonic or spore-forming phase (Figure 1-9 e) (Cali and Takvorian, 2014).

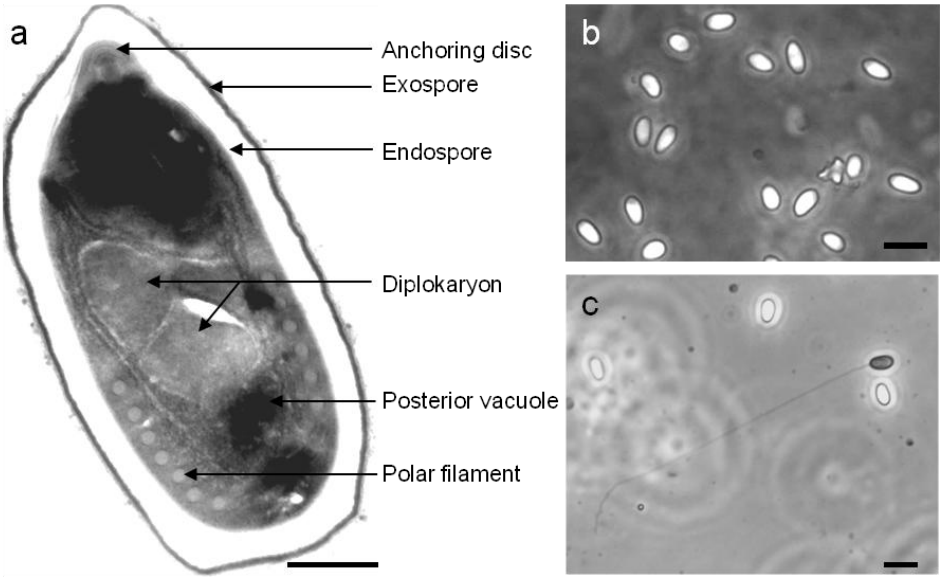

Figure 1-8: Microsporidian spores in electron and light microscopy.
(a) Electron micrograph of the ultrathin section of a microsporidian spore showing the internal structure. Scale bar=500 nm. (b) Refractive *Tubulinosema* sp. spores in phase contrast microscopy. (c) *Tubulinosema* sp. spore with extruded polar filament. Scale bars (b, c)=5 µm.

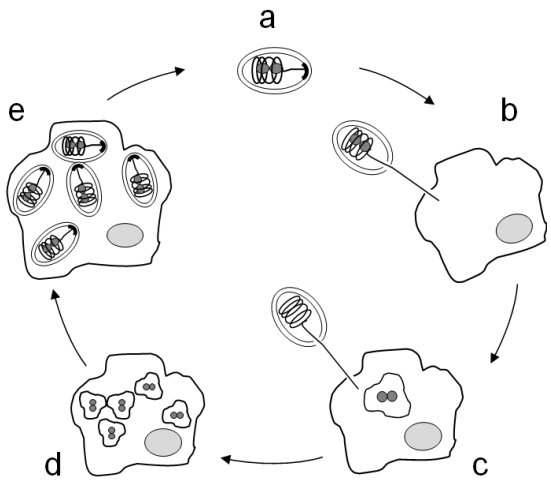

Figure 1-9: Generalized microsporidian life cycle.
(a) Infective spore in the environment is orally ingested by host. (b) Spore polar filament extrusion and penetration of the host cell. (c) Spore releases the infective sporoplasm to the host cell. (d) Multiple replication cycles generate new developmental stages, which are variable in size and shape for different microsporidia. (e) New spores are produced inside host cell and afterwards released to the environment. Illustration was adapted from Keeling and McFadden (1998).

AIM OF THIS THESIS

When *D. suzukii* was firstly observed in Germany in 2011, the need of efficient control tools to reduce economic losses of growers as shown for other European and American invaded regions became urgent. Especially for the organic growers, the evaluation of already available BCA with effective range including Diptera seemed to be the first option to test. As *Bacillus thuringiensis* serovar. *israelensis* products with efficacy towards dipteran species like mosquitoes and blackflies were already registered, it was obvious to primarily test the active ingredients of three EU-available *B.t.i*. products Solbac (Andermatt Biocontrol, Switzerland), Stechmückenfrei (formerly: Neudomück) (Neudorff, Germany), and BioMükk WDG (BIOFA AG, Germany) (**Chapter II**).

Additionally, the plant extract of the Neem tree is widely used for control of several arthropod pests, such as thysanopterans, spider mite and whitefly in ornamental plants, fruit crops and horticulture. The moulting-inhibitor effect of the product Naturen Bio Schädlingsfrei Neem (Scotts Celaflor GmbH, Germany) by direct larval application but also a potential repellent effect on adult SWD by using choice tests were examined and discussed in **Chapter III**.

If a new pathogen is needed for pest control, wild insects are typically collected via regional or global monitoring, whereby insect pathologist are investigating some of these samples for natural occurrence of entomopathogenic bacteria, viruses, fungi, and microsporidia. Due to the global occurrence of *D. suzukii*, wild flies are collected all over the world, in countries where the fly is endemic and invaded. Flies from a laboratory colony derived from wild caught flies sampled in Oregon (USA) were sent to the Julius Kühn-Institut for pathogen diagnosis, where some flies were positively screened for microsporidia infection. In **Chapter IV**, the presumably unknown microsporidium was determined by using ultrastructural, genetic and pathological investigations. Thus, a new species *Tubulinosema suzukii* and the first microsporidium appearing in SWD was identified and characterized.

Concerning the ability of *T. suzukii* to weaken the host, a comprehensive study on the biological effects longevity, fecundity, fertility of progeny, vertical transmission, spore concentration-depending survival and infection progress were analysed (**Chapter V**). Furthermore, potential side effects on non-host species *Drosophila melanogaster* and *Drosophila willistoni* were determined.

The final **Chapter VI** will discuss different possibilities for BC of SWD with microbial antagonists with regard to the current research status and future strategies, including other potential microbial antagonists and plant extracts.

CHAPTER II: *BACILLUS THURINGIENSIS* SEROVAR. *ISRAELENSIS* HAS NO EFFECT ON *DROSOPHILA SUZUKII* MATSUMURA

This chapter has been published with few modifications in:

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ABSTRACT

The spotted wing drosophila (SWD), *Drosophila suzukii*, is an invasive species to the USA, and Europe and biological control methods are urgently sought for. In this study, the potential of commercial microbial control products based on the dipteran-specific *Bacillus thuringiensis* serovar. *israelensis* (*B.t.i.*) were evaluated in laboratory experiments. These products were tested on SWD larvae and adults but neither one showed more than 10% mortality. A repellent effect of the products to SWD adults was also ruled out. It can be concluded that *B.t.i*. products are not suitable for SWD control.

INTRODUCTION

Drosophila suzukii Matsumura is endemic in East Asia and has been introduced to the USA and Europe nearly 10 years ago (Hauser, 2011; Walsh et al., 2011; Cini et al., 2012). In Germany, it has become to one of the most harmful invasive pests to commercially grown soft-skinned fruits, such as stone fruits and nearly all kind of berries, while it prefers ripe and overripe fruits (Mitsui et al., 2006; Briem et al., 2015). SWD can be controlled by pyrethroids, spinosyns and organophosphates (Bruck et al., 2011), but efficient biological control agents are not yet available (Cini et al., 2012). However, for reasons of environmental protection, safety of beneficial arthropods and the consumer demand for organic products, biological control strategies of SWD are imminent. The gram-positive bacterium *Bacillus thuringiensis* serovar. *israelensis* (*B.t.i.*) is highly specific for mosquitoes and black flies (Nematocera, Diptera) and some studies have discussed its *in vivo* toxicity to *D. melanogaster* and other fruit fly species (Martinez et al., 1997; Toledo et al., 1999; Khyami-Horani, 2002). Therefore, an efficacy against SWD cannot be excluded. Cossentine et al. (2016b) detected different *B. thuringiensis* strains as effective against SWD with over 70% larval mortality for both, whereas *B.t.i.* had marginal pathogenic effect causing less than 10% mortality. However, recent observations suggested an efficacy of commercial *B.t.i.* products in the field (Lambion and Klink, 2014). To test the effect of *B.t.i.* on *D. suzukii* under controlled laboratory conditions, three commercially available *B.t.i.* products with different formulations were

applied to *D. suzukii* larvae and adults and their mortality rates were examined. In addition, the olfactory effect on the oviposition frequency of the formulation and the active ingredients of *B.t.i.* products was tested.

MATERIAL AND METHODS

Insect rearing

Drosophila suzukii

Adult *D. suzukii* were kept in rearing containers with 25 °C, 60–70% relative humidity (r.H.) and a 16/8 hours light/dark photoperiod. To ensure a synchronized age of larvae and adults for bioassays, rearing cups with modified *Drosophila* oviposition medium (DOM) (Chabert et al., 2012) were placed into the rearing containers for 24 hours for oviposition. DOM was modified by using apple puree (400 g), apple juice (450 ml) and apple vinegar (20 ml) instead of bananas. Second instar (L2) larvae were either used for the larval bioassays or further reared until imago for the adult bioassays in an acclimated room with conditions described above. For adult rearing, a diet containing a mixture of sugar and brewer's yeast (each 1 g) and a water source was added.

Culex spp.

Mosquito larvae (*Culex pipiens* and/or *Culex torrentium*) were obtained from KABS e.V. Hessen (Germany) and reared in 25 cm diameter Petri dishes filled with tap water. Larvae could feed on aquatic dry food "TetraMin XL flakes" (Tetra GmbH, Germany) during rearing.

Bacillus thuringiensis serovar. *israelensis* (*B.t.i.*) products

Three *B.t.i*. products were used in the bioassays: BioMükk WDG (BIOFA AG, Germany) serotype H14 (37.4% active ingredient (a.i.))=Bio, Stechmückenfrei (formerly: Neudomück) (Neudorff, Germany) serotype H14 strain SA3A (1.6% a.i.)=Neu, Solbac (Andermatt Biocontrol, Switzerland), serotype or strain not specified (1.2% a.i.)=Sol. The recommended concentrations (RC) were 0.3% (BioMükk), 1.5×10^{-6} % (Stechmückenfrei) and 0.5% (Solbac), respectively. These as well as two, ten and hundred times higher the concentrations as recommended were used in the laboratory trials.

Larval bioassay

Larval bioassays were performed in 96-well microtiter plates (Greiner, Germany). Each cavity was filled with 200 µl DOM (see above) without Agar-Agar. Additionally, 20 µl of the *B.t.i.* suspension were filled in each cavity and mixed with the medium. One SWD L2 larva was placed into each cavity. For negative controls, medium was mixed with sterile tap water. The microtiter plates were closed with three layers of filter paper to ensure gas exchange and a lid. Larval mortality (6-day post-inoculation, dpi) as well as development of pupae (12 dpi) and adults (14–20 dpi) were recorded. The tests were repeated four to eight times. As a positive control for *B.t.i* performance, assays with L1 larvae of *Culex* spp. were included. Therefore, cups (6 cm diameter) were filled with 50 ml sterile table water either containing one of the three *B.t.i.* products in recommended concentrations, double recommended concentrations or just sterile table water for controls. Fifteen cups with each three L1 larvae were prepared per product.

Adult bioassay with (1) *B.t.i.* products and (2) empty formulation

(1) A total of 150 µl of the 10×RC *B.t.i.* product was filled into a bisected tubule of a cotton bud, which was placed in a plastic box (10×6 cm) containing 1 g of DOM. Five male and five female SWD (age: 14 days from start of development, freshly hatched) were placed in the plastic box for 7 days. In negative controls, the cotton bud was filled with sterile tap water. The cotton bud was refilled each day of the experiment with the treatment or control suspension. Mortality rates were recorded after 7 dpi. Three replicates with 60 adults each were conducted for treatment and control.

(2) To separate the formulation from *B.t.i.* spores and crystals, 10×RC of each product was centrifuged at 12,000 rpm (2,210 × *g*) for 5 min (Avanti J-HC, Beckman Coulter, rotor JA-14, Germany). A volume of 150 µl of the supernatant was directly transferred into the cotton bud, and the treatment was the same as mentioned above.

Arena choice test with (1) *B.t.i.* products, (2) *B.t.i.* bacteria and (3) empty formulation

(1) Two blueberry fruits were offered to five male and five female adult SWD (age: 21 days from start of development, 8-day-old adults) in one cage (R(Bio)=15, R(Neu)=19, R(Sol)=13). One fruit was dipped into *B.t.i.* suspension of the 100-fold concentration than recommended for mosquitoes (100×RC), and the other fruit was dipped in sterile table water as control (C). Both fruits were placed in a cylindrical arena of 35 cm height × 20 cm in diameter, containing an additional water source. Both, berries and flies were kept in the arena for 24 hours at

25 °C, 60% r.H. and 16/8 hours light/dark photoperiod. After 24 hours, each berry was placed in a separate plastic box and kept under same conditions for 2 weeks to observe how many adults emerged from the berries. Flies from the arena were removed after each choice test, and the cage was cleaned with 70% ethanol to remove bacteria and odours that could affect following experiments.

(2) Smears of 100 µl of 1:10 dilution of *B.t.i.* products were incubated at 25 °C for 48 hours on TSA plates containing 30 g/l Tryptic Soy Broth (BD, Germany) 15 g/l Agar-Agar (Roth, Germany) and 0.05 g/l Streptomycin (Sigma-Aldrich, Germany). One colony was picked and transferred into liquid 3% Tryptic Soy Broth medium containing 0.15 mM manganese (II) sulphate (Roth, Germany) for sporulation. The medium was incubated at 25 °C for 24 hours at 180 rpm (GFL Shaker 3017, Germany). The bacterial cell number was counted with a Thoma hemocytometer using phase contrast light microscopy (Leica DMRB, Germany). *B.t.i.* suspensions were prepared containing 10^8 cells/ml in sterile distilled water. Each treatment fruit was dipped into the *B.t.i.* suspension, and each control fruit was dipped into sterile distilled water (R(Bio)=7, R(Neu)=8, R(Sol)=9).

(3) To separate formulation from *B.t.i.* spores and crystals, the *B.t.i.* products with 100×RC was centrifuged, and the supernatant was directly applied on the fruits as mentioned in the choice tests above (R(Bio)=9, R(Neu)=8, R(Sol)=9).

Statistics

Data are shown as mean ± standard error (SE) if not indicated otherwise. Mortality data were corrected according to Abbott (1925). The number of replicates is indicated with R, and the number of tested individuals is indicated with N. All statistical analyses were performed with R version 3.2.2 (2015-05-11) (R Core Team, 2015; RStudio Team, 2015). Variance analysis was applied for multiple comparisons of the observed mortality of larvae and adults treated with three different *B.t.i.* products and three concentrations. Kruskal–Wallis ANOVA and One-way ANOVA, respectively, were applied according to normal distribution followed by Tukey HSD post hoc test to determine significances among the different samples if needed. Significant differences in the preference for oviposition on *B.t.i.* treated and untreated blueberry fruits were determined with Wilcoxon signed rank test. A level of significance α=0.05 was applied if not indicated otherwise.

RESULTS

Positive controls of L1 larvae (N=45) of *Culex* spp. showed 100% mortality for all products and concentrations within 3 hours, whereas all negative controls (N=45) treated with water survived. Table 2-1 shows the Abbott corrected mortality of SWD L2 larvae on day 6 post-inoculation; the mortality was below 1.5% when treated with two concentrations of three *B.t.i.* products. However, there was no significant difference between negative control and treatments and among treatments (Kruskal–Wallis ANOVA: Chi^2=8.569, *DF*=6, *P*>0.05). At 12 dpi, mortality of pupae resulting from the surviving larvae after 6 dpi was similarly low (Table 2-1); no significant difference between the treatment mortality and control mortality nor among the treatments was observed (One-way ANOVA: Adjusted R^2=−0.121, *F(*6,38)=0.209, *P*>0.05). SWD adults treated with ten times higher concentrations did not significantly differ in their mortality rates compared to the control, although they showed up to 20% mortality when treated with empty formulations (Kruskal–Wallis ANOVA: Chi^2=6.33, *DF*=3, *P*>0.05) (Table 2-2). The arena choice tests with one untreated control fruit and one fruit dipped into either *B.t.i.* product (Wilcoxon test: Neu: *W*=190.5, *P*>0.05; Bio: *W*=128, *P*>0.05; Sol: *W*=77, *P*>0.05), or *B.t.i.* suspension (Wilcoxon test: Neu: *W*=30.5, *P*>0.05; Bio: *W*=19, *P*>0.05; Sol: *W*=41.5, *P*>0.05), or empty formulation (Wilcoxon test: Neu: *W*=23.5, *P*>0.05; Bio: *W*=36.5, *P*>0.05; Sol: *W*=33, *P*>0.05) did not show any significant preference of *D. suzukii* adults to any fruit (Table 2-3). Thus, none of these treatments was preferred by *D. suzukii* for oviposition.

Table 2-1: Mortality in larval bioassays 6 dpi and pupal bioassays 12 dpi.
Mortality was recorded after application of commercial *B.t.i.* products at recommended (RC) and double recommended (2×RC) concentration.

Product	Concentration	R/N	Larval mortality [%][a]	± SE	Pupal mortality [%][a]	± SE
Bio	RC	8/96	1.05	0.37	0.54	2.28
Neu	RC	7/96	-0.04	0.18	1.36	3.24
Sol	RC	8/96	0.92	0.34	2.52	1.65
Bio	2×RC	5/96	0.68	0.60	-2.63	3.46
Neu	2×RC	4/96	1.57	0.92	-0.54	4.90
Sol	2×RC	5/96	1.31	0.71	6.31	3.49

Table 2-2: Mortality in adult bioassays after 7 dpi.

Mortality was recorded after application of commercial *B.t.i.* products and their empty formulations both at 10-times recommended (10×RC) concentration.

Product	Concentration	R/N	Adult mortality [%][a] with *B.t.i.* product	± SE	Adult mortality [%][a] with empty formulation	± SE
Bio	10×RC	3/60	-3.57	0.55	23.91	0.70
Neu	10×RC	3/60	-1.43	0.34	4.35	0.54
Sol	10×RC	3/60	-5.00	0.44	11.96	0.55

Table 2-3: Mean number of larvae per fruit in an arena choice test.

Two blueberries either treated with water as control or with *B.t.i.* products or empty formulations with 100-times recommended concentration (100×RC) or *B.t.i.* suspension (10^8 cells/ml) were presented to ten flies for one day. T=*B.t.i.* treated blueberry; C=control blueberry.

	B.t.i. product			*B.t.i.* suspension			*B.t.i.* empty formulation		
Product	**R**	**Mean number of larvae fruit^{-1}**	**± SE**	**R**	**Mean number of larvae fruit^{-1}**	**± SE**	**R**	**Mean number of larvae fruit^{-1}**	**± SE**
Bio (T)	15	3.33	1.43	7	3.00	1.31	9	3.38	1.08
Bio (C)		2.80	0.94		1.57	0.97		4.50	1.48
Neu (T)	19	2.26	0.72	8	1.75	0.77	8	3.33	1.31
Neu (C)		2.31	0.78		3.13	2.07		0.89	0.51
Sol (T)	13	4.15	1.48	9	4.22	2.29	9	7.56	2.32
Sol (C)		4.08	2.05		3.78	1.79		5.00	1.28

[a] Corrected according to Abbott (1925).

N, total number of tested individuals; R, number of replicates; SE, standard error.

DISCUSSION

To clarify the potential effect of commercial *B.t.i.* products on larvae and adults of *D. suzukii*, laboratory experiments on artificial *Drosophila* medium and blueberry fruits were conducted. There was hardly any effect of the three tested *B.t.i.* products on larval and adult mortality of SWD. Moreover, an alternative hypothesis that *D. suzukii* flies may be able to distinguish between *B.t.i.* treated and untreated fruits, was examined with arena choice tests. Recent studies verified the olfactory sensors of SWD adults and their preference for ripening fruits (Keesey et al., 2015) and their avoidance of aversive odours (Wallingford et al., 2015). On the other hand, Mazzetto et al. (2016a) showed a preference of SWD flies for special volatile metabolic products of acetic acid bacteria, like butyric acid, on artificial diet. In the present studies, neither avoidance nor preference for *B.t.i.* treated fruits was observed in choice tests suggesting that the volatiles produced by *B.t.i.* or *B.t.i* products are not recognizable for adult *D. suzukii*. Hence, it is unlikely that SWD flies would recognize and avoid *B.t.i.* sprayed fruits which could explain any effects observed in the field by Lambion and Klink (2014). In addition, *B.t.i.* has a strong selectivity for mosquitoes of the family Culicidae and black flies (Tyrell et al., 1979; Mulla, 1990; De Barjac and Sutherland, 1990). In conclusion, our laboratory experiments did not show any evidence that these products have any direct or indirect effect on larvae and adults of SWD. Hence, *B.t.i.* is not a candidate for biological control of SWD.

CHAPTER III: NEEM OIL INHIBITS LARVAL AND PUPAL DEVELOPMENT OF *DROSOPHILA SUZUKII* IN FEEDING TRIALS UNDER LABORATORY CONDITIONS

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ABSTRACT

Neem oil is widely used for biological control of nearly all kind of leaf mining and sucking pest insects. Its effects, repressing reproduction and inhibiting moulting, could be advantageous for control of *Drosophila suzukii* if applicable. In larval bioassay with a commercially available Neem product, mortality of up to 100% could be achieved when applying a ten times higher concentration than recommended for target insects.

INTRODUCTION

The spotted wing drosophila (SWD), *Drosophila suzukii* Matsumura, is native in South East Asia and Japan but has spread to Europe and North America during the last decade. Today it can be found in many regions in Europe where it causes severe damages to commercially grown crop plants like stone fruits, berries and grapes. Because the infestation occurs on ripening fruits just before harvest, the application of chemical insecticides is difficult for residue reasons. Therefore, effective biological control strategies would be highly desirable. The oil extract from seeds of the Neem tree (*Azadirachta indica*) is widely used as biological control agent against leaf-mining, sucking and biting pest insects and arthropods like blackflies, thrips (Thysanoptera), spider mites and white flies (Schmutterer, 1990). The secondary metabolite Azadirachtin has an insecticidal effect as it inhibits moulting (ecdysis) of insects. In this study "Naturen Bio Schädlingsfrei Neem" (Scotts Celaflor GmbH, Germany) with active ingredient Azadirachtin A was tested against SWD larvae. In laboratory bioassays, the Neem oil product caused up to 98% mortality of SWD larvae and pupae until 12 dpi, however with an applied concentration that was 10-times higher than recommended for black flies and other target pest insects.

MATERIALS AND METHODS

Insect rearing

Adult SWD were kept in rearing containers with 25 °C, 60-70% r.H. and a 16/8 hours light/dark photoperiod. To ensure a synchronized age of larvae and adults for bioassays, rearing cups with modified *Drosophila* oviposition medium (DOM) (Chabert et al., 2012) were placed into the rearing containers for 24 hours for oviposition. DOM was modified by using apple puree (400 g), apple juice (450 ml) and apple vinegar (20 ml) instead of bananas. Second instar larvae (L2) were either used for the larval bioassays or further reared until imago for the adult bioassays in an acclimated room with conditions described above. For adult rearing, a diet containing a mixture of sugar and brewer´s yeast (each 1 g) and a water source was added.

Larval bioassay

A single *D. suzukii* L2 larva was placed per cavity of a 96-well microtiter plate containing *Drosophila* apple medium and either Neem oil ("Naturen Bio Schädlingsfrei Neem", Scotts Celaflor GmbH, Germany; active ingredient: Azadirachtin, concentration=10.6 g/l) with the recommended concentration (RC) for mosquitoes or a 10-times higher concentration. Plates were closed with filter paper and a lid and kept for two weeks under standard *Drosophila* breeding conditions (16/8 hours light/dark photoperiod, 60% r.H., 25 °C). Dead larvae were recorded after 6 dpi and non-hatched pupae on 12 dpi. Three replicates (=three microtiter plates) per concentration were conducted containing each 96 larvae per plate.

Choice test

One blueberry treated with 10-times recommended concentration of the Neem oil product and one treated with water as control were offered to five *D. suzukii* males and females each for 24 hours in an arena. Afterwards, the blueberries were laid into 3% saline solution to wash out and count the larvae inside the fruit. Additionally, some fruits were kept separately for 12 days to record successful development of pupae. Eleven replicates were conducted.

All statistical analyses were performed with R version 3.2.2 (R Core Team, 2015; RStudio Team, 2015).

RESULTS AND DISCUSSION

Neem oil achieved only 2% mortality of *D. suzukii* larvae by application of a concentration recommended for target insects and up to 20% of pupae within 12 dpi. A 10-times higher concentration of Neem oil led to 98% mortality of SWD larvae within 6 days (Figure 3-1). For the choice test neither the number of larvae per fruit, nor the number of pupae per fruit differed significantly from the *D. suzukii* infestation rates of control blueberries (Figure 3-2). Hence, SWD did neither avoid nor prefer any of the different treatments. The application of the commercially available Neem oil product "Naturen Bio Schädlingsfrei Neem" with concentrations recommended for most of the common target insects was not effective against *D. suzukii*, whereas a 10-times higher concentration led to up to 100% mortality in early larval stages. As the L2 larvae could directly feed on the Neem oil mixed with artificial diet, further analyses of effectiveness in field trials are needed. Possibly, direct application of high doses on fruits is needed for that an effective dose affects the larvae inside the fruit. This may entail very high costs and environmentally adverse effects. Other studies confirmed the achieved effect of Neem oil against *D. suzukii*, although lower doses where applied with other products than for this study (Pavlova et al., 2017). An avoidance of treated fruits could not be determined in arena choice tests, so that Neem oil is most likely not inhibiting the fruit attractiveness which would favour spray application as for other target insects. However, a repellence effect can therefore also be excluded.

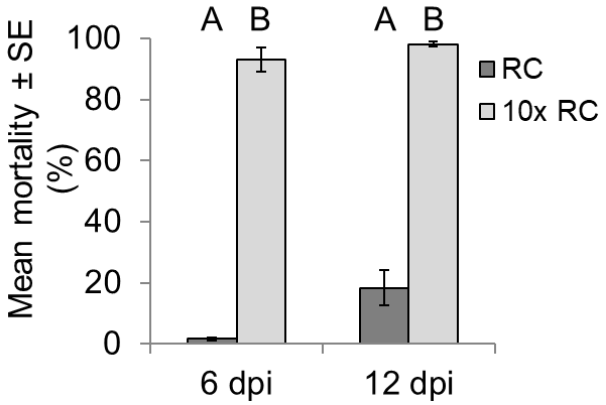


Figure 3-1: Efficacy of Neem oil exposed to SWD larvae and pupae.
Mean mortality ± standard error (SE) [%] of SWD L2 larvae (6 dpi) and pupae (12 dpi) treated with Neem oil with recommended concentration (RC) for target insects and ten times (10×RC) higher than recommended. Significant differences are indicated by different letters (6 dpi: Kruskal-Wallis ANOVA: Chi^2=8.757, *DF*=2, *P*=0.013, R/N=3/288; 12 dpi: Chi^2=7.560, *DF*=2, *P*=0.023, R/N=3/288).

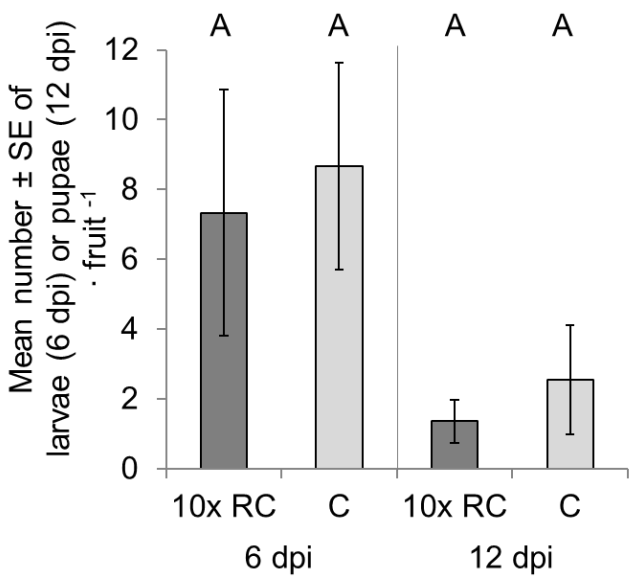


Figure 3-2: Choice test for adult SWD with Neem oil-treated blueberries.

Mean number ± standard error (SE) of SWD larvae (6 dpi) and pupae (12 dpi) per fruit either treated with Neem oil in 10-times higher concentration than recommended for target insects (10×RC) or water as control (C). Significant differences are indicated by different letters (6 dpi: Wilcoxon test *W*=5, *P*=1, N=3; 12 dpi: *W*=57, *P*=0.824, N=11).

CHAPTER IV: MOLECULAR AND MORPHOLOGICAL CHARACTERISATION OF A NOVEL MICROSPORIDIAN SPECIES, *TUBULINOSEMA SUZUKII*, INFECTING *DROSOPHILA SUZUKII* (DIPTERA: DROSOPHILIDAE)

ABSTRACT

In 2015, a microsporidial infection was discovered in a *Drosophila suzukii* laboratory colony. The microsporidium showed morphological characteristics typical of a *Tubulinosema* species. All developmental stages were diplokaryotic and grew in direct contact with the host cell cytoplasm. Spores from fresh preparations were ovoid to slightly pyriform and measured 4.29×2.47 µm in wet mount preparations. The spore wall consisted of a 125 nm thick endospore covered by a 39 nm exospore with an additional 18 nm exospore layer. The polar filament measured 67 µm in length, was slightly anisofilar and was arranged in ten coils in one or rarely two rows. The two posterior coils were 95 nm in diameter while the anterior coils were 115 nm in diameter. Early developmental stages were surrounded by electron-dense, 35.3 nm diameter, surface ornaments scattered over the membrane. Tubular elements with diameters of approximately 75 nm were attaching to the periphery of meronts and sporonts. Tissues infected included fat body, midgut and muscle. A 1915 bp rDNA fragment, covering the 3' end of the small subunit (SSU), the internal transcribed spacer (ITS) and the 5' end of the large subunit, was amplified by PCR and sequenced. Phylogenetic analyses of the SSU rDNA fragment revealed closest relationship to *Tubulinosema pampeana* (Host: *Bombus atratus*, South America) and *Tubulinosema loxostegi* (Host: *Loxostege sticticalis*, ubiquitous), but using the complete dataset of SSU-ITS-LSU rDNA genes revealed *T. hippodamiae* (Host: *Hippodamiae convergens*) as next neighbour. Based on the morphological and genetic features a new species, *Tubulinosema suzukii* sp. nov., is proposed for this microsporidium isolated from *D. suzukii*.

INTRODUCTION

Microsporidia are single-celled obligate intracellular eukaryotic parasites of many animal species (Sprague, 1977a, 1977b; Sprague and Becnel, 1999). The evolutionary origin of Microsporidia has been a matter of debate for many years, as several studies suggest they are a sister group to Fungi or an early branching of Fungi while others claim the Microsporidia to be basal to the Zygomycota (Hirt et al., 1999; Tanabe et al., 2002; Keeling, 2003; Thomarat et al., 2004; Gill and Fast, 2006; Lee et al., 2008b; Corradi and Keeling, 2009; Koestler and Ebersberger, 2011; Keeling, 2014). Today the close fungal relationship supported by phylogenetic analyses based on several genetic markers and is widely accepted

(Keeling et al., 2000; Tanabe et al., 2002; Gill and Fast, 2006; Capella-Gutiérrez et al., 2012; Xiang et al., 2014).

Microsporidia infect nearly every taxon of invertebrates and vertebrates, including humans (Sprague, 1977b; Canning and Lom, 1986; Sprague et al., 1992; Weber et al., 1994; Didier, 1998; Didier et al., 1998). Due to their more intensive study, a high proportion of microsporidial species descriptions are from insects and fish (Weiser, 1976; Sprague et al., 1992; Keeling and McFadden, 1998). Microsporidia have highly reduced genomes in comparison to other Eukaryotes, presumably due to the availability and utilization of host metabolic products (Keeling and Fast, 2002; Keeling and Slamovits, 2004; Texier et al., 2010).

More than 200 genera, including 1300 species of microsporidia have been described. Ninety-three of these genera have been described from insects, of which 57 species are parasites of Diptera (Becnel and Andreadis, 2014; Becnel et al., 2014). Prior to the advent of nucleotide sequencing, microsporidian species descriptions were based on morphological and ultrastructural characteristics of life cycle stages. These included size and shape of the spores, number of nuclei in the spores and meronts, number of coils in the polar filament and host tissue pathology (Larsson, 1999). In the late 1980s and early 1990s, rDNA sequences rapidly unravelled phylogenetic relationships among the microsporidia (Vossbrinck et al., 1987, 1993). DNA sequences of small subunit (SSU) ribosomal DNA (rDNA) were initially used for phylogenetic analyses at the family and genus levels which led to a taxonomic classification system separating microsporidia into five clades based on SSU rDNA sequences of 71 species (Vossbrinck and Debrunner-Vossbrinck, 2005) and later for 125 species (Vossbrinck et al., 2014). Since there had been no objective reason for previous primary divisions of the microsporidia (Sprague, 1977a), the idea of correlating SSU with host type and host habitat was added to cultivate discussion (Baker et al., 1995; Vossbrinck and Debrunner-Vossbrinck, 2005). Cheney et al. (2000) suggested that the use of SSU rDNA to distinguish among very closely related species or sub-species was less definitive possibly due to sequence differences among rDNA copies within a single genome. Therefore, some studies extend their analyses by comparing other genes such as elongation factors (EF) 1α and 2, β-tubulin, or the largest subunit of RNA polymerase II (RPB1), to discriminate among species (Cheney et al., 2001; Vossbrinck and Debrunner-Vossbrinck, 2005; Gill and Fast, 2006). RPB1 is a relatively large single copy, housekeeping gene consisting of eight core regions. It has been used to determine relationships among eukaryotes due to its high sequence similarities and its single-copy occurrence in the genome (Sidow and Thomas, 1994; Stiller and Hall, 1997; Hirt et al., 1999). Although several universal oligonucleotide primers covering the different core regions are available for PCR amplification and sequencing the RPB1 gene of *Nosema* and *Vairimorpha* species (Stiller and Hall, 1997; Hirt et al., 1999; Cheney et al., 2001; Kyei-Poku and Sokolova, 2017), RPB1 sequences for many

microsporidia are not available, making comparative analyses impossible for a number of genera.

Several well described entomopathogenic microsporidia are economically important as they infect beneficial arthropods such as honeybees, bumble bees, silkworms and parasitic wasps valuable for pest insect control (Nägeli, 1857; White, 1919; Sajap and Lewis, 1988). A large number (150 species) of insect-infecting microsporidia belong to the *Nosema-Vairimorpha* clade (Becnel and Andreadis, 2014; Tokarev et al., 2020). This group is characterized as being diplokaryotic throughout their life cycle with the formation of uninucleate octospores in some species. Use of molecular data for phylogenetic reconstruction has revealed that some of the *Nosema* species did not belong in the *Nosema-Vairimorpha* clade and have been reassigned to new genera. These new genera include *Brachiola, Paranosema, Vitaforma* and *Anncaliia* (Lowman et al., 2000; Canning et al., 2002; Sokolova et al., 2003; Vossbrinck and Debrunner-Vossbrinck, 2005). Moreover, a putative *Nosema* species with the unique ultrastructural feature of small microtubules on the surface of the meronts (apparently establishing a direct connection to the host cell cytoplasm for exchange of metabolites) led to the founding of the new genus *Tubulinosema* (Franzen et al., 2005b). A new family Tubulinosematidae was established that consists of eight exclusively insect-infecting species. Common to all *Tubulinosema* species are the oral ingestion of spores and the predominant infestation of host adipose tissue. Two *Tubulinosema* species have been discovered in *Drosophila* species, namely *Drosophila melanogaster* and *Drosophila willistoni*.

Drosophila suzukii (spotted wing drosophila, SWD), native to Asia and Japan, is an invasive species in Europe and North America where it causes major damage to many fruit crops, such as raspberries, blueberries, grapes, cherries and other stone fruits (Dukes and Mooney, 1999; Chown et al., 2007; Walsh et al., 2011; Cini et al., 2012). Flies obtained from a laboratory population of *D. suzukii* collected in Oregon, USA, contained a microsporidial infection. In this study, wild caught *Drosophila suzukii* Matsumura (Drosophilidae, Diptera) were screened with the Oregon isolate to assess its possible role as a biological control agent of *D. suzukii*. This study presents the isolation and molecular and morphological characterization of this new microsporidian species of *Tubulinosema* designated here as *Tubulinosema suzukii* sp. nov.

MATERIAL AND METHODS

Insect host rearing

A microsporidia-free *Drosophila suzukii* laboratory colony was established at the Institute for Biological Control of the Julius Kühn-Institut in Darmstadt (Germany). Adult SWD were

collected from a wild population in Baden-Württemberg (Germany) in the year 2013 by colleagues of the Institute for Plant Protection in Fruit Crops and Viticulture of the Julius Kühn-Institut in Dossenheim (Germany). Adult flies were incubated at 22±1 °C, 50% r.H. with a 16/8 light/dark photoperiod in 30×30×30 cm cages. Each cage contained a water source, diet for adult flies (brewer´s yeast and sugar, each 1 g) and one cup of culture medium. The culture medium consisted of 10 g wholemeal flour, 20 g sugar, 50 g brewer´s yeast, 400 g pureed apple, 450 ml apple juice and 20 ml apple vinegar, mixed with 20 g Agar-Agar boiled in 500 ml tap water. After cooling the culture medium was topped with 0.6 ml of fresh, dissolved yeast (modified from Chabert et al., 2012). The culture medium was changed weekly, except for experiments in which the culture medium was changed every 4 hours to obtain synchronously aged offspring.

An additional colony of *D. suzukii*, originally caught in Oregon, USA, was maintained at the Medical University of Vienna, Austria, and is referred to as the SAM rearing (Kaur et al., 2017). Rearing conditions are described elsewhere (Kaur et al., 2017). In 2015, microsporidia from live flies obtained from the SAM rearing were inoculated into German *D. suzukii* (see Inoculation of *D. suzukii*, below) to obtain a microsporidia-infected laboratory population for *in vivo* spore production with rearing conditions as described above for microsporidia-free SWD colony.

Microsporidia spore isolation

To isolate and purify *Tubulinosema suzukii* spores, adult *D. suzukii* from the SAM rearing facility were homogenized with a micro pestle in 500 µl sterile tap water and filtered through 4 layers of gauze bandage and additionally through a cotton filter disc with 12-15 µm particle retention (Grade 1288, Ø 90 mm, Sartorius AG, Göttingen, Germany). Spores were pelleted at 10,000 × *g* for 2 min (Centrifuge 5424 R, rotor Nr. FA-452411, Eppendorf, Hamburg, Germany) and resuspended in 500 µl sterile tap water. Spore concentration and purity was determined with a Thoma hemocytometer using phase contrast microscopy. All light microscopy was accomplished with a Leica DMRB microscope (Leica Microsystems GmbH, Wetzlar, Germany). Spores were then diluted in sterile tap water to a final concentration of 2,000 spores per µl as required for subsequent inoculation.

Inoculation of *D. suzukii* with microsporidia spores

To produce *T. suzukii* spores, SWD larvae were placed individually in 96-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) containing 440 µl sterile apple puree in each cavity. Ten µl of the microsporidia spore suspension containing 2,000 spores

per µl were added to each cavity. The microtiter plate was kept under rearing conditions in cages to allow free movement of freshly hatched adults until use for microsporidia spore isolation.

Light and transmission electron microscopy

Wet mount preparations of *T suzukii* infected SWD adults and larvae were examined for the presence of spores with phase contrast microscopy at 400- to 640-fold magnification. The size of the spores was determined using a Sony SMC 178-C camera system and software (EHDView). Methanol fixed smears of infected *D. suzukii* tissue were stained with the Giemsa (Merck KGaA, Darmstadt, Germany) protocol as modified by Huger (1964). Spores, meronts, sporonts and sporoblasts were observed under bright field illumination. Measurements are shown as mean ± standard deviation (SD) if not indicated otherwise. Sample size is indicated as N.

Tissues infected were determined by fixating adult SWD in Dubosq-Brazil´s alcoholic Bouin solution followed by embedding in Histosec (Merck KGaA, Darmstadt, Germany) (Romeis, 1989). Eight µm sections were prepared with a rotary microtome (1212, Leitz, Wetzlar, Germany) and stained with Heidenhain´s iron haematoxylin, then counterstained with erythrosine (Langenbuch, 1957; Huger, 1961; Eberle et al., 2012b) and analysed with bright field microscopy using EHDView software.

For transmission electron microscopy, SWD adults were fixed using 3% Karnovsky solution (Karnovsky, 1965) (12 hours pre-fixation, 68 hours post-fixation after removal of head and legs), post-fixed in 2% osmium tetroxide (69 hours), stained with 1% uranyl acetate (5 hours), dehydrated in graded ethanol (70-100%) and embedded in Spurr epoxy resin (Spurr low viscosity embedding kit, Sigma Aldrich/Merck KGaA, Darmstadt, Germany) (Spurr, 1969; Plattner and Zingsheim, 1987). After polymerisation for 8 hours at 70 °C, semi-thin sections of 0.4 µm were prepared, stained with 0.3% Toluidine blue in 2.5% Na_2CO_3 buffer (Böck, 1984) and inspected for microsporidia-positive tissues using light microscopy (Leica DMRB, Leica Microsystems GmbH, Wetzlar, Germany). Microsporidia-positive tissue was then selected for ultrathin sections (50-70 nm) with a diamond knife (MT15905, Diatome, Nidau, Switzerland) and a Leica Ultracut S (Reichert Ultracut S; Leica Microsystems GmbH, Wetzlar, Germany). The sections were stained with 6% lead citrate for 2 min followed 2% uranyl acetate for 10 min. Sections were examined in a Zeiss TEM 902, transmission electron microscope. The sections were stained with 6% lead citrate for 2 min followed 2% uranyl acetate for 10 min. Sections were examined in a Zeiss TEM 902, transmission electron microscope and imaged with a CCD camera system using Image SP software (SYSPROG & TrS, Minsk, Belarus).

Molecular analysis

Genomic DNA extraction and PCR

About ten adult SWD flies from infection rearing were grinded in 500 µl distilled water in with a micro pestle to isolate about 10^6 to 10^7 spores from infected tissue. The suspension was filtered through four layers of a gauze bandage and finally through nylon nets with 10 µm pore size using a vacuum pump (Merck Millipore/ Merck KGaA, Darmstadt, Germany). The filtrate containing spores was pelleted by centrifugation u at 15,000 × *g* for 10 min (Centrifuge 5424 R, rotor Nr. FA-452411, Eppendorf, Hamburg, Germany). The spore pellet was resuspended in 100 µl STE buffer (10 mM Tris, 1 mM EDTA, and 100 mM NaCl with pH 8.0) and inspected for purity by phase contrast microscopy using a Thoma haemocytometer. After mixing with 100 µg glass beads (0.25-0.5 mm size, Roth, Karlsruhe, Germany) the spores were fragmented in a tissue disruptor at 24 mHz for 20 sec (MP FastPrep ®-24 Tissue and Cell Homogenizer, MP Biomedicals, Eschwege, Germany), heated at 95 °C for 15 min and bead disrupted again for 20 sec. The solution containing genomic DNA from disrupted spores was further subjected to PCR.

Amplification of the rDNA region shown in Figure 4-8 was accomplished in two fragments using the Taq core kit (QIAGEN, Hilden, Germany). The region from 530 forward to 580 reverse was amplified using the universal oligonucleotide primers 530 F (10 mM, 5′-GTGCCAGC(C/A)GCCGCGG-3′) and 580 R (10 mM, 5′-GGTCCGTGTTTCAAGACGG-3′) (Vossbrinck et al., 1993, 2004), and the 5′ region of the small subunit rDNA was amplified using the primers 18 F 5′-CACCAGGTTGATTCTGCC-3′ (Vossbrinck et al., 2004) or the newly designed Tn37 F 5′-CGAAGATTTAGCCATGCATGCT-3′, and the reverse primer Tn562 R 5′-CCGCTTCGAATATAAGCATTGA-3′. A 50 µl reaction contained 5 µl 10× reaction buffer, 1 µl forward primer, 1 µl reverse primer (both 10 mM), 1 µl dNTP (20 mM), 0.5 µl $MgCl_2$ (25 mM), 0.25 µl Taq polymerase (5 units/µl) and 5 µl genomic DNA isolated as described above. The reactions were performed in a BioRad thermocycler (T100, BioRad Laboratories GmbH, Feldkirchen, Germany) with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 sec, 53 °C for 30 sec and 72 °C for 90 sec and a final elongation step at 72 °C for 5 min. PCR products were analysed by gel electrophoresis at 90 V for 45 min through 1.5% agarose gel in 1×TAE buffer (40 mM Tris, 20 mM Acetate, 1 mM EDTA, pH 8.3), prestained with Midori Green (0.005%, Biozym Scientific GmbH, Hessisch Oldendorf, Germany). The PCR products were purified using a QiaQuick PCR purification kit (QIAGEN, Hilden, Germany) according to manufacturer´s instructions. Furthermore, a PCR for amplification of the D-F (Hirt et al., 1999) and A-G region (Stiller and Hall, 1997; Cheney et al., 2001) of the largest subunit of RNA polymerase II (RPB1) was performed using the oligonucleotide primers Md-RPB1-F1 (5′-AAGCCCGATCTTAATGCCATTTGG-3′)/Md-RPB1-R1 (5′-GGCGTAATCTTCTCTGGAAACG-3′) (Kyei-Poku and Sokolova, 2017), AF1 (5′-GAKTGTCCKGGWCATTTCGG-3′) and AF3 (5′-GGWCATTTCGGWCACATIGA-3′)/GR1 (5′-

TGRAAMGTRTTIAGIGTCATYT-3´) (Stiller and Hall, 1997; Cheney et al., 2001) and RPB1-F1 (5´-CGGACTTYGAYGGNGAYGARATGA-3´)/RPBI-R1 (5´-CCCGCKNCCNCCCATNGCRTGRAA-3´) (Hirt et al., 1999). However, efforts to sequence the RPB1 gene failed in this study.

Cloning and sequencing of PCR fragments

Purified PCR fragments were cloned using the pGEM®-T Easy Vector System I (Promega GmbH, Mannheim, Germany) according to manufacturer´s instructions (see Figure 4-1). Electro-competent *Escherichia coli* DH10β cells were transformed using the ligation mixture and plated on LB plates mixed with XGAL (40 mg/l)/IPTG (100 mg/l)/Ampicillin (1 mg/l). Single white colonies were picked and propagated over night at 37 °C in 2 ml liquid LB medium (containing Ampicillin 1 mg/l). Plasmid was isolated from cultures using a Zyppy™ Plasmid Mini Prep kit (Zymo Research Europe GmbH, Freiburg im Breisgau, Germany).

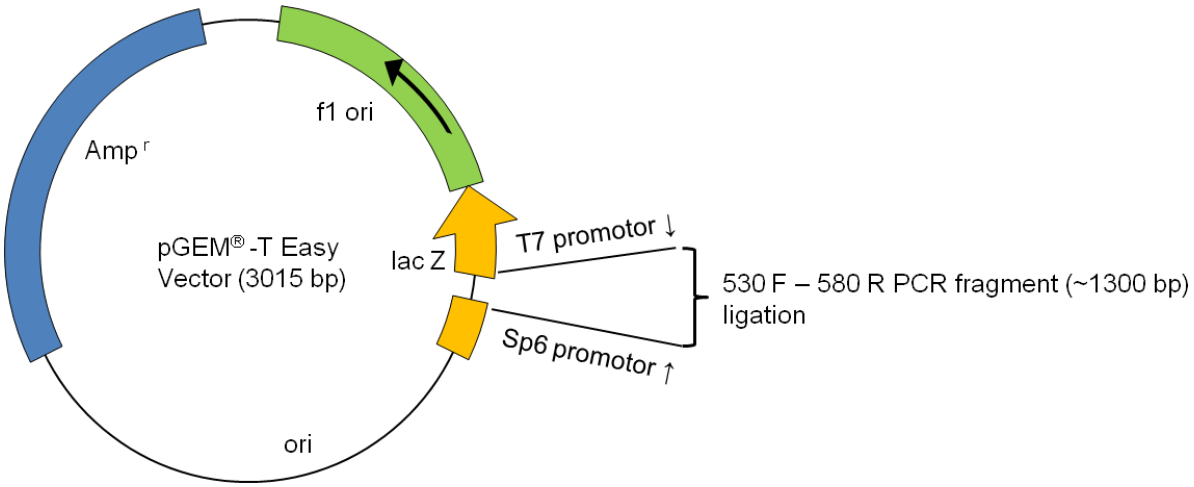


Figure 4-1: Schematic illustration of pGEM®-T Easy Vector (adapted from Promega GmbH, Mannheim, Germany) and ligation sites (T7 and Sp6 promotor region) for PCR fragment ligation. *Tubulinosema suzukii* SSU-ITS-LSU rDNA was amplified using the 530 F and 580 R universal primer designed by Vossbrinck et al. (2004).

DNA was subjected to commercial sequencing (Sanger method) (StarSEQ GmbH, Mainz, Germany) using microsporidia-specific oligonucleotide primers 530 F and 580 R and oligonucleotides primers specific for Sp6 and T7 promoter regions of the cloning vector. The obtained DNA sequences were assembled and a consensus sequence was compiled using Geneious Mapper implemented in Geneious version 8.1.9 (http://www.geneious.com; Kearse et al., 2012). The consensus sequence was analyzed for sequence similarities using

basic length alignment search tool BLAST (Altschul et al., 1990). Due to first hints to the family Tubulinosematidae additional oligonucleotide primers were designed with BioEdit Sequence Alignment Editor version 7.2.5 (Hall, 1999) using multiple sequence alignment tool ClustalW (Thompson et al., 1994) of the seven best sequence matches of *Tubulinosema* species from NCBI: *Tubulinosema kingi* (DQ019419), *Tubulinosema hippodamiae* (JQ082890), *Tubulinosema ratisbonensis* (AY695845), *Tubulinosema pampeana* (KM883008), *Tubulinosema loxostegi* (JQ906779), *Tubulinosema acridophagus* (AF024658), *Tubulinosema* sp. LS4M1 (KX379714). A 50 µL PCR reaction was performed with forward primer 18 F 5´-CACCAGGTTGATTCTGCC-3´ (Vossbrinck et al., 2004) or the newly designed Tn37 F 5´-CGAAGATTTAGCCATGCATGCT-3´, and the reverse primer Tn562 R 5´-CCGCTTCGAATATAAGCATTGA-3´ using the same PCR conditions as described above resulting in about 500 bp fragments. The fragments were purified with QiaQuick PCR purification kit and directly sequenced at StarSEQ GmbH. The obtained sequences were assembled with the first consensus sequence from cloned PCR fragments (see above). The resulting DNA sequence was assembled with the initial consensus sequence obtained from vector-cloning. The new consensus sequence included the partial DNA sequence of the 3' end SSU rDNA gene, the complete DNA sequence of the ITS, and the partial DNA sequence of the 5' end of the LSU rDNA gene and was submitted to GenBank database as *Tubulinosema suzukii* with Accession number MN631017.

Phylogenetic reconstruction

Two different data sets were used for phylogenetic analyses using the extracted sequence of the 1402 bp SSU rDNA and the complete 1915 bp ribosomal sequence including SSU, ITS and LSU rDNA.

1) Microsporidian clade assignment using SSU sequences

The SSU rDNA (~1500 bp) sequences of 23 selected representatives of all microsporidia clades were extracted from NCBI GenBank and used in ClustalW alignment together with the *T. suzukii* sequence (BioEdit Sequence Alignment Editor version 7.2.5) (Vossbrinck and Debrunner-Vossbrinck, 2005; Vossbrinck et al., 2014). Aligned sequences were trimmed according to the 1402 bp SSU sequence of *T. suzukii* and analysed with the Maximum Likelihood (ML) method based on the Kimura 2-parameter model (Kimura, 1980) implemented in MEGA 6 software (Hall, 2013; Tamura et al., 2013) using 500 bootstrap replications. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. *Basidiobolus ranarum* (JQ014008) was set as the outgroup.

2) Tubulinosema spp. phylogeny using ribosomal genes

(a) SSU. Available SSU rDNA sequences of eight *Tubulinosema* species were extracted from NCBI GenBank, aligned using ClustalW, trimmed according to the 1402 bp sequence of *T. suzukii* and analysed using a ML method based on the Kimura 2-parameter model with 500 bootstrap replications. Furthermore, the Maximum Parsimony (MP) and Neighbor-Joining (NJ) methods were applied with 500 bootstrap replications, and the Pairwise Genetic Distance based on nucleotide differences was analysed.

(b) SSU-ITS-LSU. The 1915 bp nucleotide sequence was submitted to the NCBI GenBank database as a BLAST search resulting in 6 closest matches with *Tubulinosema* species (98% identity) Sequences were aligned using ClustalW, the comparative analyses were accomplished with ML, MP and NJ methods using 500 bootstrap replications and furthermore, the Parsimony informative positions were extracted.

The most closely (75% identity) related non-*Tubulinosema* species *Brachiola* (=*Anncaliia*) *algerae* (AY230191), was set as the outgroup for both analyses.

RESULTS

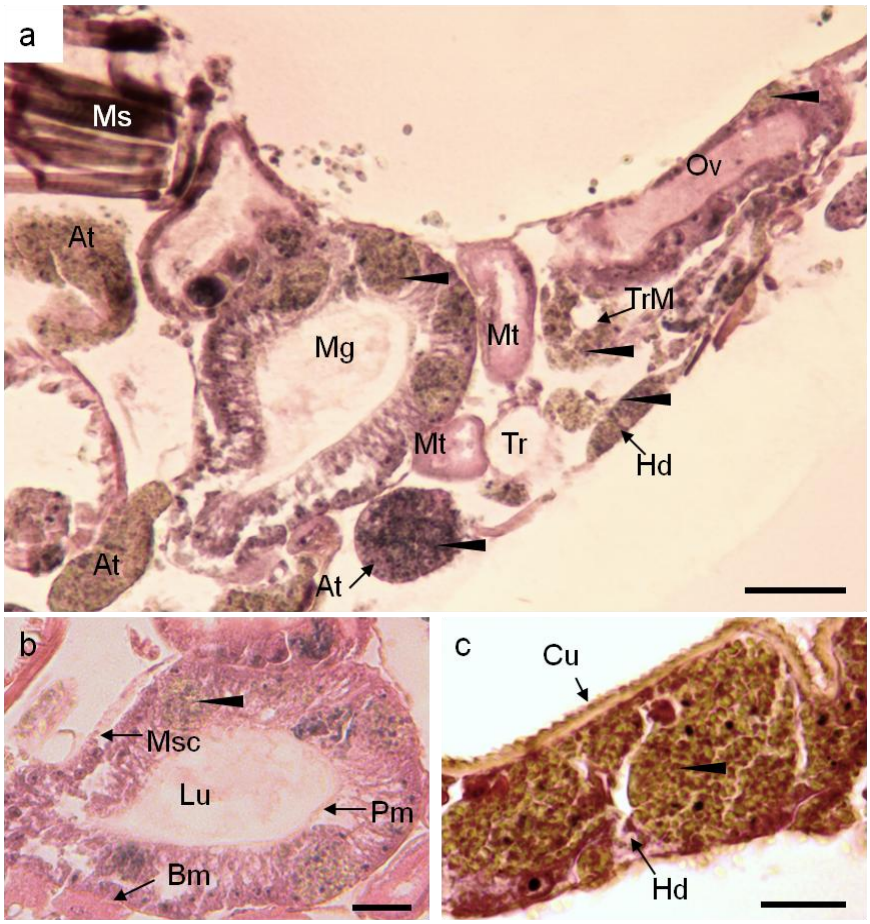
The morphology, pathology and rDNA sequences of a new microsporidium infecting *D. suzukii* were examined. Based on these analyses, this microsporidium is designated as *Tubulinosema suzukii*.

Light microscopy

When healthy SWD larvae were fed *T. suzukii* spores, the emerging adults were heavily infected showing a microsporidian infection of adipose tissue (100%, N=16), midgut muscularis and epithelial cells (75%, N=16), muscle tissue (62.5%, N=16), hypodermis (43.75%, N=16) and tracheal matrix (31.25%, N=16) (Figure 4-2 a-e). Spores were found inside immature ovaries in 36.4% (N=11) of female SWD (Figure 4-2 a, e). The adipose tissue surrounding the ovary was infected in 27.3% (N=11) of the female samples. Infection of the ventral nerve cord was recorded in 43.75% (N=16) of the samples. No data are available for infection of the male testes due to low sample size (5 individuals). Effects on fitness parameters and survival, transmission routes and development-dependent susceptibility will be described elsewhere (Chapter V).

The first occurrence of meronts and sporonts was observed three days after inoculation of the L2 larval stage at the earliest (Figure 4-3 a-h). Mononucleate meronts were not detected.

Diplokaryotic meronts were observed within 6 days post infection of SWD larvae and pupae (Figure 4-3 a) as well as meronts with two and four diplokarya (Figure 4-3 b, c). Diplokaryotic meronts were occasionally building irregular chains or clusters of three meronts (Fig 4-3 d, e). Sporonts with one or two diplokarya (Figure 4-3 f, g) and sporonts close to or in division with four diplokarya were observed (Figure 4-3 h-j). Merogony appeared to occur through binary fission of meronts with two separated pairs of nuclei migrating to each pole of the cell (Figure 4-3 j). Heavy infections were detected in L3 SWD larvae (6-7 dpi), where sporoblasts and spores could be found, most frequently released from adipose tissue (Figure 4-3 k). Sporoblasts were oval measuring 5.13±0.32 × 2.61±0.15 µm (length × width, mean ± SD, N=46) in wet mount preparations. Spore shape was oval to slightly pyriform (Figure 4-3 l-n) and size ranged between 4.29±0.32 × 2.47±0.18 µm in wet mount preparations (N=143, Figure 4-3 m) and 3.84±0.29 × 2.40±0.21 µm after methanol fixation and Giemsa-staining (N=140, Figure 4-3 l, Table 4-1). Extruded polar filaments (Figure 4-3 n) of 30 analysed spores measured 67.80±13.20 µm (mean ± SD) ranging from 45.72 to 96.28 µm. Other characteristics of developmental stages are shown in Table 4-1, 4-2 and 4-7.



(Figure 4-2 continues on the following page)

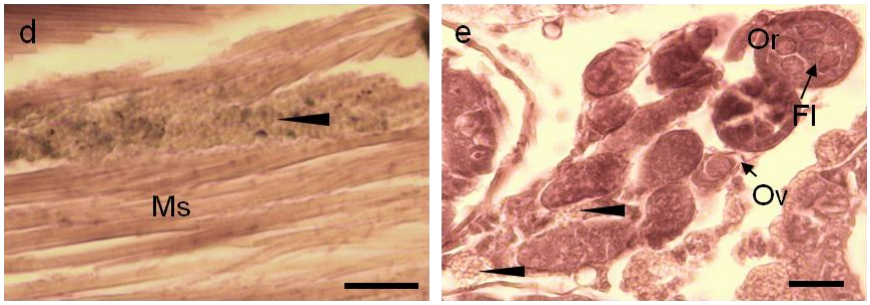


Figure 4-2: Light microscopic images of *T. suzukii* infections sites in SWD.

(a) Section of a female *D. suzukii* adult with heavily infected adipose tissue (At), hypodermis attached to cuticle (Hd), adipose tissue surrounding ovary (Ov), tracheal matrix (TrM) and midgut epithelial cells (Mg). Spores found in respective tissues are indicated with arrowheads. Abbreviations: Tr, trachea; Mt, Malpighian tubules; Ms, muscle cells. (b) Spores (arrowhead) in microsporidian-infected midgut epithelial cells. Abbreviations: Bm, basal membrane; Lu, midgut lumen; Msc, muscularis; Pm, peritrophic membrane. (c) Spores (arrowhead) in microsporidian-infected hypodermis (Hd) attached to cuticle (Cu). (d) Spores (arrowhead) in microsporidian-infected muscle cells (Ms). (e) Spores (arrowheads) in microsporidian-infected ovary (Ov, arrow). Abbreviations: Or, Ovar; Fl, follicle cells (arrow). Scale bar=20 µm. Hematoxylin Heidenhain-stain.

Table 4-1: Size of developmental stages with mean diameter (Ø), minimum and maximum size, standard deviation (SD), sample size (N) and corresponding image in Figure 4-3.

Developmental stage	Ø (µm)	Min (µm)	Max (µm)	SD (µm)	N	Figure 4-3
Meront Giemsa-stained						
binucleate	4.61	3.56	5.66	0.54	27	a
Sporont Giemsa-stained						
diplokaryon	6.05	5.25	7.22	0.53	16	f
2 diplokarya	6.02	5.30	6.77	0.58	9	g
Sporoblast wet mount						
length	5.13	4.64	5.99	0.32	46	k
width	2.61	2.29	2.98	0.15		
Spores wet mount						
length	4.29	3.49	5.18	0.32	143	m, n
width	2.47	2.04	2.94	0.18		
Spores Giemsa-stained						
length	3.84	3.60	4.65	0.29	140	l
width	2.40	1.96	3.11	0.21		

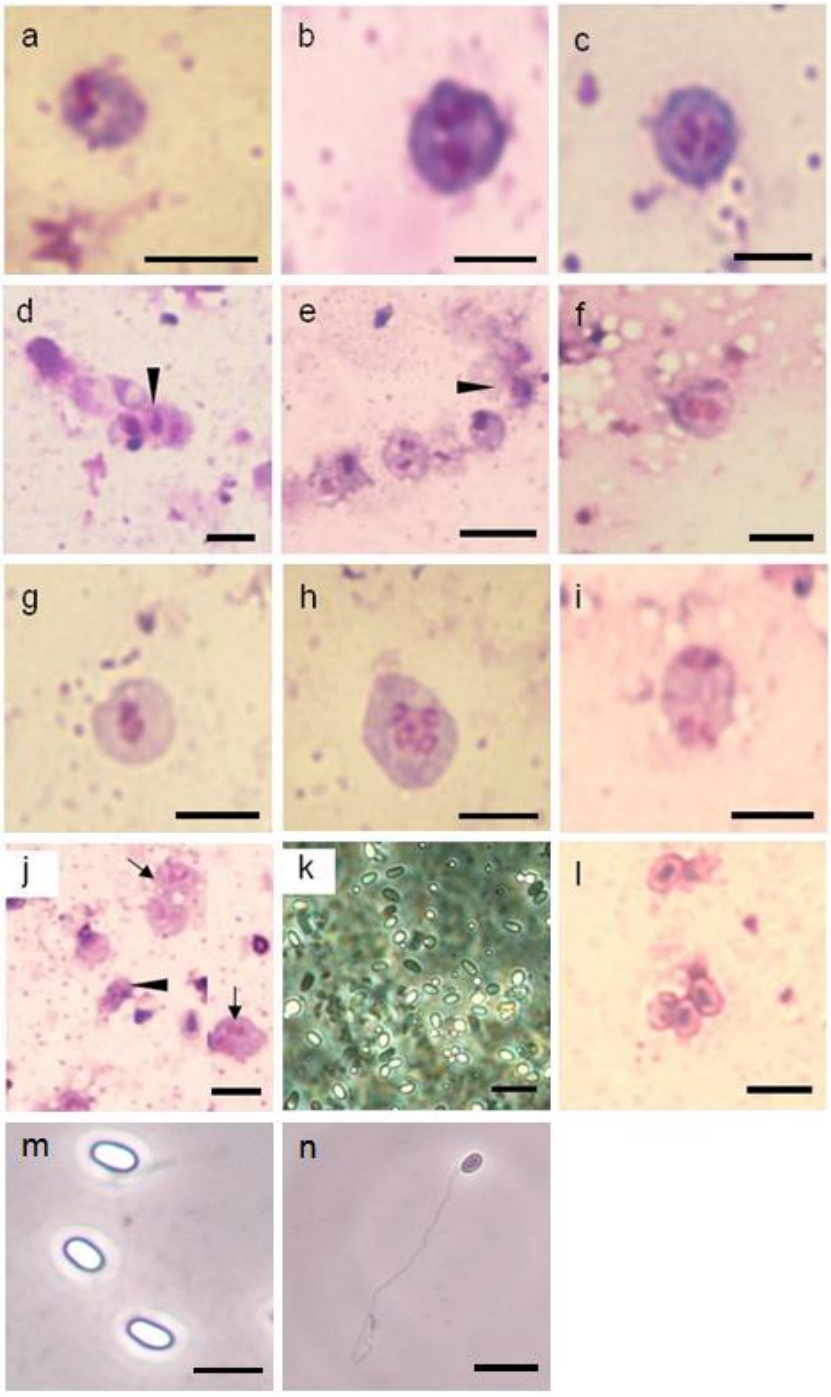


Figure 4-3: Developmental stages of *T. suzukii*.

a-j, l bright field micrographs of Giemsa-stained smears; k, m, n phase contrast micrographs of wet mount preparations. (a) Meront with one diplokaryon with densely packed chromatin. (b) Meront with two diplokarya and (c) meront with four diplokarya ready to divide. (d, e) Meront chains of three meronts (arrowheads). (f) Sporont with diplokaryon. (g) Sporont with two diplokarya. (h) Sporont with four diplokarya. (i) Sporont with four diplokarya and fission septum. (j) Binary fission of a meront (arrowhead) and of two sporonts in division (arrows). (k) Spores (white, oval) and sporoblasts (dark) of heavily infected SWD L3 larvae with lipid droplets (white, round). (l) Giemsa-stained spores. (m) Refractive spores with a slightly pyriform shape from a moderately infected

SWD pupa. (n) Spore with extruded polar filament after treatment with 5% acetic acid. Images a-d, f-j, l, m scale bars=5 µm and images e, k, n scale bars=10 µm.

Ultrastructural analyses

Early developmental stages observed with electron microscopy had typical characteristics shared by Tubulinosematidae (Figure 4-4 a-f). Meronts (Figure 4-4 a, b) had an irregular or round shape with dot-like electron-dense ornaments on the surface, which were evenly scattered around the outer membrane (Fig 4-4 a, b, arrowheads), later developing to the more electron-dense membrane of sporonts (Figure 4-4 c-e). Characteristic microsporidian features were measured in size and summarized in Table 4-2. The diameter of membrane ornaments was about 35.26±1.09 nm (mean ± SE, N=35). Tubules on the membrane surface characteristic for the Tubulinosematidae were observed in late meronts and sporonts occurring in isolation or packed in bundles attached the membrane (Figure 4-4 c, e, arrowheads), sometimes reaching far into the cytoplasm periphery. Tubular wall structure appeared to have an electron-density similar to that of the membrane, whereas the inner space was electron-lucent. The average outer diameter was about 74.08±1.32 nm (based on 43 measured tubules from 17 meronts/sporonts) and 35.31±1.35 nm for the inner diameter (24 tubules from 14 meronts/sporonts). Meronts often occurred together with other developmental stages in host cells and a direct contact to host cytoplasm was observed. As occasionally observed in light microscopy, some meronts formed chains consisting of three meront. Most meronts contained a single diplokaryon with two nuclei surrounded by double membrane and separated by a perinuclear space (Figure 4-4 a). The early meront cytoplasm contained numerous free ribosomes. Late meronts contained some rough endoplasmic reticulum as well (Figure 4-4 a, b).

Sporonts (Figure 4-4 c-e) consisted of an electron dense membrane appearing thicker than in merontal stage (Figure 4-4 c, e). During transition from meront to sporont, apparent surface ornaments on the merontal membrane seem to fuse, building a thickened membrane at several locations on the membrane (Figure 4-4 d, e). Tubules on the sporont membrane frequently formed clusters or bundles (Fig 4-4 e). Size and shape of the sporonts were similar to those of the meronts, also showing a diplokaryon and numerous free ribosomes but more rough endoplasmic reticulum than was present in the meronts.

Sporogony appears disporoblastic (Figure 4-4 f). Early sporoblasts have unarranged early coil units in their cytoplasm (Fig 4-5 a). Late sporoblasts have a shape similar to that of the mature spores but without the thick endospore and with an abundance of rough endoplasmic reticulum (Figure 4-5 b).

Spores were ovoid or slightly pyriform and smaller than the sporoblasts and contained typical organelles in the anterior region and polaroplast, sporoplasm, polar filaments, manubrium, anchoring disc, diplokaryon, posterior vacuole, endospore and exospore (Figure 4-6 a, b; 4-7 a, b). The thicker endospore measured 125.33±2.69 nm (mean ± SE, N=13) and the exospore was 39.29±1.67 nm (N=13). No surface ornaments were observed like in meronts, but exospore of immature spores often had a second uniform layer (18.39±0.91 nm, N=4 spores, Figure 4-6 a, b; 4-7 b) which disappeared as the spores matured (Figure 4-7 a). The anchoring disc is forming an indentation of the endospore (Figure 4-7 a). The anterior polaroplast shows uniform lamellar and occasionally tubular structures, latter ones of 40.14±4.44 nm diameter (N=3, Figure 4-7 a, b). The polar filament is slightly anisofilar and has about 8 to 11 coils (10.1±0.22, N=30 spores, Figure 4-7 c, d). The anterior coils with an average diameter of 115.36±1.64 nm (N=10 spores, 40 coils) usually consisted of five concentric rings with different electron translucency: an electron-lucent first layer, an electron-dense second one, a more lucent third, a very dense fourth layer and a lucent layer, followed by the translucent coil nucleus of granular or amorphous structure (Figure 4-7 d). About two to three posterior coils were smaller (95.35±0.87 nm, N=10 spores, 23 coils, Figure 4-7 c, asterisk) and had an electron-dense instead of a translucent nucleus structure like anterior ones. The coils were mostly orientated in one row or rarely two rows if maturation was not completed.

Table 4-2: Summary of *T. suzukii* features from TEM.

Size or diameter with standard error (SE), sample size (N/cell=measured features/number of cells) and corresponding image in Figure 4-4, 4-6 and 4-7.

Feature	Size (nm)	SE (nm)	N/cells	Figures
Membrane ornaments (diameter)	35.26	1.09	35/4	4-4 a
Tubules meronts/sporonts (diameter)	74.08	1.32	43/17	4-4 b, c, e
Spore				
Endospore (thickness)	125.33	2.69	13/13	4-6, 4-7 a, b
Exospore (thickness)	39.29	1.67	13/13	4-6, 4-7 a, b
Layer on exospores (thickness)	18.39	0.91	4/4	4-6 a, b; 4-7 b
Tubular structures polaroplast (diameter)	40.14	4.44	3/3	4-7 a, b
Polar filaments				
Anterior coils	115.36	1.64	40/10	4-7 c, d
Posterior coils	95.35	0.87	23/9	4-7 c

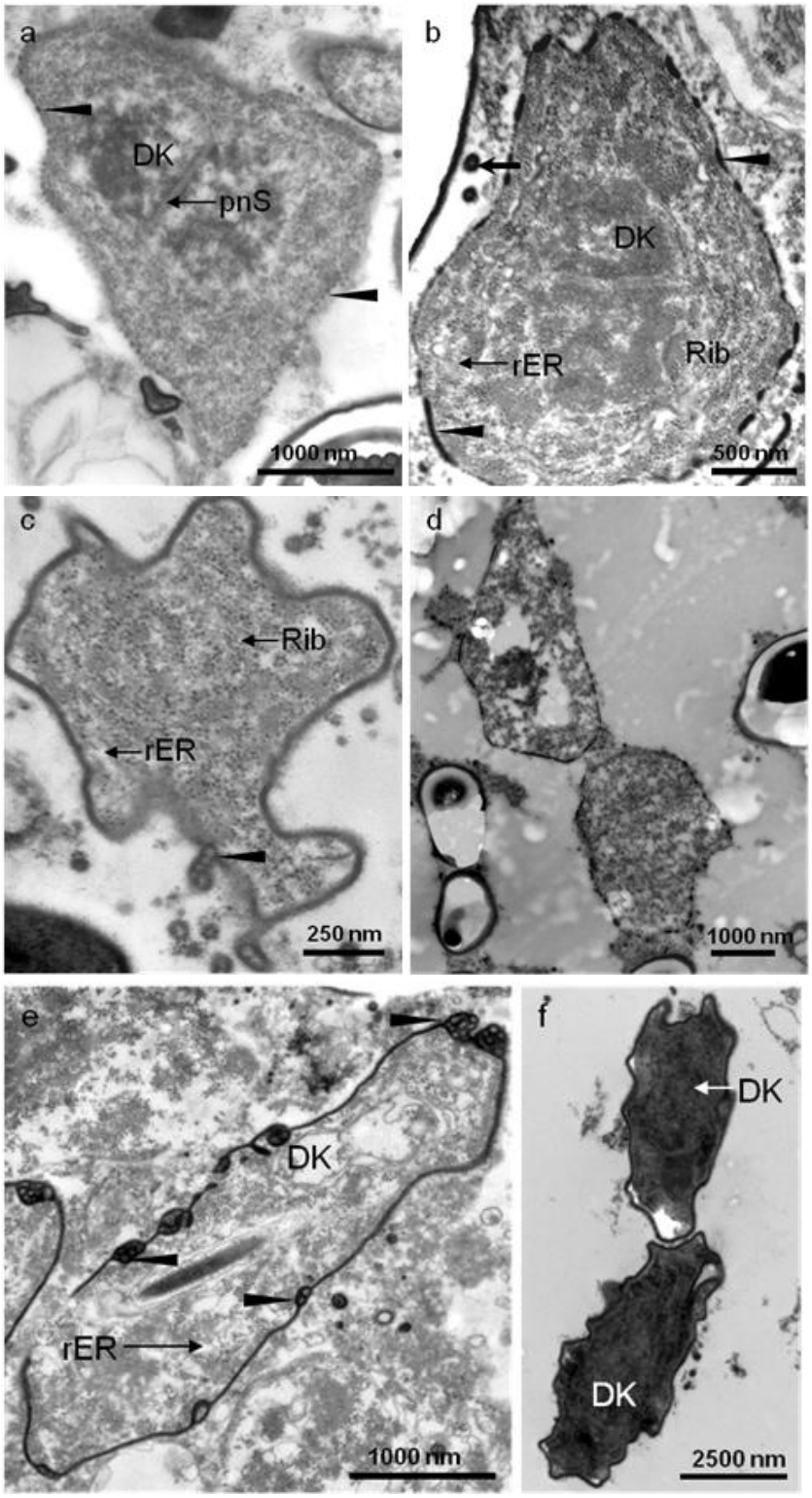


Figure 4-4: Transmission electron micrographs of *T. suzukii* early developmental stages with ultrastructure.

(a) Early meront with ornamental structures on membrane surface (arrowhead), diplokaryon (DK) and perinuclear space (pnS, arrow) between the two nuclei. (b) Late meront with diplokaryon (DK), small tubules in periphery (thick arrow), thickening membrane (arrowheads), rough endoplasmic reticulum (rER) and free ribosomes (Rib) in cytoplasm. (c) Early sporont with thickening membrane and rough endoplasmic reticulum (rER, arrow) and numerous free ribosomes (Rib, arrow) and

longitudinal section of a tubule (arrowhead). (d) Sporonts in division still connected through membrane and cytoplasm bridge. (e) Elongated diplokaryotic sporont with electron-dense membrane and bundles of tubules on surface (arrowheads) and ribosome-rich cytoplasm with rough endoplasmic reticulum (rER). (f) Disporoblastic sporogony: diplokaryotic sporoblast after division.

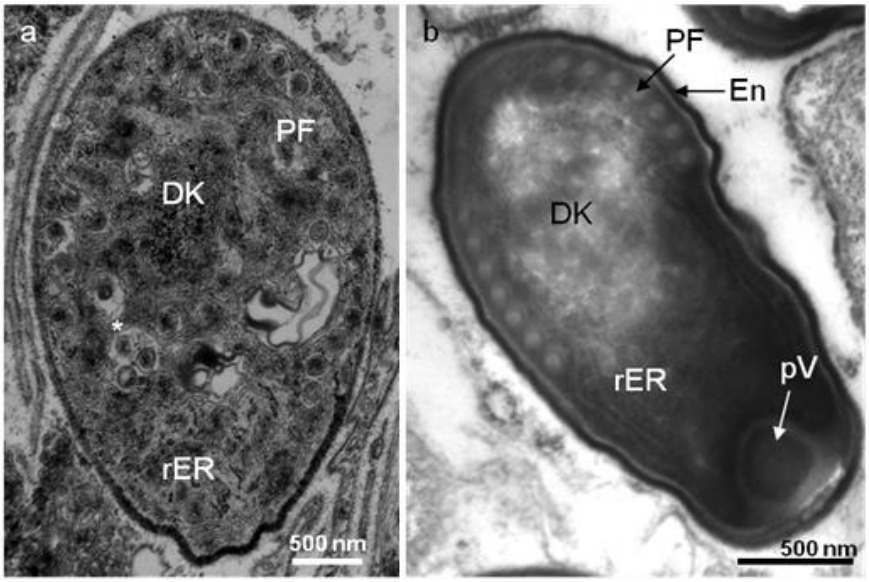


Figure 4-5: Transmission electron micrographs of *T. suzukii* sporoblasts with ultrastructure.
(a) Early sporoblast with unarranged polar filament (PF), rough endoplasmic reticulum (rER) and diplokaryon (DK). (b) Late sporoblast, size and shape similar to immature spore, endospore much thinner (arrow, En). Polar filament coils (PF) and posterior vacuole (pV) arranged.

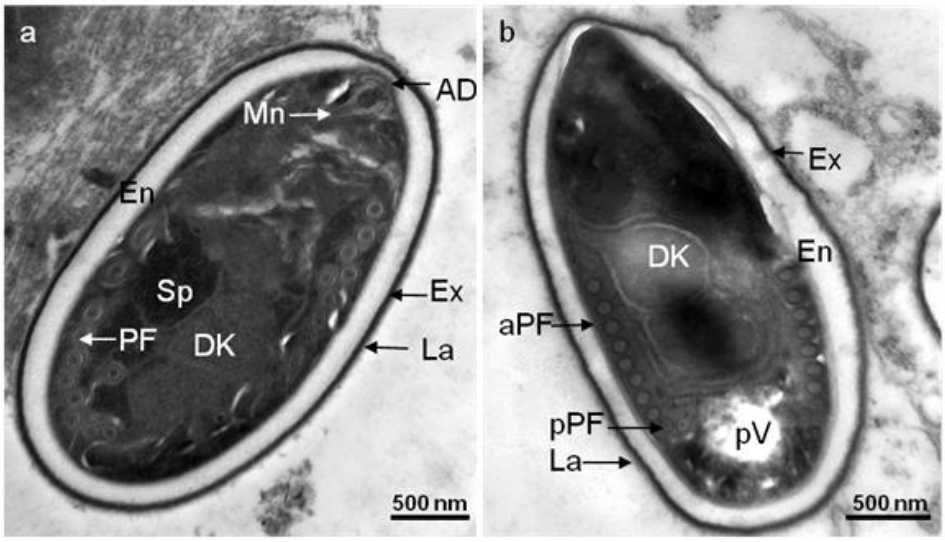


Figure 4-6: Transmission electron micrographs of *T. suzukii* spores with ultrastructure.
(a) Immature spore and (b) mature spore consisting of endospore (En), exospores (Ex) and a second layer (La), posterior vacuole (pV, here broken during embedding), diplokaryon with two membranes

(DK, arrow), polar filaments (PF), smaller posterior and larger anterior ones (pPF, aPF), anchoring disc (AD), and manubrium (Mn).

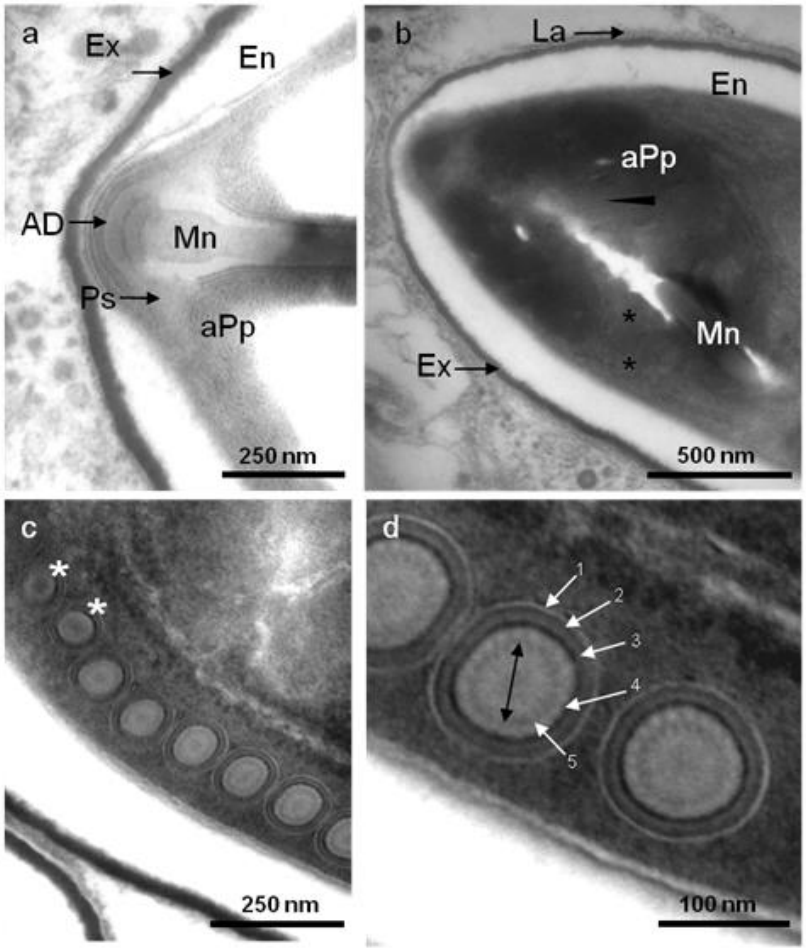


Figure 4-7: Transmission electron micrographs of *T. suzukii* spore internal structure and details.
(a) Spore details: Anchoring disc (AD) forming an indentation at the endospore (En), polar sac (Ps), manubrium (Mn) and anterior polaroplast (aPp) of mature spore. (b) Additional layer (La) on the exospores (Ex), lamellar (arrowhead) and tubular (asterisk) structures in anterior polaroplast (c) Cross sections of polar filaments (PF) at higher magnification showing smaller posterior coils (asterisk) and (d) five ring zones of different electron density consisting of a whitish electron-lucent outer layer (1), an dark electron-dense second (2), a more lucent third (3), a very dense fourth (4) and a lucent fifth layer (5); the centre of the coil is indicated by a black double arrow.

Molecular analysis

PCR fragments amplified using the 530 F and 580 R universal oligonucleotide primers covering SSU-ITS-LSU rDNA were cloned in the pGEM®-T Easy Vector and sequenced with forward primer SP6. Sequencing of two clones resulted in two sequences of different length, which were assembled into an 842 bp sequence. Two sequences which were 798 bp in length were obtained from two clones with the T7 reverse primer. The two sequences overlapped by 84 bp and were merged to form a 1476 bp sequence after removal of the vector backbone sequences. PCR with the universal primer 18 F and the Tubulinosematidae-specific primer Tn562 R yielded a 584 bp fragment which overlapped with the first sequence by 145 bp. Assembly of the two sequences resulted in a 1915 bp sequence fragment with a GC content of 41.62%, containing SSU rDNA (1402 bp), ITS rDNA (52 bp) and the 5´end of the LSU rDNA (461 bp) (Figure 4-8).

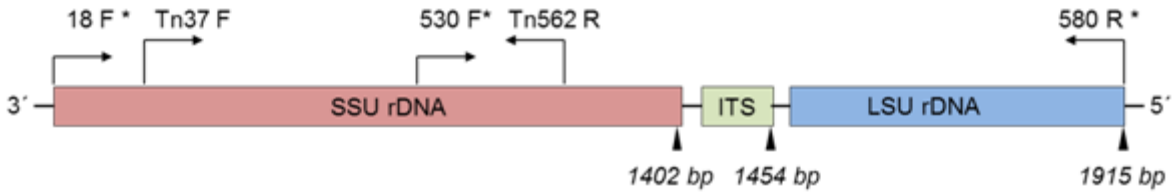


Figure 4-8: Schematic illustration of the ribosomal gene arrangement in *T. suzukii* (SSU-ITS-LSU), created by using three universal oligonucleotide primer (asterisk) and two *Tubulinosema*-specific oligonucleotide primers designed in this study.

BLAST search of the GenBank database using the *T. suzukii* SSU rDNA sequence (1402 bp) revealed close matches with other microsporidia, in the family Tubulinosematidae. The SSU rDNA showed a 99.7% sequence identity (1392/1396 bp) to *T. loxostegi* (JQ906779) with four nucleotide differences, consisting of two base exchanges of guanine : adenine, one thymine : cytosine and one adenine : thymine base changes. A 98% sequence identity to the complete 1402 bp sequence was achieved by *T. pampeana* (KM883008), whereby 1378 out of 1378 bp showed 100% identity except to the first 24 nucleotides of the 3´ end of SSU rDNA sequence that is not available for *T. pampeana*. Comparing the SSU sequence with those of *Drosophila*-infecting *Tubulinosema* spp., the sequence similarity was higher for *T. ratisbonensis* (AY695845) than for *T. kingi* (DQ019419) having 1389 out of 1396 bp (99.5%) identical for *T. ratisbonensis* with 7 bp difference and 1379 out of 1399 bp were identical (98.6%) for *T. kingi* with 20 bp different nucleotides including 10 gaps, respectively. The ITS region had only one BLAST hit with *T. hippodamiae* (KM883009) with 94% similarity and 49 out of 52 bp identity. The LSU rDNA showed most similarity (96.8%, 449/464 bp) with *Nosema* sp. isolate 7 (AF240356) and differed in 15 bp including five gaps. About 96.6%

similarity was given with *Tubulinosema* sp. isolate Bpas2 (MF998087) with 439 out of 454 bp identity and 15 nucleotides difference. The complete 1915 bp sequence showed most similarity to *T. loxostegi* with 98.7% (1854/1878 bp) followed by 98.4% similarity to *Tubulinosema* sp. isolate Bpas2 (1849/1880 bp).

The Maximum Likelihood (ML) phylogenetic analysis of aligned SSU rDNA (~1400 bp) sequences of 23 representatives of five microsporidia clades (Table 4-3) clustered *T. suzukii* with *Thelohania (Kneallhazia) solenopsae* in clade III Aquasporidia (Figure 4-9) in agreement with Vossbrinck et al. (2014), although *T. solenopsae* was originally designated to clade V Marinosporidia but marked as Terresporidia.

ML phylogenetic analysis of SSU rDNA sequences of other available *Tubulinosema* species (Table 4-4) with high BLAST scores and clade III microsporidian species *Brachiola algerae* as outgroup clustered *T. suzukii, T. loxostegi* and *T. pampeana* to one group based on the high identity scores of the partial SSU rDNA sequence (Figure 4-10 a). The according distance matrix (Table 4-5) showed lowest genetic distance of the SSU rDNA sequence to *T. pampeana* and another *Bombus*-infecting *Tubulinosema* sp. (*T.* sp. isolate Bpas2), which is shown by the lowest nucleotide count difference.

Furthermore, phylogenetic analysis of all available Tubulinosematidae SSU-ITS-LSU sequences in NCBI GenBank revealed *T. hippodamiae* and *T. suzukii* forming a sister group (Figure 4-10 b). Moreover, *T. loxostegi* appeared as paraphylum to *T. suzukii*. Here, *Drosophila*-infecting *T. kingi* and *T. ratisbonensis* were forming a sister group but also the *Bombus* sp.-infecting *Tubulinosema* were forming one cluster based on high identity scores. Differently to SSU rDNA phylogeny, both groups were less close related to *T. suzukii.* The small subunit rDNA region contained only five parsimony informative positions, the ITS rDNA contained 14 and the 580 reverse region in the large subunit rDNA contained 19 parsimony informative positions (Table 4-6). Overall, the topology of ML, NJ and MP trees was identical for both datasets SSU and SSU-ITS-LSU with varying bootstrap values.

Table 4-3: GenBank Accession number of microsporidia representatives of all five clades.

Microsporidian species	**Accession number**	**SSU Sequence length (bp)**	**Clade**
Amblyospora opacita isolate 1	AY090052	1300	I
Amblyospora sp. clone 1	AJ252949	1331	I
Amblyospora weiseri isolate 1	AY090048	1296	I
Basidiobolus ranarum strain FSU 770	JQ014008	1066	Outgroup
Endoreticulatus schubergi	L39109	1251	IV
Hyalinocysta chapmani	AF483837	1303	I
Kabatana takedai	AF356222	1371	V
Marssoniella elegans isolate 1	AY090041	1283	I
Microsporidium prosopium	AF151529	1346	III
Nosema apis	X73894	1198	IV
Nosema bombycis	L39111	1233	IV
Nosema carpocapsae	AF426104	1234	IV
Nosema pyrausta	AY958071	1207	IV
Nosema vespula	U11047	1244	IV
Orthosomella operophterae, Oxford isolate	AJ302317	1279	IV
Paranosema whitei	AY305323	1335	II
Parathelohania anophelis	AF027682	1286	I
Polydispyrenia simuli isolate 1	AY090069	1375	II
Schroedera plumatellae	AY135024	1341	III
Septata intestinalis	L39113	1294	IV
Thelohania (Kneallhazia) solenopsae	AF031538	1380	III
Trichonosema pectinatellae	AF484695	1368	III
Tubulinosema suzukii	MN631017	1402	III
Vavraia oncoperae	X74112	1326	III

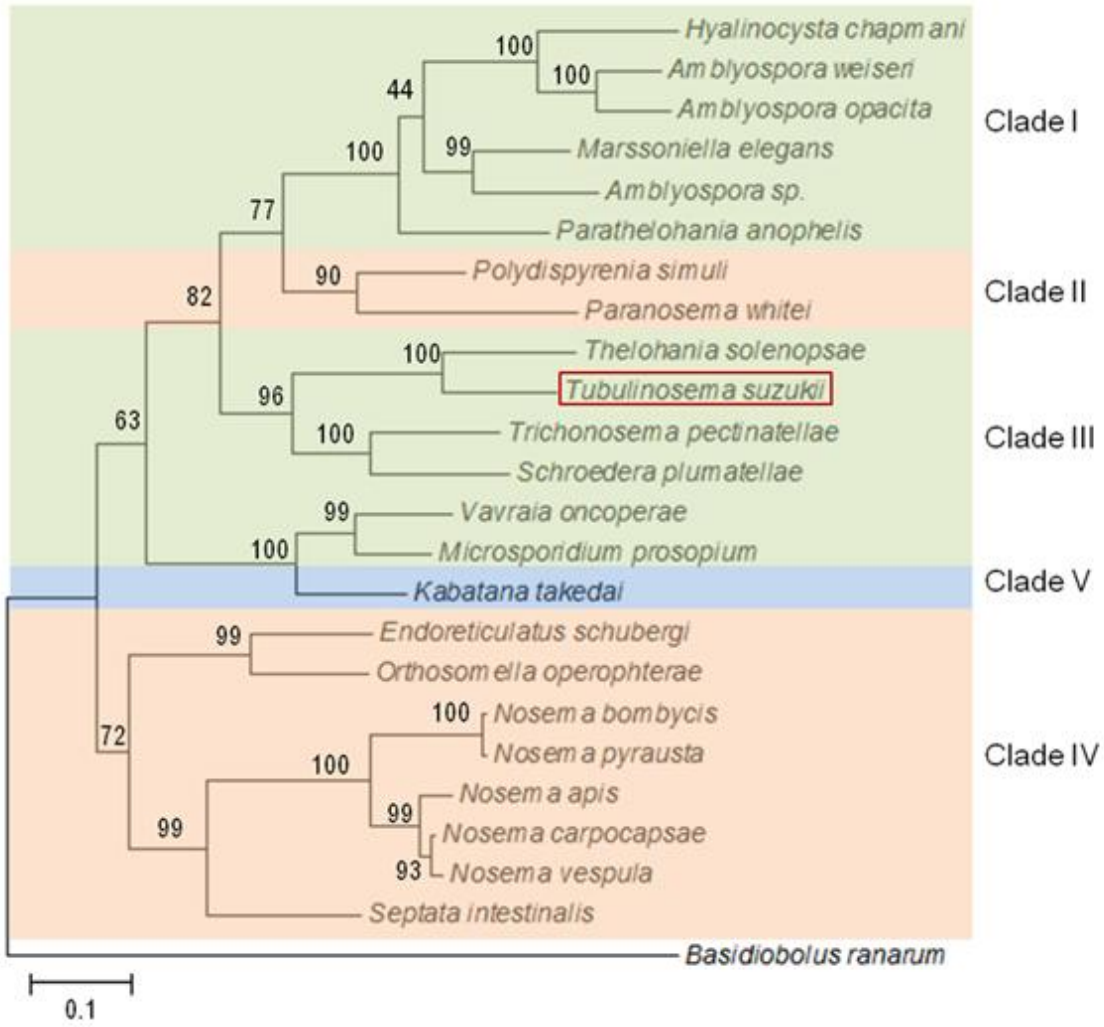


Figure 4-9: Maximum Likelihood analysis of 1402 bp SSU rDNA of *T. suzukii* showing its relationship to other microsporidia representatives from clade I and III (Aquasporidia, green), II and IV (Terresporidia, orange) and V (Marinosporidia, blue).

The analysis involved 24 nucleotide sequences with a total of 1542 positions in the final dataset. The tree with the highest log likelihood (-21831.1) is shown. The percentage of trees in which the associated taxa clustered together in 500 bootstrap replicates is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Basidiobolus ranarum* was set as outgroup.

Table 4-4: Microsporidian species, host and accession number of the whole SSU-ITS-LSU region of Tubulinosematidae used for the phylogenetic analysis in Figure 4-10.

T. hippodamiae sequences were aligned and compiled to one consensus to achieve the largest possible fragment. Length of sequence fragments used for alignments and phylogenetic analyses are given in the right-hand column.

Microsporidian species	**Host**	**Order**	**Accession number**	**Sequences length (bp) used for † SSU, ‡ SSU-ITS-LSU phylogeny**
Brachiola (Anncaliia) algerae	*Homo sapiens*	Primates	AY230191	† 1390, ‡ 1879
Tubulinosema acridophagus	*Schistocerca americana*	Orthoptera	AF024658	† 1398
Tubulinosema hippodamiae	*Hippodamia convergens*	Coleoptera	JQ082890	† 1398
Tubulinosema hippodamiae	*Hippodamia convergens*	Coleoptera	KM883009	Consensus created with JQ082890: ‡ 1866
Tubulinosema kingi	*Drosophila willistoni*	Diptera	DQ019419	† 1392, ‡ 1876
Tubulinosema loxostegi	*Pyrausta/ Loxostegi sticticalis*	Lepidoptera	JQ906779	† 1398, ‡ 1876
Tubulinosema pampeana	*Bombus atratus*	Hymenoptera	KM883008	† 1378, ‡ 1825
Tubulinosema ratisbonensis	*Drosophila melanogaster*	Diptera	AY695845	† 1398, ‡ 1855
Tubulinosema sp. isolate Bpas2	*Bombus pascuorum*	Hymenoptera	MF998087	† 1381, ‡ 1877
Tubulinosema sp. LS4M1	not determined (n.d.)	n.d.	KX379714	† 896
Tubulinosema suzukii	*Drosophila suzukii*	Diptera	MN631017	† 1402, ‡ 1915

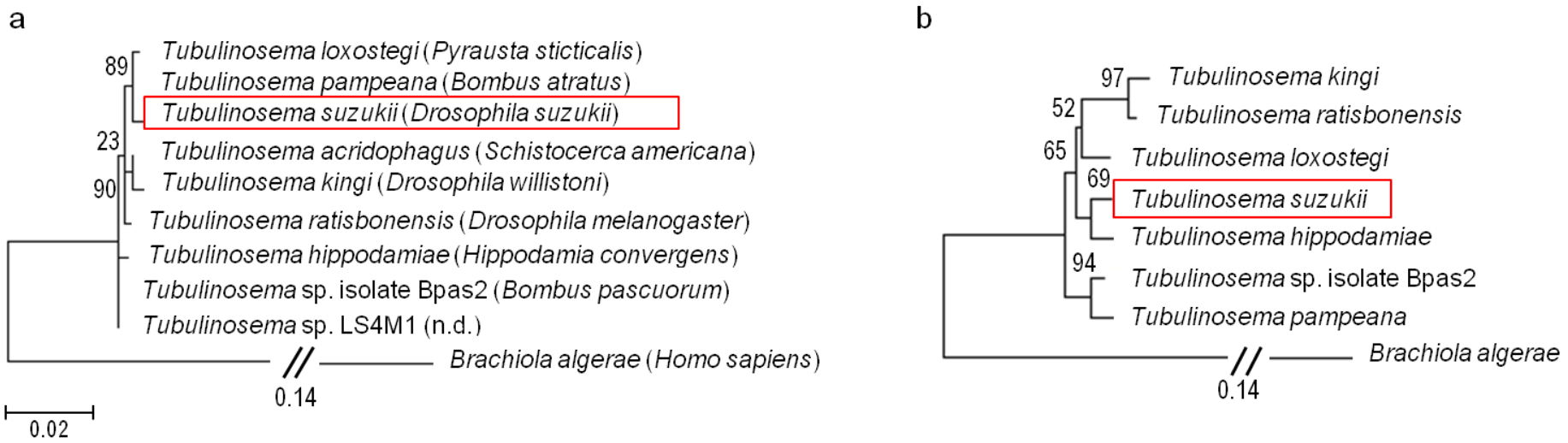


Figure 4-10: Maximum Likelihood phylogenetic analyses based on the small ribosomal subunit gene (a) of Tubulinosematidae (with host type) and (b) the complete ribosomal genes (SSU-ITS-LSU).

(a) includes 1402 bp of the SSU and (b) 1915 bp SSU-ITS-LSU of *T. suzukii*. The analyses involved (a) 10 sequences with a total of 1416 positions and (b) 8 sequences with a total of 1936 positions in the final dataset. The trees with the highest log likelihood (-3138.1/-4848.6) are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. *Brachiola algerae* was set as the outgroup for both trees.

Table 4-5: Estimates of evolutionary divergence between sequences.

The number of nucleotide differences per sequence between sequences is shown. The analysis involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1416 positions in the final dataset. Two species were identified which include each one undetermined nucleotide that is different to all other species if it is not guanine (underlined, probability for sequence difference is shown as *P*: *T. hippodamiae* R=A/G, *P*=0.5, and *Tubulinosema* sp. isolate Bpas2 N=A/G/T/C, *P*=0.75). Matrix includes lowest and highest (bracket) possible nucleotide differences.

Species	Sequence length (bp)	*T. suzukii*	*T. hippodamiae*	*T. loxostegi*	*T. acridophagus*	*T. ratisbonensis*	*T.* sp. isolate Bpas2	*T. pampeana*	*T.* sp. LS4M1	*T. kingi*
T. suzukii	1402									
T. hippodamiae	1398	9 (10)								
T. loxostegi	1398	5	8 (9)							
T. acridophagus	1398	7	7 (8)	6						
T. ratisbonensis	1398	8	8 (8)	7	5					
T. sp. isolate Bpas2	1381	3 (4)	3 (5)	5 (6)	4 (5)	4 (5)				
T. pampeana	1378	0	6 (7)	2	4	5	3 (4)			
T. sp. LS4M1	896	3	2 (3)	2	1	2	0 (1)	0		
T. kingi	1392	12	10 (11)	9	3	8	7 (8)	7	3	
B. algerae	1390	291	289 (290)	290	288	287	287 (288)	288	179	288

Table 4-6: Parsimony informative positions of SSU-ITS-LSU rDNA alignment of 7 *Tubulinosema* species including the 1915 bp of *T. suzukii* and *Brachiola algerae* as outgroup. In total, 38 positions in the 1915 bp sequence were informative.

Species	**SSU**					**ITS**														**LSU**																		
T. suzukii	C	A	T	T	G	A	T	A	A	A	A	A	T	A	T	A	G	G	G	A	T	A	A	G	T	A	G	A	A	C	A	T	C	T	T	A	T	G
T. hippodamiae	.	G	A	G	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	T	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.
T. sp. *Bpas2*	.	G	A	G	.	C	A	.	T	T	.	T	A	G	A	G	T	T	T	G	G	T	.	T	.	.	.	.	.	T	T	C	A	A	.	G	A	.
T. kingi	.	G	.	A	A	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	A	C	T	A	G	G	T	T	C	T	.	.	G	G	T
T. ratisbonensis	T	G	C	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	A	C	T	A	G	G	T	T	C	T	.	.	G	G	T
T. pampeana	.	.	.	.	.	C	A	.	T	T	.	T	A	G	A	G	T	T	T	G	G	T	.	T	.	.	.	.	.	.	.	.	A	.	C	.	.	.
T. loxostegi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	A	.	.	G	.	.	T	A	.	.	.	.	G	T	T	C	A	A	.	G	A	.
B. algerae	T	G	A	.	A	-	.	T	.	.	.	G	.	T	A	.	T	.	T	.	C	.	.	T	G	.	A	G	.	-	T	C	G	.	C	T	A	.
Position	887	1050	1164	1165	1409	1421	1426	1427	1430	1431	1434	1436	1438	1439	1440	1441	1442	1444	1447	1678	1679	1680	1682	1683	1685	1687	1688	1690	1692	1733	1734	1735	1736	1737	1739	1743	1747	1749

DISCUSSION

This study reports a new microsporidium isolated from infected *D. suzukii* larvae and adults, which has clear ultrastructural and sequence characteristics of the family Tubulinosematidae (Franzen et al., 2005b). Based on the morphological and ecological character states and the comparative sequence analyses presented here, a new *Tubulinosema* species is proposed, *Tubulinosema suzukii* sp. nov.

The occurrence of tubules of various sizes and shapes on the surface of the meront and sporont is the most striking feature of the genus *Tubulinosema* (see Table 4-7, Figure 4-4 c). Other common features of the genus *Tubulinosema* include diplokaryotic nuclei, slightly anisofilar polar filaments with diameters ranging between 64-157 nm and arranged in coils of 10-18 turns. Furthermore, *Tubulinosema* spores are ovoid or slightly pyriform and range in size 3.9-4.3 × 2.4-2.6 µm. The greatest variability among *Tubulinosema* species is in the form and size of meronts and sporonts and nuclear ploidy (Kramer, 1964; Franzen et al., 2005b, 2006). However, these data are lacking for some *Tubulinosema* and cannot be used for comparative purposes (Table 4-7). In some species of *Tubulinosema* the tubules of late sporonts are packed in clusters on the membrane surface. The tubules of previously described *Tubulinosema* species have been reported to be in the range of 20 to 60 nm being equally or scanty distributed over membrane surface (Armstrong et al., 1986; Streett and Henry, 1993; Franzen et al., 2005b, 2006; Plischuk et al., 2015). In contrast, the tubules of *T. suzukii* are larger than other species being approximately 75 nm in diameter, occasionally reaching the periphery of the host cell cytoplasm. *Tubulinosema suzukii* and *T. maroccanus* show tubules to be at a distance from the membrane surface of the meront or sporont (Issi et al., 2008). And both have a bipartite polaroplast and a second exospore layer (Figure 4-6 a, b; 4-7 a, b). Regular chains of four meronts in Giemsa-stained smears as observed for many *Nosema* spp. (Brooks et al., 1985) were not found in light microscopy, but chains and clusters of three (Figure 4-3 d, e) as described for *T. maroccanus* (Issi et al., 2008).

When discovering *T. suzukii* it appears not to be closely related to previously described microsporidia in drosophilids. Comparison with two *Tubulinosema* spp. from *Drosophila willistoni* (*T. kingi*, formerly known as *Nosema kingi*) (Burnett and King, 1962) and *D. melanogaster* (*T. ratisbonensis*) (Franzen et al., 2005b) showed limited shared characteristics (Figure 4-10, Table 4-7). Genetic distance estimation by nucleotide differences in the SSU rDNA showed a far relatedness compared to other Tubulinosematidae except for *T. pampeana* that is lacking some nucleotides of the SSU sequence (Table 4-5). Some morphological characteristics like form and size of mature spores are similar to *T. loxostegi*, whereby the combination of the number and arrangement of polar filament coils was different (Table 4-7). Furthermore, the *T. loxostegi* exospores structure with a spiky

double layer was different to *T. suzukii* and no teratoid sporogony was observed (Malysh et al., 2013b).

Tissue tropism, on the other hand, is similar among all described *Tubulinosema* spp. typically showing heavily infected adipose tissue with infection of the midgut cells, tracheal matrix, muscle cells and ventral nerve cord (Table 4-7). Franzen et al. (2005b, 2006) found infection of gonads, ovary and testis in *D. melanogaster* and *D. willistoni* as well as infected neural tissue in *D. melanogaster*. Data on infected testis of male *D. suzukii* could not be provided for *T. suzukii* in this study, but seven out of eleven female flies showed spores inside ovaries or in the ovary-surrounding adipose tissue. Furthermore, hypodermis tissue heavily packed with spores was firstly described here and has been never observed for other *Tubulinosema* spp.

Phylogenetic analysis of SSU rDNA sequence revealed *Thelohania solenopsae* as next neighbour to the *T. suzukii* forming a sister group in clade III microsporidia (Aquasporidia, microsporidia with aquatic origin). Nucleotide BLAST searches revealed close relatedness to other *Tubulinosema* species. The sequence similarity of the *Tubulinosema* genus is high with low genetic variability (Malysh et al., 2013a), clearly indicating its assignment to this genus.

Comparing the SSU rDNA sequence with those of other *Drosophila*-infecting *Tubulinosema* spp., the sequence similarity was higher for *T. ratisbonensis* than for *T. kingi*, but the best BLAST match was achieved by *T. loxostegi.* BLAST search of the ITS region gave only a hit with *T. hippodamiae*. Phylogeny of the SSU rDNA showed *T. suzukii* formed a cluster with *T. pampeana* and *T. loxostegi* (Figure 4-10 a). Similar findings with a *T. loxostegi*/*T. pampeana* cluster were described by Plischuk et al. (2015). The sister clade to *T. pampeana*/*T. loxostegi*, containing *T. acridophagus* and *T. kingi,* was previously confirmed (Franzen et al., 2006). Moreover, SSU-ITS-LSU showed a different pattern to SSU in the phylogenetic analyses, whereby *T. suzukii* formed a sister group with *T. hippodamiae* instead of *T. pampeana* and *T. loxostegi*. However, the position of *T. ratisbonensis* is not robust comparing all phylogenetic trees. Some authors argued that the close relationship and the low genetic variability of *Tubulinosema* species make it challenging to distinguish them only by the SSU rDNA sequence (Malysh et al., 2013a). Here it was shown that SSU phylogeny was not fully consistent with SSU-ITS-LSU phylogenies.

The complete 1915 bp sequence including the partial SSU, the complete ITS and partial LSU rDNA gene showed most similarity in BLAST search to *T. loxostegi* (sequence identity 98.7%) also applicable for the small ribosomal subunit, but only SSU phylogeny confirmed the close relatedness. The small subunit rDNA region contained only five parsimony informative positions, the ITS contained 14 and the 580 reverse region in the large subunit rDNA contained 19 parsimony informative positions (Table 4-6). From the information by running comparative analyses on the complete dataset it can be concluded with a high degree of

certainty that *T. kingi* and *T. ratisbonensis* are sister taxa and, not surprisingly, are both parasites of drosophilid flies. There is a good level of support for *T. pampeana* and *Tubulinosema* sp. isolate Bpas2 having a close affinity as illustrated in Figure 4-10 b. Both species are parasites of bees in the genus *Bombus* and therefore may be important factors in plant pollination. Although the SSU sequences for *T. suzukii* and *T. pampeana* are identical, it appears that *T. hippodamiae* to be the most closely related species to the new species *T. suzukii*, described here (Figure 4-10 b). *T. hippodamiae* and *T. suzukii* have unrelated insect hosts. It is interesting to note that although they appear to be closely related based on rDNA analysis, the *Tubulinosema* species included in this study infect five different orders of insects (Orthoptera, Coleoptera, Hymenoptera, Lepidoptera and Diptera).

As stated by Cheney et al. (2001) and Ironside (2013), *inter alia*, an additional criterion for molecular species discrimination is the amplification and phylogeny of supplementary marker genes such as β-tubulin (Lee et al., 2008a), RPB1 and elongation factor 1α. Single-copied protein-coding regions with high sequence diversity are preferred for species demarcation, as ribosomal gene operons are highly conserved among species but appear multi-copied in microsporidia genome (Cheney et al., 2000). Here, the RPB1 gene was chosen, although it also appears in multiple copies in *Nosema bombycis* (Ironside, 2013). However, RBP1 could not be amplified with any primer combination, as most degenerate oligonucleotide primers were designed on more distantly related microsporidia *Vairimorpha* sp. and *Nosema* sp. (Hirt et al., 1999; Kyei-Poku and Sokolova, 2017) or based on conserved amino acid motifs from plant, animal and fungal sequences (Stiller and Hall, 1997). Since no RPB1 sequences are available for other *Tubulinosema* spp., the RPB1 sequence of *T. suzukii* alone would not be of use for species demarcation. Microsporidia species definition is still under construction, so that SSU rDNA sequence differences as well as phenotype differences and host type are used for species demarcation until now (Vossbrinck et al., 2014). As more species are discovered and more genes are sequenced a clearer picture will emerge.

In summary, based on light and electron microscopy as well as phylogenetic analyses, it is evident that the microsporidium found in *D. suzukii* is a member of the genus *Tubulinosema* but it is sufficiently different from other *Tubulinosema* species to be considered as a new species *Tubulinosema suzukii* sp. nov.

Table 4-7: Comparison of morphological and ultrastructural features of *Drosophila*-infecting Tubulinosematidae *T. kingi*, *T. ratisbonensis* and *T. suzukii* and closely related *T. hippodamiae*, *T. loxostegi* and *T. pampeana.*

Features	*T. suzukii*	*T. hippodamiae*	*T. loxostegi*	*T. pampeana*	*T. kingi*	*T. ratisbonensis*
Host	*D. suzukii* several *Drosophila* spp.	*Hippodamia convergens*	*Pyrausta/ Loxostege sticticalis*	*Bombus atratus*	*Drosophila willistoni* several *Drosophila* spp. braconid wasps	*D. melanogaster* several *Drosophila* spp.
Tissue infected	A, H, Mg, Ms, N, Ov, Tr	A, CT, Hg, Ms, Mt, N, Ov	A, CT, Ms	A, Mg, Ms, Mt, N	A, G, Mg, Mt	A, G, Mg, N
Meronts (M) **Size (µm) Ø** **Nuclei** **Abundance of tubules**	Round or irregular 3.8–4.6 Two or four Occasionally, M and St	Round to slightly ovoid One or two Not observed	No data Prominent, St, young Sp	No data Prominent surrounding St	Round to oval 4-5 One, two or four Scanty, M and St	Round, oval or irregular 4-5 One, two or four Prominent, M and St
Sporonts (St) **Size (µm) Ø** **Nuclei**	Round to irregular Tubules occasionally present 6 Two or four	Round to ovoid	No data	No data	Oval to irregular 8-10 Two or four	Round to oval or irregular 4 Two or four
Spore (Sp) shape **Size (µm) fresh** **Size (µm) fixed** **Ratio length: width**	Ovoid to slightly pyriform 4.29×2.47 (length × width) 3.84×2.40 1.73 (0.58)	Ovoid-pyriform 3.90×2.50 3.58×2.06 1.5-1.6	Wide-oval 4.2×2.4 1.8	Ovoid 4.0×2.37 3.98×1.88 1.6-1.7	Ovoid to slightly pyriform 4.3×2.6 3.6×2.4 1.7-1.8	Slightly pyriform 4.18×2.48 3.67×2.06 1.6-1.7
Number of coils (*) **Arrangement**	8-11 (median: 10), (2) One or two rows	10-14 One or two rows	10-14 (2-3)	14-18 One or two rows	13 (3-4) One or two rows	9-14 (3-4) One row
Polar filament diameter (posterior-anterior)	Slightly anisofilar (95–115 nm)	Slightly anisofilar	Slightly anisofilar (49–64 nm)	Isofilar (103.4 nm)	Isofilar to slightly anisofilar (117/104–119/140 nm)	Slightly anisofilar (125–157 nm)
Polaroplast	Bipartite: Lamellar and tubular structures	Lamellar	Bipartite: thin and thick lamellae	Lamellar	Lamellar	No data
SSUrDNA **Similarity to** **Similarity to**	MN631017 JQ906779 99.71% KM883008 100%	JQ082890, KM883009 AF024658 99.0% AY695845 99.0%	JQ906779 AF024658 99.6% AY695845 99.4%	KM883008 JQ906779 98.4% AF024658 98.3%	DQ019419 AY695845 99.0% AF024658 98.9%	AY695845 DQ019419 99.0% AF024658 99.6%
Reference	This study, *ibidem*	Bjørnson et al. (2011)	Malysh et al. (2013b)	Plischuk et al. (2015)	Burnett and King (1962) Kramer (1964) Armstrong et al. (1986) Franzen et al. (2006)	Franzen et al. (2005b)

A, adipose tissue, CT, connective tissue; H, hypodermis; G/OV, gonads/ovary, Mg, midgut, Ms, muscle, Mt, Malpighian tubules, N, neural tissue. (*) coils of less electron density.

TAXONOMIC SUMMARY

Type host: *Drosophila suzukii*

Other hosts: *Drosophila willistoni*, *Drosophila melanogaster*, *Trichopria drosophilae* under laboratory conditions.

Locality: Infected *D. suzukii* were obtained from the laboratory strain SAM (*D. suzukii* America, sampled in Oregon, USA) from Rupinder Kaur and Wolfgang Miller, Medical University of Vienna, Austria.

Site of infection: Fat body, midgut epithelium, midgut muscularis, thoracic and abdominal muscles, hypodermis, tracheal matrix, ventral nerve cord, adipose tissue surrounding ovary and inside immature ovary. No data available for spermatheca or testes infection.

Transmission: Horizontally (per os) through cadaver and putatively vertically through infected adipose tissue surrounding ovary (trans-ovum), described in Chapter VI. Spores inside immature ovary were observed. Autoinfection within the host.

Merogony: Direct contact to host cell cytoplasm by tubular elements. Meronts divide by binary fission. Meronts with one, two and four diplokarya were observed. Tubules were present on late meront membrane surface, often observed in periphery.

Sporogony: Direct contact to host cell cytoplasm by tubular elements. Diplokaryotic sporonts divide into two sporoblasts by binary fission (disporoblastic sporogony), no plasmodia or sporophorous vacuoles observed. Sporonts with small tubules on membrane surface, packed in bundles. Single tubules equally distributed on the membrane surface were not observed.

Spores: Ovoid to slightly pyriform, diplokaryotic. Spore size about 4.29±0.32 × 2.47±0.18 µm (mean ± SD, N=143) in wet mount preparations. Lamellar and tubular structures of polaroplast, slightly anisofilar polar filament with 8-11 (median: 10) coils in one or two rows each having about five layers. Extruded polar filament size about 67.80±13.20 µm (mean ± SD, N=30) in wet mount preparations.

Specimen deposition: Infected *D. suzukii* flies, slides with smears and flies embedded in paraffin wax or in Spurr resin are stored at the Institute for Biological Control, Federal Research Centre for Cultivated Plants, Julius Kühn-Institut, Heinrichstraße 243, 64287 Darmstadt, Germany. SSU-ITS-LSU rDNA: GenBank accession number: MN631017.

Etymology: Species name after the host species.

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Author´s contribution:

S. Biganski, R.G. Kleespies, J.A. Jehle and C.R. Vossbrinck conducted the design of the study and wrote the final manuscript. S. Biganski performed the experiments, analyzed the data and drafted the first version of the manuscript. R.G. Kleespies carried out transmission electron microscopy. J.T. Wennmann and C.R. Vossbrinck contributed to the molecular analyses. R. Kaur contributed to the Drosophila breeding lines.

CHAPTER V: FITNESS EFFECTS OF THE NEW MICROSPORIDIAN SPECIES *TUBULINOSEMA SUZUKII* INFECTING *DROSOPHILA SUZUKII*

ABSTRACT

Microsporidian infections of insects are important natural constraints of population growth, often reducing lifespan, fecundity and fertility of the infected host. The recently discovered *Tubulinosema suzukii* infects *Drosophila suzukii* (spotted wing drosophila, SWD), an invasive pest to many fruit crops in North America and Europe. In laboratory tests, fitness effects on larval and adult stages were explored. High level infection after larval treatment caused up to 70% pupal mortality, a decreased lifespan and a 70% reduced oviposition of emerging adults in biparental infection clusters. A clear sex-linkage of effects was noted; females were specifically impaired, as concluded from fecundity tests with only infected female parents. Additive effects were noted when both parental sexes were infected, whereas least effects were found with only infected male parents, though males where mostly negatively affected in their survival if they were fed with *T. suzukii* spores in adult stage. Although most negative effects on fitness parameters were revealed after larval treatment, offspring infection did never reach more than 4%, suggesting a limited vertical transmission. For that reason, a self-reliant spread in natural SWD populations would probably only occur by spore release from cadavers or frass.

INTRODUCTION

Microsporidia are spore-forming, obligate intracellular parasites belonging to Fungi kingdom (Sprague et al., 1992; Hirt et al., 1999; Keeling et al., 2000). They infect a huge range of arthropod species but also vertebrates and even humans (Sprague and Becnel, 1999). Spores enter the host cell cytoplasm either by oral ingestion or other infection routes, such as vertically trans-ovarial: inside egg or embryo (Dunn et al., 2001), trans-ovum: outside egg shell (Goertz and Hoch, 2008; Becnel and Andreadis, 2014), venereal: paternal (Kellen and Lindegren, 1971) or horizontally via injection of contaminated ovipositors by vectors like parasitoids (Siegel et al., 1986; Futerman et al., 2006). To spread and reproduce within the host tissue, microsporidia undergo several morphological changes from meronts (proliferation) to spores (sporogony) (Becnel and Andreadis, 2014; Vávra and Larsson, 2014). Severe infections located in one or more host tissues or organs can lead to highly negative health effects on the host, as it is known for *Nosema* disease in honeybees causing heavy intestine disorders (Bailey, 1955; Higes et al., 2006; Mudasar et al., 2013). Though, microsporidia are rarely fast-killing pathogens, they are able to reduce populations by weakening the host fitness while reducing fertility and offspring, growth rate, and lifespan.

Moreover, horizontal and vertical transmission can lead to self-dissemination within one and towards other host populations. Horizontal transmission often depends on host death caused by high virulence and usually correlates with high density of host populations, e.g. in honeybee hives or salmon hatcheries (Hauck, 1984; Lipsitch et al., 1995). In contrast, vertical transmission is frequently found with microsporidia with low virulence, depending on host survival, fertility and number of infected offspring to ensure their own reproduction (Kellen et al., 1965; Dunn and Smith, 2001). Vertical transmission has been noted for at least fourteen microsporidian taxa (Terry et al., 2004). Some are known to reduce fecundity and fertility in several amphipod and lepidopteran hosts (Mercer and Wigley, 1987; Bauer and Nordin, 1989; Ebert, 1995; Futerman et al., 2006; Goertz et al., 2008). Although there are some microsporidia showing increased mortality of their insect host, their usage as pest control agent in plant protection has been rarely considered. *Paranosema locustae*, formerly named *Nosema locustae*, infecting acridids, is an example of efficient but also problematic use of microsporidia in biological control (BC) (reviewed by Lockwood et al., 1999).

Due to human activity, travel and worldwide transport of agricultural products, but also climate change, the spread of pest insects from cultivated plants is highly facilitated, resulting in the invasion of new pests and diseases to previously unaffected regions (Kiritani and Yamamura, 2003). About 10 years ago, the spotted wing drosophila (SWD) *Drosophila suzukii* (Walsh et al., 2011) has become a major pest in commercial orchards for soft-skinned fruits in Europe, North and South America and Asia (Walsh et al., 2011; Cini et al., 2012; Tochen et al., 2014). Naturally occurring antagonists, macroorganisms (Chabert et al., 2012; Gabarra et al., 2015) and microorganisms may play an important role for BC of SWD but efficient microbial antagonists have not been found yet (Gabarra et al., 2015; Woltz et al., 2015; Cuthbertson and Audsley, 2016). Different antagonistic fungi and bacteria have been screened but also critically discussed due to inefficiency and/or inappropriate application (Woltz et al., 2015; Haye et al., 2016; Biganski et al., 2018) and some viruses have been identified from SWD (Medd et al., 2017; Carrau et al., 2018).

Recently, a microsporidian infection was discovered in SWD flies originated from Oregon, USA (Kaur et al., 2017) and a new species. *Tubulinosema suzukii* (family: Tubulinosematidae) was described (Chapter IV). To explore the potential effect of *T. suzukii* infection on fitness parameters of SWD hatching and survival rates, egg production and offspring rates for infections either starting in larval or adult stages were tested in laboratory experiments. *T. suzukii* had strong impact on every parameter when infecting larvae.

MATERIAL AND METHODS

Insect host rearing

Microsporidia-free *D. suzukii* (SWD) flies were kept in cages of 30×30×30 cm (BugDorm, MegaView Science Co., Taiwan) with tap water, a diet for adult flies (brewer´s yeast and sugar, each 1 g) and artificial oviposition medium as described elsewhere (Chapter IV, modified from Chabert et al., 2012). The oviposition medium was replaced weekly. If synchronous larvae were needed, the medium was replaced every 4 hours. Insect rearing and subsequent biotests were performed under following conditions: 22±1 °C, 50% r.H., 16/8 hours light/dark photoperiod. Microsporidia-free *Drosophila melanogaster* (DM) and *Drosophila willistoni* (DW) were kept under same conditions as described for SWD.

Preparation of *Tubulinosema suzukii* spores for SWD inoculation

Tubulinosema suzukii was first isolated from a SWD rearing originated from flies caught in Oregon, USA (Kaur et al., 2017). Adult SWD were grinded with a micro pestle, dissolved in sterile water and filtered through four layers of gauze and additionally filtered through cotton filter disc with 12-15 µm particle retention (Grade 1288, Ø 90 mm, Sartorius AG, Göttingen, Germany). Spores were spun down at 10,000 × *g* (Centrifuge 5424 R, rotor Nr. FA-452411, Eppendorf, Hamburg, Germany) and resuspended in 500 µl sterile tap water. Spore concentration and purity was determined with a Thoma hemocytometer under phase contrast microscope (DMRB, Leica, Wetzlar, Germany) following spore dilution in sterile tap water to final concentrations as required for subsequent biotests.

Preparation of standards in real-time quantitative PCR (qPCR)

Tubulinosema suzukii spores were extracted from adult SWD carrying an infection with spores (about 3 weeks after initial inoculation of L2 larvae with 10 µl containing 1.5×10^4 *T. suzukii* spores). To produce standard curves, 10 flies were grinded with a micro pestle. About 1×10^7 spores were purified with one filtration step through four layers of gauze mesh and a final purification with Percoll. For this purpose, 400 µl spore suspension was overlaid on 1.6 ml 75% Percoll (Merck, Darmstadt, Germany) dissolved in 1×PBS in a 2 ml reaction tube and spun down for 20 min at 12,900 × *g* and 15 °C in an Eppendorf centrifuge (5424 R, rotor Nr. FA-452411, Eppendorf, Hamburg, Germany). The spores were forming a band close to the bottom of the reaction tube. The spore band was washed twice in 1×PBS at 15,000 × *g* for 5 min, and the resulting pellet was dissolved in distilled water. Afterwards, spores were inspected for purity under phase contrast microscope (DMRB, Leica, Wetzlar, Germany). For

preparation of a qPCR standard, serial dilutions of purified spores were prepared with 10^1-10^6 spores in 100 µl distilled water.

SWD inoculation and DNA extraction for qPCR

L2 larvae of SWD were exposed to 10 µl spore suspension with 4×10^4 spores in a microtiter plate overlaid on 440 µl pureed apple. Every 2-3 days three larvae and later pupae or adults were removed and euthanized with ethyl-acetate and surface sterilized with 0.05% sodium hypochlorite. One was examined visually for infection by light microscopy with 400-fold magnification (DMRB, Leica, Wetzlar, Germany) and modified Giemsa-staining according to Eberle et al. (2012b). Two larvae per replicate were used for genomic DNA extraction as described above and following qPCR. This was repeated for adult SWD which were starved for 3 hours followed by bulk feeding for 18 hours with a spore suspension containing in total 5×10^5 *T. suzukii* spores mixed with blue food colour (modified droplet feeding method from Hughes and Wood, 1981). To each group of ten flies, 10 µl spore suspension were given. The time frame for euthanizing each four flies per replicate was kept larger than for larval inoculation, as infection could also appear delayed in adults (day 3, 5, 10, 18, 28, 38). Two flies were prepared for microscopy and Giemsa-staining and two others were used for genomic DNA extraction after surface sterilization with sodium hypochlorite.

Sample and standard spore preparations (see Preparation of standards in real-time PCR) were spun down at 15,000 × *g* for 10 min. The pellet was dissolved in 200 µl CTAB lysis buffer (AppliChem, Darmstadt, Germany). Following addition of 200 mg of glass beads (0.25–0.5 mm diameter, Roth, Karlsruhe, Germany) spores were broken by bead beating in a tissue disrupter at 24 mHz for 1 min (MP FastPrep ®-24 Tissue and Cell Homogenizer, MP Biomedicals, Eschwege, Germany). Lysis was performed, after adding 2 µl proteinase K (200 ng/µl, BIORON GmbH, Römerberg, Germany), by incubation at 56 °C on a thermo shaker mixing with 250 rpm for 18 hours. DNA was extracted with a two-step phenol-chloroform extraction with 25:24:1 phenol:chloroform:isoamyl alcohol. DNA preparations were washed twice in chloroform and finally subjected to ethanol precipitation (96% ethanol). DNA pellets were dissolved in 30 µl distilled water. Quantitative PCR reactions were carried out with 2 µl of DNA dissolved in distilled water and Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific GmbH, Darmstadt, Germany) using 12.5 µl Master Mix, 1 µl 10 mM forward primer Tn37 F and 1 µl 10 mM reverse primer Tn562 R and 8.5 µl Nuclease-free water per reaction with following reaction conditions: 94 °C 3 min initial denaturation, followed by 35 cycles of 94 °C for 45 sec, 50 °C for 30 sec, 72 °C for 90 sec and stepwise temperature increase from 50 °C to 94 °C in 0.5 °C every 5 sec (CFX96 Touch™ Real Time PCR Systems, Bio-Rad Laboratories GmbH, Feldkirchen, Germany).

Larval inoculation for lethal concentration (LC_{50}), survival and fecundity tests

A single L2 larva (three days-old) was placed in each cavity of a 96-well microtiter plate (Greiner Bio-One GmbH, Frickenhausen, Germany) filled with 440 µl pureed apple and overlaid with 10 µl spore suspension containing **a)** a concentration of 10^1 to 10^6 spores per µl (five concentrations in logarithmic scale) to determined LC_{50}, **b)** in total 5×10^2, 5×10^3 and 5×10^4 *T. suzukii* spores for hatching and survival experiments, **c)** in total 1.5×10^4 *T. suzukii* spores for fecundity experiments, and **d)** in total 5×10^4 *T. suzukii* spores for host range testing with *D. melanogaster* and *D. willistoni.* Untreated controls contained the equivalent amount of sterile tap water added to pureed apple.

Experimental design for LC_{50}, hatching and survival tests

Microtiter plates containing inoculated larvae were transferred into cylindrical cages (30 cm height, 25 cm diameter, closed with a nylon membrane) containing a water source, adult diet and oviposition medium (see Insect host rearing) changed twice a week. Mortalities in LC_{50} tests (18 days post inoculation) and hatching (19 dpi) and survival of adults (63 dpi) were recorded daily until all SWD died. The lethal concentration of 50% mortality after 18 dpi was calculated using Probit analysis. Mortality data were corrected for control mortality (Abbott, 1925).

Experimental design for fecundity tests

For fecundity experiments, inoculated larvae hatched and the adults were separated into male and female groups directly after hatching for three days to avoid premature mating. One three-day old naive male and female adult were then placed together for mating and oviposition They were kept in boxes (6 cm height, 10 cm diameter) containing a Petri dish (3 cm diameter) with oviposition medium prepared as described above and in chapter IV. After 48 hours, the Petri dish with oviposition medium was replaced by a new one and eggs were counted from the oviposition medium. Afterwards, the oviposition medium was placed in a separate box and eggs were reared until hatching. This procedure was repeated every two days until the last *T. suzukii* inoculated SWD pair died. Hatched offspring was determined 18 days after oviposition and sex ratios were recorded. Microsporidian-treated flies were post-hoc inspected for established infections and assigned to different groups in analyses: MF=male and female SWD infected, F=female infected/male healthy, M=male infected/female healthy. Transmission was examined in two separate trials (18 pairs) with both infected parents. Oviposition medium was changed every two days as described above and the oviposition rate was recorded. Eggs were transferred to fresh medium for

development. Eggs were not surface sterilized to avoid manipulation of the respiratory filaments resulting in higher mortality. Experiments were carried out in incubator (Rumed 3501, Rubarth, Laatzen, Germany) under rearing conditions: 22±1 °C, 60% r.H., 16/8 hours light/dark photoperiod.

Inoculation of adults and design of survival and fecundity experiments

Three days-old (survival experiment) or one day-old (fecundity experiment) SWD adults were placed together in groups of four (survival experiment) or separated into male groups and female groups (fecundity experiment) in a plastic box, where they were starved for 3 hours followed by bulk feeding overnight (about 18 hours) with 100 µl containing in total 3×10^5 *T. suzukii* spores mixed with blue food colour (modified droplet feeding method from Hughes and Wood, 1981). Only flies with blue abdomen were selected for the experiments, whereby one male and one female were placed together in a cage (containing diet and water). In survival experiments, daily survival was recorded until all adults died. For fecundity experiments, egg deposition was recorded every two days and hatched offspring was determined 18 days post oviposition. Microsporidia-treated flies were post-hoc microscopically inspected on developed infections.

Statistical analyses

Estimation of the median lethal concentration (LC_{50}) and slope of the concentration–mortality curve were calculated by Probit analysis using ToxRat® software (ToxRat Solutions, 2003). All other statistical analyses were conducted with R version 3.5.1 (2018-07-02) (R Core Team, 2018; RStudio Team, 2018). For test on normal distribution, Shapiro Wilk test was chosen with $\alpha=0.05$ level of significance. Numbers of independent replicates and sample size are indicated by R/N. For all statistical tests, a level of significance of $\alpha=0.05$ has been set if not indicated otherwise.

RESULTS

Median Lethal Concentration (LC_{50})

Inoculation of second instar SWD larvae with suspensions of five different spore concentrations of *T. suzukii* resulted in a LC_{50} of about 6.9×10^3 spores/µl (95% confidence limits (CL)=3.4×10^3-1.7×10^4 spores/µl, N=631, slope=0.915, Chi^2=21.683) (Figure 5-1). Mortality rates were determined by failure of hatching of imagines since death of larvae could not be recorded within the growth medium. Control mortality was 28.2% after 18 dpi. Maximum mortality was 80% at the highest concentration applied.

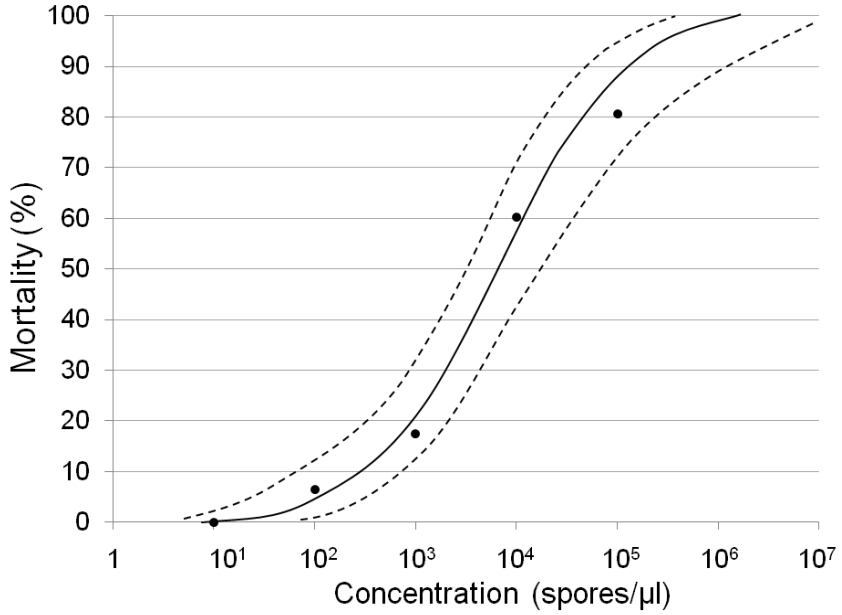

Figure 5-1: Concentration-mortality response of SWD L2 larvae 18 days after exposure to *T. suzukii* spores.

Dots=observed mortality, solid line=calculated concentration, dashed lines=95% upper and lower confidence limits (CL). Concentration response: *F*=76.966, *DF*=3, *P*(F)=0.003.

Quantification of infection process

To study the infection process, SWD L2 larvae were inoculated with 4×10^4 spores, followed by DNA extraction from larvae, pupae and adults and real-time quantitative PCR (qPCR) (Figure 5-2) and light microscopic examination (Figure 5-3). In third instars (L3) at 3-5 dpi, an infection equivalent to the DNA copies of 1.33±1.22 $\times10^6$ (mean ± SE) extracted spores was recorded (Figure 5-2). In early pupal stage, microsporidial DNA increased to 8.54±7.12 $\times10^6$ DNA copies per pupa and was stable until late pupal stage (8-13 dpi) with 6.8±2.97 $\times10^6$ copies. After the transition between late pupal stage to adulthood (13-20 dpi), the DNA amount increased by the factor of 20 in early adult stage with 13.3±7.91 $\times10^7$ copies per

individual and was significantly increased compared to 3-5 dpi DNA copies (*F*=4.444, *DF*=3, *P*(F)=0.02, Tukey HSD: 3-5 dpi-13-20 dpi: *P*=0.01). Correspondingly, Giemsa-stained early larvae (6 dpi) revealed just a few free developmental stages consisting of meronts and sporonts (Figure 5-3 a). Early pupal stage (8 dpi) showed meronts containing two nuclei as well as diplokaryotic sporonts and sporonts close to division/separation of nuclei (Figure 5-3 b). Later in pupal stage (10 dpi), few spores and predominantly (dividing) sporonts were observed (Figure 5-3 c). In late pupal stage (13 dpi), mainly spores could be found, but also sporoblasts (Figure 5-3 d). Adult stages (15 dpi) showed mostly binucleate sporoblasts and single spores (Figure 5-3 e), whereby the amount of spores was increasing with time (17 dpi, Figure 5-3 f).

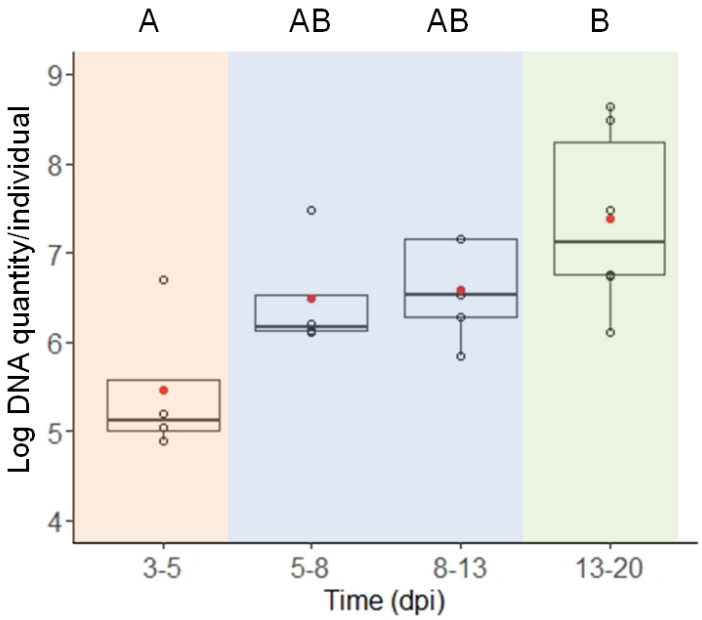

Figure 5-2: SSU rDNA copies of *T. suzukii* from single SWD larvae (orange), pupae (blue), adults (green).

The course of infection is starting from L2 larvae inoculated with 4×10^4 spores. Replicates/numbers (R/N): larvae 3-5: R/N=2/4, pupae 5-8: R/N=2/4, pupae 8-13: R/N=3/5, adult 13-20: R/N=3/6. One-way ANOVA: *F*=4.444, *DF*=3, *P*(F)=0.02, Tukey HSD: 3-5 and 13-20: *P*=0.01. Different letters indicate statistical difference. Boxplot indicates the median logarithmic DNA quantity per individual with mean (red dots), lower hinge=Q1 (25^{th} percentiles), upper hinge=Q3 (75^{th} percentile), lower whisker=Q1-1.5×interquantile range (IQR), upper whisker=Q3+1.5×IQR, and open dots=fluctuation (jittering).

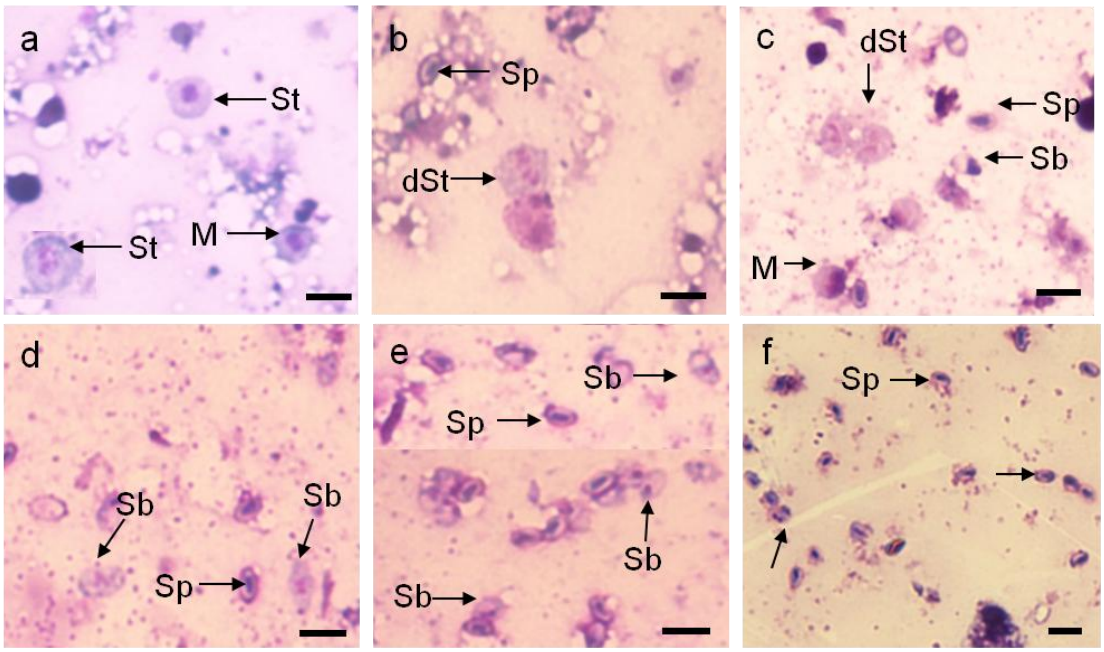

Figure 5-3: Bright-field light microscopy of Giemsa-stained smears. Developmental stages of *T. suzukii* isolated from SWD larvae, pupae and adults on different time points post inoculation. SWD were inoculated in L2 larval stage with 4×10^4 *T. suzukii* spores. (a) Larval stage (6 dpi). (b) Early pupal stage (8 dpi). (c) Pupal stages (10 dpi). (d) Late pupal stage (13 dpi). (e) Early adult stage (15 dpi). (f) Adult stage (17 dpi). Abbreviations: (M) meront containing two nuclei, (St) sporont, (Sp) spore, (dSt) dividing sporont, (Sb) sporoblast. Scale bar=5 µm.

Hatching and survival rate of inoculated SWD larvae

L2 larvae exposed to 5×10^4 *T. suzukii* spores showed 71.2% reduced hatching at 19 dpi (mean ± SE=19.84±8.41%) compared to the control (69.1±4.14) (Figure 5-4 a). Hatching at lower spore amounts did not statistically differ from the control group, though an intermediate mortality between those of the control and 5×10^3 spores was observed for the treatment with 5×10^2 spores (Chi^2=15.195, *DF*=3, *P*=0.0016, Dunn´s test: Control-5×10^1: *P*=1, Control-5×10^2: *P*=0.502, Control-5×10^3: *P*=0.002, 5×10^1-5×10^3: *P*=0.0043).

For host range testing, L2 larvae of *D. melanogaster* (DM) and *D. willistoni* (DW) were exposed to 5×10^4 of *T. suzukii* spores/well. This spore amount did not significantly reduce hatching in DM and DW compared to their corresponding controls (Chi^2=7.441, *DF*=3, *P*=0.059) (Figure 5-4 b).

Kaplan Meier survival analysis was performed with individuals from the same experiment, which achieved adulthood (Figure 5-5). Hatching appeared from day 13 to day 19, which was owed to some age differences of inoculated L2 larvae. Survival analysis was performed using

log rank test with Bonferroni adjustment and revealed significant differences in the lifespan of SWD adults for every treatment compared to the control (Survival formula: Chi^2=534, *DF*=3, *P*<2e-16) (Figure 5-5, Table 5-1). It was striking that the survival curves of the control group and the 5×10^2 was similar until day 35, then mortality increased for 5×10^2, indicating a delayed effect on the survival of adults when inoculated with very low spore concentration.

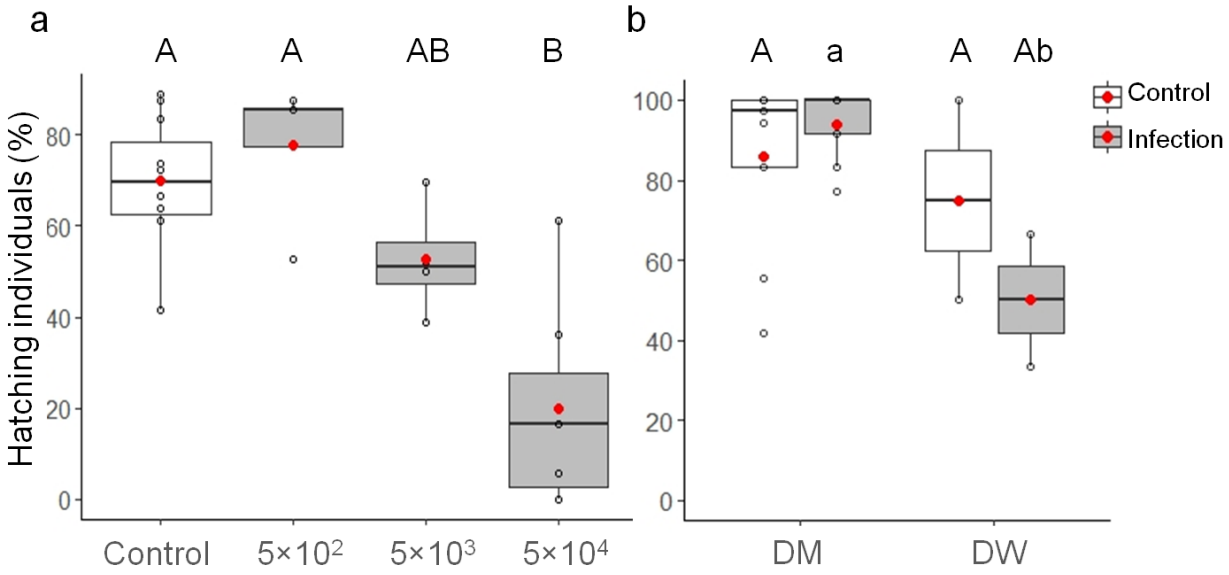

Figure 5-4: Hatching of SWD after exposure to *T. suzukii* spores.

(a) SWD hatching (%) at 19 dpi after exposure to different *T. suzukii* spore amounts (5×10^2, 5×10^3, 5×10^4) in L2 larval stage and control. Replicate/number (R/N): C=11/408, 5×10^2=4/180, 5×10^3=4/204, 5×10^4=7/264. Chi^2=15.195, *DF*=3, *P*=0.0016. (b) Hatching (%) of *D. melanogaster* (DM) and *D. willistoni* (DW) controls and exposed to 5×10^4 *T. suzukii* spores in L2 larval stage (R/N: DM Control =9/287, Infection=9/276; DW Control=3/48, Infection=3/48, Chi^2=7.441, *DF*=3, *P*=0.059). Different letters indicate statistical difference. Boxplot indicates the median percentage of hatching individuals (for boxplot description see Figure 5-2).

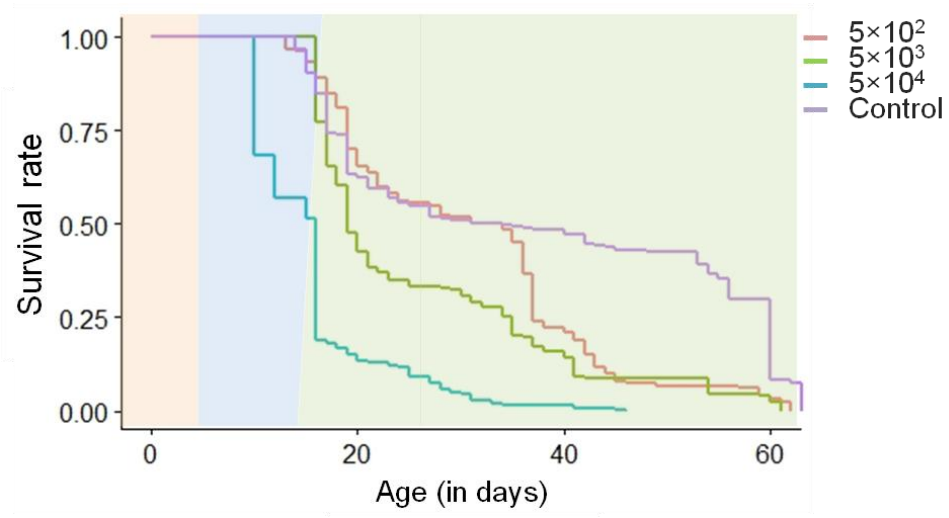

Figure 5-5: Kaplan-Meier survival curves of SWD, shown as complete lifetime from larval stage (day 0-6, orange field) to pupal stage (day 7-19, blue field) up to hatching and death in adult stage (green field).

Second instar larvae were either exposed to 5×10^2 spores (red line, replicates/number (R/N)=4/180), 5×10^3 spores (green line, R/N=4/204), 5×10^4 spores (turquoise line, R/N=7/264) or sterile water as control (purple line, R/N=11/408). Log rank test: Chi^2=534, *DF*=3, *P*<2e-16.

Table 5-1: Pairwise comparison of SWD survival after treatment in L2 larval stage using Log Rank test with Bonferroni adjustment.

P-values among all treatments are shown: control, L2 larvae treated with 5×10^2, 5×10^3 or 5×10^4 *T. suzukii* spores in 10 µl.

	5×10^2 spores	**5×10^3 spores**	**5×10^4 spores**
5×10^3 spores	0.0023	-	-
5×10^4 spores	< 2e-16	< 2e-16	-
Control	5.8e-09	< 2e-16	< 2e-16

Survival rate of inoculated SWD adults

To study the effect of late infection initiation, freshly hatched SWD adults were treated with 3×10^5 spores in 100 µl. In Kaplan Meier analyses (Figure 5-6), control males showed significantly higher survival rates to *T. suzukii*-inoculated (infected) males (*P*=0.027), infected females (*P*=0.0008) and control females (*P*=0.004), whereas control females had similar survival curves as both infection treatments for male and female. (Chi^2=13.1, *DF*=3, *P*=0.004, Table 5-2).

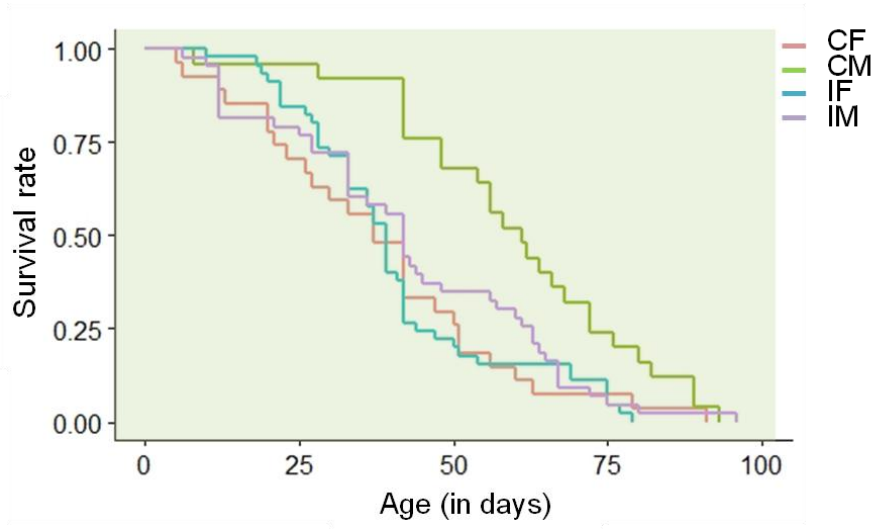

Figure 5-6: Kaplan-Meier survival curves of adult SWD inoculated with *T. suzukii* three days after hatching.

Separated male and female SWD could either feed on sterile water as control (CF=control female, red, replicates/number (R/N)=4/27; CM=control male, green, R/N=4/25) or a spores suspension with 3×10^5 *T. suzukii* spores (IF=inoculated female, turquoise, R/N=4/45; IM=inoculated male, purple, R/N=4/43). Log rank test: Chi^2=13.1, *DF*=3, *P*=0.004.

Table 5-2: Pairwise comparison of SWD survival after treatment in adult stage using Log Rank test with Bonferroni adjustment.

P-values among all treatments are shown: control females, control males, *T. suzukii*-infected females, and infected males.

	Control females	**Control males**	**Infected females**
Control males	0.004	-	-
Infected females	0.879	0.0008	-
Infected males	0.448	0.027	0.528

Fecundity and fertility of SWD treated with *T. suzukii*

When SWD were infected in larval stage, the mean number (± SEM) of laid eggs during lifetime was 65.37±22.83 for pairs with an infected parental female, 70.37±19.91 for infected parental males, 39.77±4.52 when both parents were infected, and 135.97±8.81 for the untreated control (Figure 5-7). Oviposition of pairs with at least one infected parent differed significant from the untreated control, indicating a strong effect of microsporidian infection on the fecundity of SWD (Chi^2=48.84, *DF*=3, *P*<0.05, Dunn´s test: F-C: *P*=0.006, M-C:

P=0.019, MF-C: *P*<0.001). The mean lifetime (± SE) of pairs did not differ from each other (Control=35.9±2.07 days, F=32.75±3.5, M=26.75±5.7, MF=29.6±1.5; Chi^2=2.6825, *DF*=3, *P*=0.44). Comparing the number of deposited eggs and the resulting offspring within each treatment, no reduced hatching rates could be determined (C: *T*=1.09, *DF*=77.818, *P*=0.279, MF: *W*=732.5, *P*=0.160, M: *T*=0.044, *DF*=14, *P*=0.965, F: *T*=0.027, *DF*=14, *P*=0.979). Indeed, the number of viable offspring was very similar to oviposition, thus the primary effect was on fecundity and not on fertility (Chi^2=41.336, *DF*=3, *P*<0.05, Dunn´s test (Bonferroni adjustment): F-C: *P*=0.037, M-C: *P*=0.073, MF-C: *P*<0.001). Considering oviposition within the first 10 dpi, already 45% of the control eggs where laid compared to the rest of the lifetime or the experimental duration (mean ± SE: C=61.3±5.4, F=30.4±10.6, M=40±12.3, MF= 19.4±2.8) (Figure 5-8). But again, treatments differed significantly from the untreated control (*F*=13.96, *DF*=3, *P*<0.001, Tukey HSD: F-C: *P*<0.05, M-C: *P*>0.05, MF-C: *P*<0.001). Interestingly, oviposition of the first 10 days showed a similar pattern as observed for total oviposition shown in Figure 5-7.

The sex ratio of the offspring was not affected in any combination; male to female ratio was always close to 50% for each. Only for the mating experiment, infected male and healthy female showed a slight, but not significant shift to increased female offspring production, though this experiment was only carried out once (Table 5-3). Microscopic inspection of 227 offspring individuals from transmission experiments with both infected parents (R/N=2/18) revealed 8 adult flies having a microsporidian infection (3.52%) three weeks after egg deposition.

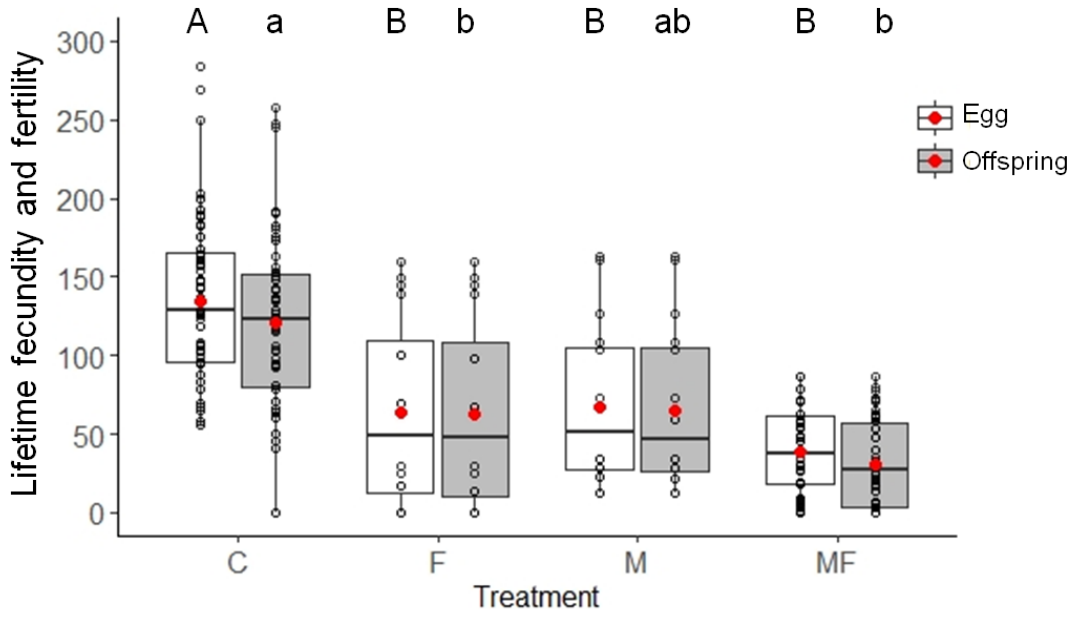

Figure 5-7: Fecundity (eggs, white boxes) and fertility (offspring, grey boxes) during lifetime of SWD adult pairs inoculated with *T. suzukii* spores in L2 larval stage.

(Figure caption continues on the following page)

Pairs consisted of either one infected individual or both infected individuals described as follows: C=untreated control, replicate/number (R/N)=3/40; F=female infected/male healthy, R/N=3/8; M=male infected/female healthy, R/N=3/8; MF=both infected, R/N=3/35. Kruskal Wallis test: Chi^2=48.84, *DF*=3, *P*<0.05, Dunn´s test: F-C: *P*=0.006, M-C: *P*=0.019, MF-C: *P*<0.001. Different letters indicate significance difference. Boxplot indicates the median number of eggs or offspring (for boxplot description see Figure 5-2).

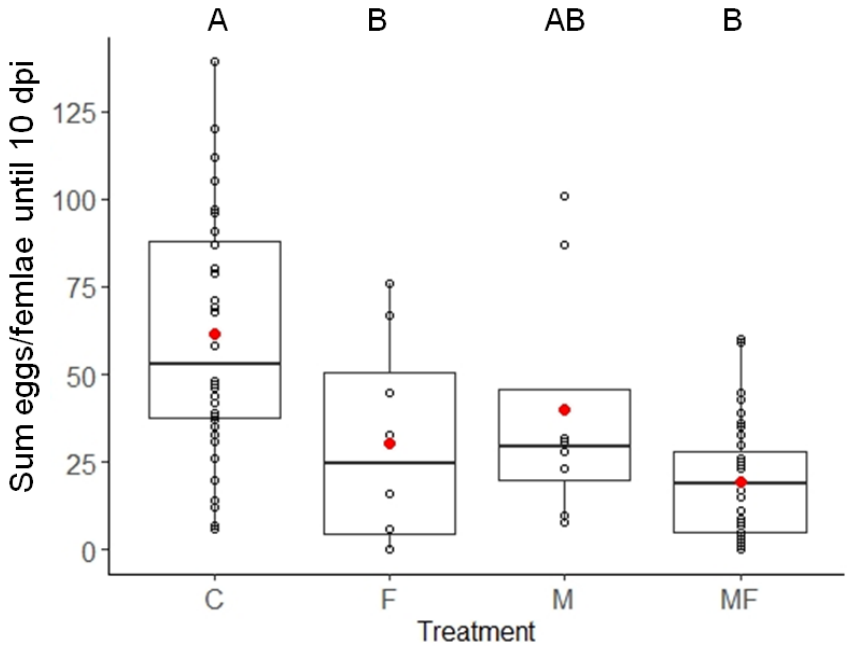

Figure 5-8: Total number of laid eggs during the first 10 days for untreated control (C), pairs with female infected parent (F), male infected parent (M) and both infected parental flies (MF).
One-way ANOVA: *F*=13.96, *DF*=3, *P*<0.001, Tukey HSD: F-C: *P*<0.05, MF-C: *P*<0.001. Different letters indicate significant difference. Boxplot indicates the median number of eggs with mean (for boxplot description see Figure 5-2).

Table 5-3: Sex ratio of the resulting offspring from replicate trials (replicate number/number of pairs=R/N) with number of tested offspring individuals (N♀ Ind.), percentage of female and male and significance (Wilcoxon rank sum test).

Treatment	R/N	N♀ Ind.	% Female	% Male	*P*-value
Male/female	3/17	439	51.54	48.46	0.877
Male	1/4	121	63.63	36.37	n.d.
Female	1/5	179	47.48	52.52	n.d.
Control	3/29	3351	49.47	50.53	0.907

When SWD adults were inoculated with *T. suzukii*, mean oviposition was 107.55±12.43 eggs/pair for the microsporidian-treated group and 152.94±19.43 eggs/pair for the control group (Figure 5-9), but no statistical significance between both treatments was noted (*T*=-1.968, *DF*=29.169, *P*=0.059). Also the number of hatched offspring recorded 18 days after oviposition did not differ among treatments (*T*=-2.011, *DF*=25.89, *P*=0.055). In both treatments, fertility was about 15-20% lower than fecundity but this difference was not significant (eggs-offspring control: *T*=0.750, *DF*=32.693, *P*=0.459; eggs-offspring microsporidia: *W*=573.5, *P*=0.193).

The sex ratio of offspring from *T. suzukii* inoculated parents was close to 50% male to female, as it was for control treatment (Table 5-4). Inspection of 223 offspring adults which had derived from *T. suzukii* treatment did not show any infected offspring, indicating that this microsporidium is not vertically transmitted if infection occurs in adult stage. Microscopic inspection of 31 microsporidia-treated pairs resulted in one male fly with an established *T. suzukii*-infection.

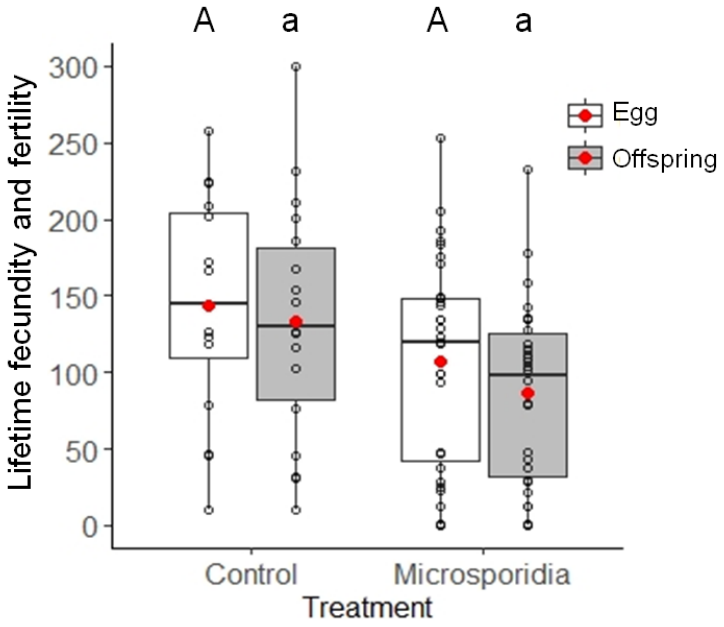

Figure 5-9: Fecundity (eggs, white boxes) and fertility (offspring, grey boxes) during lifetime of untreated control pairs (replicates/number (R/N)=4/17) and microsporidia-treated pairs (R/N=4/31).

T-test (eggs control-microsporidia; offspring control-microsporidia): *T*=-1.968, *DF*=29.169, *P*=0.059; *T*=-2.011, *DF*=25.89, *P*=0.055. T-test (eggs-offspring control): *T*=0.750, *DF*=32.693, *P*=0.459. Wilcoxon rank sum test (eggs-offspring microsporidia): *W*=573.5, *P*=0.193. Different letters indicate significant difference. Boxplot indicates the median number of eggs or offspring (for boxplot description see Figure 5-2).

Table 5-4: Sex ratio of the resulting offspring from replicate trials (R/N=replicate number/number of pairs) with number of tested individuals (N♀ Ind.), percentage of females and males and significance (Wilcoxon rank sum test).

Treatment	R/N	N♀ Ind.	% Female	% Male	*P*-value
Microsporidia	2/10	1405	54.09	45.91	0.611
Control	2/11	1139	51.45	48.55	0.913

DISCUSSION

A novel microsporidium *T. suzukii* infecting SWD was discovered in 2015 from a laboratory SWD rearing, which originally derived from field collections in Oregon (USA) (Chapter IV). In this study, the potential fitness effects of SWD larvae and adults have been elucidated, complemented by initial host range studies. When L2 larvae were inoculated with *T. suzukii* spores, concentration-dependent effects on the mortality as well as the hatching, survival, lifetime fecundity and the offspring hatching were noted.

A LC_{50} of 6.9×10^3 spores/µl was observed and sublethal concentrations were applied for following infection experiments. It was succeeded to trace the infection process and prevalence of *T. suzukii* in the life cycle of SWD using qPCR. The applied qPCR to measure infection progress in individual SWD inoculated in larval stage clearly showed that it is possible to follow the replication cycle of *T. suzukii* upon emergence of flies. Even though, some issues concerning this assay need to be solved, like the limited standard curves, in which it was not possible to extract more than 10^6 spores but results of extracted infected individuals showed partly more than 10^7 or even 10^8 DNA copies of *T. suzukii* per individual at 20 dpi.

A high larval mortality was found and the hatching declined dramatically (up to 70%) with increasing spore concentration. SWD larvae surviving the *T. suzukii* infection would die during pupal or adult stage. At high concentration, only 20-30% reached adulthood and died within 40 days. At low concentration, no difference to control group was seen until day 35, when mortality increased. Interestingly, no evidence was shown that *T. suzukii* has such strong effect on hatching of other drosophilids when inoculating L2 larval stages of *D. melanogaster* as one indigenous species and *D. willistoni*, a non-native species. Compared to controls, hatching of *D. willistoni* was reduced about 33% when inoculating larvae with 5×10^4 spores but *D. melanogaster* was not affected at all. *T. suzukii* showed low virulence to both species, but transmission to offspring as important parameter for evaluating virulence (Anderson and May, 1982) was not measured for both in this study. In contrast, in adult infection experiments control male flies had a significantly higher lifespan compared to

other treatments. This effect could be due to the experimental design where one female and one male were kept together. As shown for other drosophilids, mating is significantly reducing survival of females and the absence of rivals increases male lifetime (Aigaki and Ohba, 1984; Partridge et al., 1987; Bretman et al., 2013). The high larval and lower adult susceptibility is reminiscent for other microsporidia found in drosophilids, as was also observed for other drosophilids-infecting microsporidia, *T. ratisbonensis* and *T. kingi* of *D. melanogaster* and *D. willistoni* (Armstrong, 1976; Armstrong et al., 1986; Armstrong and Bass, 1989; Futerman et al., 2006; Vijendravarma et al., 2008; Niehus et al., 2012).

Fecundity and fertility were significantly reduced after larval inoculation with about 70% less egg deposition with biparental infections, supporting the hypothesis of high larval susceptibility (Vijendravarma et al., 2008, 2009). Females seem to be the mainly weakened, as pairs with only infected females showed the secondly highest egg reduction, driving the thought that female ovaries are involved in infection. One important fitness parameter is the oviposition rate within the first 10 days after hatching of adults, as drosophilids are laying the majority of eggs within this time frame compared to the rest of the lifetime (Robertson and Sang, 1944). *T. suzukii*-inoculated pairs laid about 46-57% (MF, M, and F) of the lifetime-eggs within the first 10 days, controls deposited 45%. The sublethal spore concentrations for this experiment (10 µl with 1.5×10^4 spores/µl) did not reduce fly survival. Hence, no higher oviposition than in controls was observed within 10 dpi, comparable with results for *T. kingi* (Armstrong and Bass, 1989). No change in fertility was found, all treatments showed only 10% reduced hatching of offspring assuming *T. suzukii* has no effect on fertility. Furthermore, it can be concluded that *T. suzukii* is not transmitted trans-ovarially, as offspring infection was rarely observed, but horizontally via cadavers or frass and trans-ovum. *T. suzukii* spores were found inside maturing SWD ovaries, possibly influencing egg development in early adult stage (see Chapter IV). Spores were not found inside mature ovaries but infecting ovary-surrounding adipose tissue, which can be transferred via egg deposition. Armstrong (1976) and Futerman et al. (2006) observed transmission rates of 1-11% in *Drosophila* showing a similar transmission pattern for *Tubulinosema* microsporidia infecting closely related drosophilids. In adult experiments, no oviposition reduction or vertical transmission was observed, suggesting a delayed infection process through behavioural, morphological or immunological reasons (Blaser and Schmid-Hempel, 2005; Solter, 2014).

In conclusion, the new microsporidian species *T. suzukii* found in SWD has a very strong impact on longevity and fecundity of SWD when spores are inoculated to early larval stages. Connecting these negative effects, SWD infection could have a population-reducing effect but adult flies are no suitable *T. suzukii* target. As this microsporidium was only found in a laboratory population thus so far, field populations should be screened for natural occurrence of the newly discovered pathogen.

CHAPTER VI: GENERAL DISCUSSION AND OUTLOOK

The need of developing adequate biological strategies beside the use of chemical insecticides to control SWD has triggered research on this topic during the last decade. Currently, only chemical control, netting and sanitary measures are successfully used to control the SWD. Effective microbial antagonists applicable as future SWD biological control agents are still lacking. Their development requires initial isolation from natural habitats and host insects and/or testing of available products for their efficacy against SWD. Several microbial biocontrol products with a target range including dipterans have been tested against SWD but mostly appeared to be not suitable because of low mortality rates or too slow efficacy (Cuthbertson et al., 2014b; Cuthbertson and Audsley, 2016). Although Cossentine et al. (2016b) showed two *Bacillus thuringiensis* serovars (*B. thuringiensis thuringiensis, B.t.t.* and *B. thuringiensis kurstaki, B.t.k.*) being effective against SWD larval and pupal stages, especially *B.t.t.* is no control option as it produces type 1 β-exotoxin which is toxic to mammals. Some *B.t.k.* strains showed significant larval mortality though the target species and application area of *B.t.k*. strains are generally lepidopteran pests (Wilcox et al., 1986; MacIntosh et al., 1990). Only one out of the several cry-toxins secreted by *B.t.k.* was shown to be toxic to Diptera (Federici et al., 2006). In contrast, strains of the *Bacillus thuringiensis* serovar. *israelensis* are known to be highly specific for Diptera and are widely used for mosquito or black fly control (De Barjac and Sutherland, 1990; Schnepf et al., 1998). The three *B.t.i.* products based on strain H14 tested in this thesis did not have any lethal effect on SWD. In Chapter II it was shown that *B.t.i.* was non-toxic to *D. suzukii*, although earlier field trials had suggested some efficacy (Lambion & Klink, 2014). The lack of efficacy of *B.t.i.* against SWD was also confirmed by Cossentine et al. (2016b). They discussed that any *B.t.* treatment previous to larval fruit infestation is uneconomical and inefficient for growers as fruit damage will still occur by oviposition.

Generally, the use of other bacteria like the α-proteobacterium of the genus *Wolbachia* spp. (Rickettsiales: Rickettsiaceae,) or the γ-proteobacterium *Serratia* spp. (Enterobacteriales: Enterobacteriaceae) for BC has been widely considered for insect pests (Steinhaus, 1959; Bouchon et al., 2011). Cattel et al. (2016a) and Fountain et al. (2018) recorded an association of SWD with *Wolbachia* (European prevalence: 46%) and *Serratia* (UK prevalence: 6.6%) by molecular approaches. For Diptera, the genus *Wolbachia* is endosymbiontically associated but is able to induce either insecticide resistance or making hosts more susceptible to insecticides (Liu and Guo, 2019). The latter point may be advantageous for pest management as infection with *Wolbachia* could weaken the target insect for other control measures. It should be therefore included in further screening efforts for entomopathogens in *D. suzukii*. The biological important characteristic of *Wolbachia* is the reproductive manipulation of the host through induction of a cytoplasmic incompatibility (CI) and also

male-killing in drosophilids (Werren et al., 2008; Veneti et al., 2012). Although early studies stated a correlation of *Wolbachia* and decreased fecundity in the fly, the *Wolbachia* found in SWD denoted as *w*Suz induced neither CI nor increased embryo mortality or sex-biasing effects (Hamm et al., 2014; Mazzetto et al., 2015). But *w*Suz has even positive influence on SWD; it appears to have a protective effect against viral infections with RNA viruses such as Drosophila C virus (Stevanovic et al., 2015; Cattel et al., 2016b). Hamm et al. (2014) stated that it is still unclear what maintains *w*Suz in *D. suzukii* but obviously reproductive manipulation is not necessary for its stable persistence and therefore, this bacterium is no candidate for *D. suzukii* control from an agricultural perspective.

Another available BCA is Azadirachtin, the effective substance of Neem oil. In Chapter III it was shown that Neem oil reached up to 100% larvicidal toxicity but only with uneconomically high concentrations, but no repellent effect in choice tests. Other studies reported about insufficient mortality with standard concentrations (Cuthbertson et al., 2014b), which was confirmed in this thesis. Considering that, Neem oil should be examined in combination with possible microbial biocontrol candidates to increase larvicidal effects at lower concentrations. Essential oils of lavender, macadamia and avocado showed oviposition deterrent effects on SWD but because of high costs, oils can only be considered as additive treatment in small-scale horticulture (Erland et al., 2015).

Viruses were not addressed in this thesis but several reports showed the abundance of RNA- and DNA-viruses in *D. suzukii* (Medd et al., 2017; Carrau et al., 2018). The nudivirus Kallithea virus (KV), originally discovered in *D. melanogaster*, was tested for fitness effects showing some promising negative impact on survival rates of adults, egg deposition frequency and adult fly mobility (Palmer et al., 2018). Nudiviruses are enveloped dsDNA viruses with genomes of 97-230 kbp length (Harrison et al., 2020). They are a phylogenetic sister group to baculoviruses which are widely used to control lepidopteran and hymenopteran pests in agriculture and forestry (Wang et al., 2007; Wang and Jehle, 2009; Jehle, 2010; Williams et al., 2017). KV is widespread in *D. melanogaster* and *Drosophila simulans* with an estimated global prevalence of 2-7% (Webster et al., 2015). Though, the above-mentioned study on fitness effects was done by intrathoracical micro-injection in *D. melanogaster*, the nudivirus KV should be evaluated on its lifespan-reducing potential versus SWD. However, several further studies on (RNA- and DNA-) viruses that were discovered in *D. suzukii* showed survival reduction after micro-injection as well (Lee and Vilcinskas, 2017). One major problem is that virus particles need to be ingested orally for pest management and therefore need enough structural stability to resist in the environment until they enter the alimentary tract. The closely related baculoviruses are protected by a robust protein matrix, the occlusion body, which is absent from most nudiviruses (“nudi”, Latin: naked). Further evaluation of these potential virus candidates through oral inoculation experiments is

therefore indispensable. Beyond that, finding a way to formulate the virus synthetically for environmental resistance and oral inoculation is another challenge.

Other authors tested the efficacy of entomopathogenic fungi and different fungal strains on *D. suzukii* and found mixed effects with moderate mortality depending on direct or indirect application on adult flies, larvae or pupae (Cuthbertson et al., 2014b; Naranjo-Lazaro et al., 2014; Woltz et al., 2015; Cossentine et al., 2016a; Becher et al., 2018). Cossentine et al. (2016a) showed sufficient mortality rates caused by *Metarhizium brunneum* strain F52 conidia at 25 °C, exceeding 70-95% mortality within 7 to 10 days after initial application. The effect was decreasing at lower temperatures. This fungal strain also successfully reduced reproduction in female SWD with a significantly reduced number of accumulated F1 pupae. In addition, a horizontal transmission between sexes was achieved. Both, the reduced lifespan and decreased fecundity suggest *M. brunneum* F52 as a candidate for further field trials for *D. suzukii* control.

In this thesis, a new microsporidian species, *T. suzukii*, was identified and characterized (Chapter IV), and evaluated for effects on fitness parameters of *D. suzukii* larvae and adults (Chapter V). Microsporidia have never been considered for SWD management, as microsporidia are controversial agents for pest control. On the one hand, they typically have no fast-killing effect on hosts but can regulate population size or even induce suppression through chronic infections with slow-killing or fitness-reducing effects as shown for the culicine *Edhazardia aedis* (Sweeney and Becnel, 1991; Becnel et al., 1995). On the other hand, production, formulation and application have ever been problematic due to some characteristic features of these pathogens. Production and propagation are only possible *in vivo*, but some species have additional intermediate hosts and complicated life cycles (Solter and Becnel, 2007). Long-term storage is limited although spores can be stored frozen for several years (Brooks, 1988; Maddox and Solter, 1996). Application is only possible with legal registration after investigating possible effects on human, vertebrate and invertebrate non-target organisms such as beneficials. Some microsporidia isolated from insect hosts are potentially pathogenic for immune-compromised humans as shown for *Brachiola* (=*Anncaliia*) *algerae* and *Tubulinosema ratisbonensis* (Lowman et al., 2000; Franzen et al., 2005a). The estimation on the potential transmission to non-target species would be needed before considering field trials of the newly discovered microsporidium *T. suzukii.* In addition, the transmission to and by beneficial predators and antagonistic parasite wasps used for *D. suzukii* control would need consideration. The susceptibility of the pteromalid wasp *Pachycrepoideus vindemiae* with *Tubulinosema kingi* from *Drosophila willistoni* resulted in decreased fecundity with distinct reduced parasitisation effects (Futerman et al., 2006). This pupal parasitoid was also examined on host acceptance and parasitisation efficacy of the alternative host *D. suzukii* ranging from low (25%) to sufficient (68%) parasitisation rates in laboratory and field trials (Chabert et al., 2012; Rossi-Stacconi et al., 2015). However, the

high hemocyte load of *D. suzukii* led to encapsulation of parasitoid eggs which resulted in decreased parasitoid development inside pupae (Rossi-Stacconi et al., 2013). Another pupal parasitoid, *Trichopria drosophilae*, was recently tested in field trials after laboratory parasitisation experiments showed promising results (Rossi-Stacconi et al., 2019). Necessarily, negative interactions of parasitoid and microsporidium need to be tested in advance to augmented release if a natural distribution of *T. suzukii* in SWD populations appears, either through application or by introduction of infected hosts from the USA. Nonetheless, the here described *T. suzukii* shows several characteristics making it suitable for natural population reduction and biological control, such as reduced fecundity with up to 70% less egg deposition and significantly shortened lifespan. It could influence the reproduction rate and generation time sufficiently if once stably introduced into a field population. Contrary, persistence in wild populations is uncertain through low vertical transmission for that species. A natural prevalence in the Northwest of the USA, where *T. suzukii*-infected flies were originally caught, was not examined so far. Whether *T. suzukii* is able to persist in nature or if it once invaded with the invasive fly to other non-indigenous countries is currently unknown and could be a topic of future investigations. On top, the fitness-suppressing outcome is high if *T. suzukii* spores are fed to larval *D. suzukii* but negligible after adult inoculation. An oral ingestion of spores by larvae inside fruits would need an enormous amount of spores sprayed on fruit skin to ensure “injection” of some pathogens to contaminate the fruit pulpa during the oviposition process by adult flies. In conclusion, *T. suzukii* is assumed to have some capacity to naturally control the invaded fly, but requires an introduction by larval inoculation and a stable manifestation in a population by sufficient self-dissemination through vertical or horizontal transmission. This issue seems universally applicable for several microbial antagonists and insecticides requiring oral ingestion instead of surface contact making the application of potential candidates challenging (Schetelig et al., 2018). Furthermore, trials with different fungal and bacterial strains showed strong evidence, that *D. suzukii* susceptibility to microbial antagonists is highly strain-depending (Schetelig et al., 2018).

In summary, the perfect candidate of a microbial antagonist that can be used for microbial control of *D. suzukii* has not been found yet. Although the microsporidian *T. suzukii* has potential for natural population reduction, further research on this pathogen is needed for evaluation. It is not only the cellular immunity of the invasive fly, which makes it resistant against many parasitic wasp species by encapsulation and melanisation of parasitic eggs. In addition, the exceptional behavioural and developmental patterns like egg deposition and larval development inside host fruits as well as the polyphagy generating numerous possible habitats and refuges, are making the fly a complex and intricate target for biological and integrated pest management.

LIST OF FIGURES

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EDUCATIONAL RECORDS

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10/2008-09/2012	Dresden University of Technology, Institute for Zoology, Advanced Zoology and Parasitology (Prof. Dr. em. Rudolf Entzeroth), Dresden Degree: Bachelor of Science, Biology Title: „Untersuchungen zur Prozessierung von heterologen *Et* Gam 56-mCherry Fusionsproteinen in *Eimeria nieschulzi* mittels Antikörpern“
09/2006-07/2008	Vocational Training: Berufliches Schulzentrum Dippoldiswalde Degree: State-certified Chemical-technical assistant, Biotechnology

06/1996-07/2005	Gymnasium, Gymnasium Dresden-Plauen Degree: Allgemeine Hochschulreife (Abitur)

ACTIVITIES AND AWARDS

2019-2021	Member-at-Large Microsporidia Division, Society of Invertebrate Pathology
2016-2018	Student Representative Microsporidia Division, Society of Invertebrate Pathology
August 2017	Travel Award Microsporidia Division, Society of Invertebrate Pathology, San Diego, California

PUBLICATIONS

Peer-Reviewed Journals

Biganski, S.; Jehle, J. A.; Kleespies, R. G. (2018): *Bacillus thuringiensis* serovar. *israelensis* has no effect on *Drosophila suzukii* Matsumura. Journal of Applied Entomology, 142, 33-36.

Biganski, S., Kurze, C., Müller, M. Y., & Moritz, R. F. (2018). Social response of healthy honeybees towards *Nosema ceranae*-infected workers: care or kill? Apidologie, 49, 325-334.

Non-peer-Reviewed Journals

Biganski, S.; Jehle, J. A.; Kleespies, R. G. (2018): Pathogens to control *Drosophila suzukii*. Journal für Kulturpflanzen, 70, 65.

Biganski, S.; Jehle, J. A.; Kleespies, R. G. (2017): Neem oil inhibits larval and pupal development of *Drosophila suzukii* in feeding trials under laboratory conditions. In: E. Tarasco, J. A. Jehle, M. Burjanadze, L. Ruiu, V. Půža, E. Quesada-Moraga, M. Lopez-Ferber, D. Stephan (Eds.), Proceedings of the 16th meeting “Is IPM ready for Microbial Control Agents?" at Tbilisi (Georgia): 11th-15th June, 2017 (IOBC-WPRS Bulletin 129), Darmstadt, pp. 3-6.

Conference Contributions

Biganski, S.; Jehle, J. A.; Kleespies, R. G. (2019): Fitness effects of the newly-discovered microsporidian species *Tubulinosema* sp. on its host *Drosophila suzukii*. In: International Congress on Invertebrate Pathology and Microbial Control & 52nd Annual Meeting of the Society for Invertebrate Pathology & 17th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate Pests"; 28th July-1st August, 2019, Valencia; Programme and Abstracts, p. 157.

Biganski, S.; Jehle, J. A.; Kleespies, R. G. (2018): Biologie und Virulenz eines neu entdeckten Mikrosporidiums aus der Kirschessigfliege (*Drosophila suzukii*). Biology and virulence of a new discovered microsporidium of the spotted wing drosophila (*Drosophila suzukii*). In: JKI (Ed.): 61. Deutsche Pflanzenschutztagung: Herausforderung Pflanzenschutz - Wege in die Zukunft; 11th-14th September, 2018, Universität Hohenheim -Kurzfassungen der Vorträge und Poster- (Julius-Kühn-Archiv 461), Braunschweig, pp. 290-291.

Biganski, S.; Jehle, J. A.; Kleespies, R. G. (2017): Microsporidia against Spotted Wing Drosophila? Antagonistic potential of underestimated pathogens in biological control. In: JKI (Ed.): 10th Young Scientists Meeting 2017, 8th-10th November in Siebeldingen - Abstracts (Berichte aus dem Julius Kühn-Institut 192), 18.

Biganski, S.; Jehle, J. A.; Kleespies, R. G. (2017): Neem oil inhibits larval and pupal development of *Drosophila suzukii* in feeding trials under laboratory conditions. Microbial and Nematode Control of Invertebrate Pests: 16th Meeting of the IOBC-WPRS Working Group; 11th-15th June, 2017, Tbilisi (Georgia), Programme and abstract book, p. 100.

Biganski, S.; Jehle, J. A.; Kleespies, R. G. (2017): First finding of a microsporidium of the family Tubulinosematidae infecting *Drosophila suzukii*.: 50th Annual Meeting and Golden Jubilee Conference of the Society for Invertebrate Pathology: Program and Abstract Book; University of California San Diego, La Jolla, California; 13th-17th August, 2017, 13.

Vogt, H.; Eben, A.; Boehnke, B.; Golla, B.; Hoffmann, C.; Herz, A.; Englert, C.; Kleespies, R. G.; Biganski, S.; Pinggera, J. (2017): Herausforderung Kirschessigfliege: Aktuelles aus der Forschung und zur Bekämpfung. In: Arbeitsgemeinschaft für Lebensmittel-, Veterinär- und Agrarwesen (Ed.): Bericht ALVA – Jahrestagung 2017 - "Zukunft Obstbau", pp. 70-72.

Biganski, S.; Jehle, J. A.; Kleespies, R. G. (2016): *Bacillus thuringiensis* subsp. *israelensis* (*B.t.i.*)-Präparate zur Bekämpfung von *Drosophila suzukii* - Chance oder Sackgasse?. In: JKI (Ed.): 60. Deutsche Pflanzenschutztagung: 20th-23rd September 2016, Martin-Luther-Universität Halle-Wittenberg; Kurzfassungen der Beiträge (Julius-Kühn-Archiv 454), Quedlinburg, pp. 455-456.

Biganski, S.; Nakai, M.; Jehle, J. A.; Kleespies, R. G. (2016): Detection of natural antagonists in *Drosophila suzukii* – a chance for biological control of the invasive insect pest?: 49th annual meeting of the Society for Invertebrate Pathology and International Congress on Invertebrate Pathology and Microbial Control: 24th-28th July 2016, Vinci Centre International de Congrès, 114.

Biganski, S.; Kleespies, R. G.; Jehle, J. A. (2015): Investigation on pathogenic antagonists of selected insect pests – an overview. In: JKI (Ed.): Achtes Nachwuchswissenschaftlerforum 2015: 19th-21st October in Quedlinburg; Abstracts (Berichte aus dem Julius-Kühn-Institut 181), Quedlinburg; Ribbesbüttel, 19.

Müller, M. Y., Biganski, S., Moritz, R. F. A. (2015): Plasticity of behavioural modulation in honey bee workers (*Apis mellifera*) infected with *Nosema ceranae* (Microsporidia). 62. Jahrestagung der AG der Institute für Bienenforschung e.V., 24th-26th March, 2015, Münster, Programm und Abstracts, p. 41

Darmstadt, 16 June 2020

EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirekt übernommene Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommene Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Darmstadt, den 16. Juni 2020

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