Importance and distribution of endosymbionts and other bacteria in economic important thrips species with special focus on *Echinothrips americanus*

Dissertation

zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der

Naturwissenschaftlichen Fakultät I – Biowissenschaften –

der Martin-Luther-Universität
Halle-Wittenberg,

vorgelegt

von Frau Julia Chuttke

geb. am 06.10.1989 in Cottbus

Gutachter/Gutachterin:

Prof. Dr. Gerald Moritz
Prof. Dr. Diane Ullman
Prof. Dr. Thomas Schmitt

Tag der Verteidigung:

04.10.2021

CONTENTS

ABBREVIATIONS

a.	antenna
aa.	antenna anlage
abd.	abdomen
abdfle.	abdominal flexure
abdg.	abdominal gland
abdggl.	abdominal ganglion
abdseg.	abdominal segment
ag.	accessory gland
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bm.	bacterial microorganisms
bp	base pair
ce.	compound eye
cho.	chorion
CI	cytoplasmic incompatibility
cm.	cibarial muscle
cytp.	cytoplasm
dATPs	deoxyadenosine triphosphate
dCTPs	deoxycytidine triphosphate
ddH_2O	double-distilled water
dGTPs	deoxyguanosine triphosphate
dH_2O	deionised water
DIG	digoxigenin
DMF	Dimethylformamide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphate
dTTPs	deoxythymidine triphosphate
dUTPs	deoxyuridine triphosphate
e.	eye
ep.	endoplasm
fb.	fat body
fc.	follicular epithelial cells
fg.	foregut
ftsZ gene	Filamenting temperature-sensitive mutant Z gene
fw.	forewing
ga.	gonadal anlage
gc.	germ cells
ger.	germarium

h.	head
ha.	head anlage
hg.	hindgut
hw.	hindwing
intest.epith.	intestinal epithelium
l.	leg/extremity
l1.–l3.	thoracic legs
la.	leg anlage
ld.	lipid droplet
L:D	light : dark
m.	muscle
mc.	mouth cone
mda.	mandible anlage
mg.	midgut
mggl.	mesothoracic ganglion
mit.	mitochondrion
mtggl.	metathoracic ganglion
mxa.	maxilla anlage
NBT	4-Nitro blue tetrazolium chloride
nt.	neuronal tissue
nuc.	nucleus
oc.	oocyte
oes.	oesophagus
oocn.	oocyte with cleavage nucleus
ov.	ovariole
ovip.	ovipositor
ovip.egg.	vitellogenic egg before oviposition
PCR	polymerase chain reaction
pggl.	prothoracic ganglion
PI	thelytokous parthenogenesis
pp.	periplasm
RH	relative humidity
rpm	rounds per minute
RT	room temperature
ser.	serosa
sg.	salivary gland
sp.	spermatozoa
spt.	spermatheca
sto.	stomodaeum
subggl.	suboesophageal ganglion

supraggl.	supraoesophageal ganglion
t.	testis
ta.	transition area between oocytes
Taq	*Thermus aquaticus*
tf.	terminal filament
thseg.	thoracic segment
vit.	vitellarium
vol.	volume
wsp gene	*Wolbachia* surface protein gene
y.	yolk
yb.	protein yolk body

CHAPTER 1

General introduction

1.1 Insect-symbiont associations

Insects live in a close relationship with their environment. The evolutionary success of insects is attributed to their genetic repertoire and rapid adaptation to changes in their terrestrial ecosystems. Numerous studies document that the presence of resident microbes is important for the performance of diverse host functions (e.g. Feldhaar 2011, Douglas 2015). Often insect-microbe symbioses occur as long-term association with a biosynthetic function for the host metabolism. Short- and long-term microbial interactions can exist as a natural outcome of an acquired or inherited function that increases the insect's fitness. Consequently, interactions with symbionts are key components for insect diversity and specification (Bordenstein 2003, Hurst et al. 2003, Moran et al. 2005).

Insects possess different physical barriers against their natural environment. The cuticular surface of the insect body and the intestinal lining is populated with beneficial microorganisms, which are known to protect from predators, parasites, and pathogens (Brownlie and Johnson 2009, Ballinger and Perlman 2019) and/or the digestion and absorption of nutrients (Buchner 1965, Baumann 2005). Insect-microbe communities of the digestive tract are fundamental to the diverse lifestyles of insects (Engel and Moran 2013). The extracellularly microbial associations are prevalent, transmitted through the environment. Another group of bacterial microorganisms is mainly maternally transmitted via the egg system or embryo and appears intracellularly in different tissue and cell types of the host insect (Moran et al. 2008). It is estimated that more than 70% of all insects exhibit associations with intracellular bacteria (also called endosymbionts) (Ishikawa 2003). These heritable organisms can occur in varied characteristics. Mutually obligate symbionts, mostly responsible for a specific function within the metabolism, demonstrably contribute to the vitality and development of the host (Moran et al. 2008, Wilson and Duncan 2015). Especially, plant sap-sucking insects possess symbionts, based on the nutritional source (i.e. xylem or phloem), with a highly-specialized function to synthesize amino acids and co-factors (Buchner 1965, Baumann 2005, Feng et al. 2019). The second class of heritable symbionts, the facultative type, is very common in insect species and can have diverse effects on the host's fitness. These bacteria appear in cells of various tissues within the insect body. Several facultative symbionts affect defence against stress or natural enemies, and others improve host metabolism or influence behaviour and thereby achieve an

increase in fitness of individuals (e.g. Brownlie and Johnson 2009, Hansen and Moran 2014). In contrast to the mutualistic impacts, secondary symbionts can function as reproductive manipulators. Within host populations, the bacteria cause an alteration of the host sexuality and reproduction (Breeuwer and Werren 1990). The endosymbionts can alter the reproduction employing different ways to the benefit of the female progeny (Rousset et al. 1992, Werren et al. 1995), and this ensures the bacteria a high transfer rate to the next generation (Werren et al. 2008, White et al. 2013). Symbioses with these bacteria can affect (1) the male-killing phenomenon, the embryonic or larval decease of infected males, (2) the feminization, the transformation of genetic males into phenotypic and functional females, (3) the thelytokous parthenogenesis, symbiont-infected females produce only daughters, and (4) the initiation of the cytoplasmic incompatibility between symbiont-infected males and uninfected females (Werren and O'Neill 1997). Considering the enormous host range, endosymbionts play a key role in the evolution of the sex-determining systems and reproductive strategies of insects (Cordax et al. 2011, Ma et al. 2014). A large number of insect taxa show endosymbiosis, induced by bacteria like *Wolbachia*, *Cardinium*, *Spiroplasma*, *Rickettsia*, and *Arsenophonus*, that are associated with the described beneficial strategies on the fitness and the adverse impacts on the reproductive mechanism (Duron et al. 2008).

1.2 Facultative symbionts

1.2.1 *Wolbachia*

Wolbachia are intracellular α-proteobacteria that occur in a variety of arthropods and nematode species (Schulenberg et al. 2000, Werren et al. 2008, Landmann 2019). These gram-negative bacteria rank among the order of Rickettsiales and are classified to the family of Anaplasmataceae. First mentioned in 1924 through Hertig and Wolbach and later described as pleomorphic, coccal rod cells in the gonads of *Culex pipiens* were referred to as *Wolbachia pipientis* (Hertig 1936). Currently, the *Wolbachia* genus is divided into 18 major clades (named supergroups A to R) (Baldo and Werren 2007, Gerth 2016, Wang et al. 2016), based on molecular phylogenetic lineages, determined by multilocus sequence typing (Baldo et al. 2006). The *Wolbachia* genome possesses high genetic flexibility. Common, the *wsp* gene and *ftsZ* gene were used for the characterization of *Wolbachia* bacteria. The *Wolbachia* surface protein is localized in the outer membrane of the bacterium (Braig et al. 1998). Its exact function is still unknown. This protein ranks among the family of pfam0617, therefore it is possibly involved in antibody detection (Noh et al. 2006), causes an antigen reaction in the host (French et al. 1999) and/or enables the penetration into host cells (Mohan Nair & Venkitanarayanan 2007). Consequently, the Wsp protein of *Wolbachia* bacteria might play a role in infection, proliferation, or pathogenicity within host cells (Serbus et al. 2008). The prokaryotic

cytoskeleton localized FtsZ protein is involved in the cell division (Dai and Lutkenhaus 1991). This protein can recruit other proteins, which are involved in septum formation during cell division. Thus, the protein is the bacterial homolog to the eukaryotic tubulin (Erickson 1995), whereby its function is analogous to the cell shape-determining actin homologue in bacteria during cell elongation. The *Wolbachia* clades A and B comprise the majority of insect (Lo et al. 2002). A meta-analysis indicates a *Wolbachia* distribution in about 65% of all insect species (Hilgenboecker et al. 2008).

The morphology of this bacterium can vary in size from 0.2 to 1.8 µm (Hertig 1936, Bordenstein et al. 2006). As already described by Hertig 1936 the bacteria possess a length from 0.5 to 1.3 µm and a diameter from 0.25 to 1.8 µm. Bordenstein et al. (2006) ascertained *Wolbachia* symbionts with a length of 0.3–1 µm and a diameter of 0.25–0.59 µm in the testes of *Nasonia vitripennis*. Furthermore, the bacteria are surrounded with up to three layers of membranes (Stouthamer et al. 1999, Bordenstein et al. 2006). The small rods can occur in almost all tissue and cell types (Pietri et al. 2016), whereby they are often localized in the ovaries and testes of the host insect (Clark and Karr 2002). *Wolbachia* are mainly vertically transmitted through the female germline. The facultative symbiont uses different mechanisms to incorporate in the germline and the precursor cells of the later embryonic, larval, and adult tissue types (reviewed in Landmann 2019). *Wolbachia* can be inherited through permanent persistence in the germline stem cells, the colonization of the somatic stem cell niches (Frydman et al. 2006, Toomey et al. 2013), the transmission from soma to a stem cell or passing from cell to cell (Pietri et al. 2016). For the distribution throughout oogenesis, the bacteria move through microtubules interactions and use the dynein and kinesin motors (Ferree et al. 2005, Serbus and Sullivan 2007). Moreover, the symbionts also use the actin cytoskeleton for dispersion (Newton et al. 2015). In filarial nematodes, an association between the localization and transmission of the actin cytoskeleton and the *Wolbachia* surface proteins was detected (Melnikow et al. 2013). During embryonic development, *Wolbachia* are moving along the cortex of the syncytial embryo through the utilization of identical mechanisms as during the female progenesis. Symmetric and asymmetric cell divisions enable the potential presence in the later larval tissue types (Albertson et al. 2009). Consequently, the distribution of *Wolbachia* in the adult tissues is associated with bacterial integration during oogenesis and embryogenesis. Numerous studies observed the bacteria in a range of somatic tissue types in both the larval and adult stages (Dobsen et al. 1999, Pietri et al. 2016). Dobson et al. (1999) first localized the bacterium in a variety of larval and adult tissues, including the nervous system, midgut, fat body, hemolymph and wings, of diverse *Drosophila* and mosquito lines. In recent decades, PCR analysis and different histological techniques documented the symbiont in a large number of germline and somatic cells and tissues during insect developmental stages (reviewed in Pietri et al. 2016). Besides the vertical transmission paths, horizontal gene transfers from

Wolbachia to the host genome also known. Andersson (2005) ascertained two models of gene transfer in eukaryotes, the transmission of genes from mitochondrion and plastids, which is described as endosymbiotic gene transfer, and the horizontal transfer of genes from kindred organisms. The genomes of *Wolbachia* vary from 1.08 to 1.7 Mb (Werren et al. 2008), whereby the broad transmission spectrum is associated with the high plasticity conditioned by flexible elements and simple repetitive DNA sections (Brownlie and O'Neill 2006, Klasson et al. 2008). A *Wolbachia* DNA sequence was first detected on the X-chromosome of the bean beetle *Callosobruchus chinensis* (Kondo et al. 2002). In the following years, elements of the symbiont DNA were found in several insect sequences (e.g. Hotopp et al. 2007, Klasson et al. 2009). The enormous spectrum to transmit vertically and the high flexibility to integrate with the host genome of the endosymbiont results in a variety of *Wolbachia*-insect associations.

Wolbachia have developed strategies to alter the reproduction and/or sex ratio (often towards a female-biased sex ratio), which were detected several insect populations. The great impact on reproduction goes back to four major strategies. One way is the feminization of genetic males (Bouchon et al. 1998, Kageyama et al. 1998). *Wolbachia* cause the loss of the male-determining chromosome and transformed males into phenotypic and reproductive females (Werren 1997). Another mechanism associated with the killing of males is induced during embryogenesis and results in an embryonic or later larval death of males (Bonte et al. 2008). A *Wolbachia* induced thelytokous parthenogenesis occurs in haplodiploid insect species (Stouthamer et al. 1990). The endosymbiotic bacteria engage in the cell division by reduplication of chromosomes, so that a diploid organism develops from unfertilized eggs (Stouthamer and Kazmer 1994, Pannebakker et al. 2004). Consequently, all offspring are females. The most common *Wolbachia*-induced phenomenon is the initiation of a reproductive incompatibility (Yen & Barr 1971). This strategy appears when an infected male mates with an uninfected female (unidirectional form). Furthermore, the cytoplasmic incompatibility (CI) can occur when the organisms exhibit different strains of the symbiont (bidirectional CI). In haplodiploid insect species leads the *Wolbachia*-induced CI to an elimination of the paternal chromosomes, so that all offspring are males or result in an embryonic death of fertilized egg (Reed and Werren 1995). Currently, the molecular mechanisms of this effect are largely unknown. Werren 1997 described the phenomenon with the mod (modification) and res (rescue) model. During the spermatogenesis, secreted *Wolbachia* proteins modify the paternal chromosomes or, eliminate required host proteins for the normal paternal chromosome condensation/decondensation (Bourtzis et al. 2003). In the early mitotic divisions, the asynchronous development of the pronuclei takes place and results in a breakdown of the nuclear envelope (Tram and Sullivan 2002), so that paternal chromosomes condense before or during zygote formation. Infected females are able to reverse the sperm modification through the production of a rescue factor so that successful fertilization takes place (Hughes and

Rasgon 2012). *Wolbachia* strain-specific patterns are due to the recruitment of host molecules that are necessary for the normal sperm-egg interaction (Bourtzis et al. 2003). The characteristics of the modification-rescue mechanisms are dependent on the respective *Wolbachia* strain (Werren 1997). Therefore, *Wolbachia* are able to alter the sex-determining systems of insects on the chromosomal and molecular level (Ma et al. 2014).
In contrast to the reproductive effects, the intracellular bacteria have an enormous variety of beneficial effects on the host's biology. For example, the presence of the symbiont in digestive and metabolic tissues can have a positive impact on the physiology through promoting and regulating of the immunity and bioenergetics (Duron et al. 2008).

1.2.2 *Cardinium*

The gram-negative *Cardinium* belongs to the order of Sphingobacteriales and in the family of Flexibacteriaceae. This bacterium was first reported in 1996 through Kurtti et al., which observed the endosymbiont in the cell cultures from the tick *Ixodes scapularis*. Afterwards, the new undescribed bacterium was often named Cytophaga-like organism (CLO, Hunter et al. 2003) or Cytophaga-Flavobacterium-Bacteroides (CFB, Weeks and Breeuwer 2003), and was observed in connection with the induction of feminization and parthenogenesis in arthropods (Weeks et al. 2001, Zchori-Fein et al. 2001). Finally, the symbiont with *Wolbachia*-like phenomena was termed as *Cardinium hertigii* (Zchori-Fein and Perlman 2004, Zchori-Fein et al. 2004). The endosymbiont occurs in an estimated 12.5% of all terrestrial arthropod species (Weinert et al. 2015). *Cardinium* are mainly transovarially transmitted via egg system or embryo to the next host generation, whereby horizontal transmission paths are also known. Six *Cardinium* genomes have currently been sequenced with a mean size of 0.88 Mb to 1.36 Mb (Penz et al. 2012, Santos-Gracia et al. 2014, Brown et al. 2018, Showmaker et al. 2018, Zeng et al. 2018, Siozios et al. 2019).
The intracellular bacterium possesses a pleomorphic morphology with parallel arrays of microfilament-like structures (MLS) inside the cell body (Zchori-Fein et al. 2004). These rods vary greatly in size from 0.42 to 2.35 µm in length and 0.31 to 0.66 µm in width (Zchori-Fein et al. 2004). The MLS are characteristic of this endosymbiont and resemble microtubules. These structures extend from the inner membrane into the cytoplasm (Bigliardi et al. 2006, Nakamura et al. 2009) and stand in connection with an anti-feeding prophage-like (“afp”) secretion system, which has an excretion function of factors outside of the cell (Penz et al. 2012, Böck et al. 2017). *Cardinium* is associated with the reproductive tissues of both female and male and contributes to host development and reproduction (Perlmutter and Bordenstein 2020). Within female's germline, the symbiont could always be observed in the cytoplasm of oocytes (Sacchi et al. 2008), in the germarium, the follicle cells and mature eggs (Matalon et al. 2007). Furthermore, *Cardinium* can occur in every somatic tissue type of arthropods, particularly in

the salivary glands and digestive system epithelium (Marzorati et al. 2006, Kitajima et al. 2007). The facultative symbiont is able to induce a thelytokous parthenogenesis, a feminization and cytoplasmic incompatibility. *Cardinium* cause the parthenogenesis in different parasitic wasps of the *Encarsia* genus (Zchori-Fein et al. 2001, 2004), and induce the feminization in mites like *Brevipalpus phoenicis* (Weeks et al. 2001, Groot and Breeuwer 2006) or scale insects (Provencher et al. 2005), and effect a cytoplasmic incompatibility in various wasps, mites, planthoppers and thrips species (Hunter et al. 2003, Gotoh et al. 2007, Ros and Breeuwer 2009, Wu and Hoy 2012, Zhang et al. 2012, Nguyen et al. 2017). A male-killing effect could not be found in single *Cardinium* infected individuals so far. Furthermore, the endosymbiont can affect the developmental time (Zhang et al. 2012) or the fecundity of host populations (Weeks and Stouthamer 2004, White et al. 2011). Thus, *Cardinium* may have a modulating effect on the biological, ecological, and evolutionary success of host populations (Douglas and Prosser 1992, Hedges et al. 2008).

Numerous studies show that *Cardinium* can co-occur with *Wolbachia* in single hosts (e.g. Zchori-Fein and Perlman 2004, Weeks et al. 2003, Duron et al. 2008, Lv et al. 2020). Coinfections with both endosymbionts are common in nature and can achieve different phenomena with diverse expression levels in a host compared to symbiont-single infected individuals. *Cardinium* and *Wolbachia* double infections mostly result in reproductive incompatibility, induced through both endosymbionts (for example Perlman et al. 2006, Zhao et al. 2013), or independent from each other (Nguyen et al. 2017). Manipulation types with different reproductive strategies are also known in co-infected organisms. For example, in the whitefly, *Bemisia tabaci*, the double infection leads to the male-killing phenomenon and cytoplasmic incompatibility (Lv et al. 2020). Moreover, coinfections can also affect other parameters of the host fitness (e.g. White et al. 2009), whereby multiple infections lead to a higher fitness effect than single symbiont infections in a host population (Frank 1998).

1.3 Thysanoptera and their endosymbionts

Thysanoptera are micro-insects, which occur in all climatic areas of the earth. Haliday (1836) grouped those insects into an order based on their characteristic wing morphology. Systematically, the order is divided into two suborders Terebrantia and Tubulifera, which are differentiated among others through the conspicuous morphological shape of the 10th abdominal segment and the associated egg deposition. There are currently 6288 known species of thrips worldwide (ThripsWiki 2020, retrieved on 31st August 2020). The insects possess a small mean body size from 0.5 mm to 3 mm, whereby some tropical and subtropical species can grow up to 15 mm. Thrips are holometamorphic insects and develop from fertilized

or unfertilized eggs over two feeding active larval stages, two (Terebrantia) or three (Tubulifera) mainly inactive resting stages to the adult stage (Mound 1996).

1.3.1 Development of thrips

Most Thysanoptera reproduce by haplodiploid parthenogenesis (arrhenotoky) so that males develop from unfertilized (haploid) and females from fertilized (diploid) eggs (Fig. 4.1A) (Evans et al. 2004). Other species of thrips possess diploid parthenogenesis (thelytoky) (Jordan 1888). During meiosis, females produce diploid eggs through the fusion of the egg-prenucleus and one of the polar bodies and, in consequence, all offspring are female (Fig. 4.1B). Changes between arrhenotokous and thelytokous parthenogenesis occurs in a few species of thrips which is called deuterotoky. Thelytokous parthenogenesis can be caused through the *Wolbachia* endosymbiont in thrips, which effect a conversion of arrhenotokous haplodiploidy to thelytoky (Arakaki et al. 2001, (Fig. 4.1C)). Otherwise, *Cardinium* and *Wolbachia* endosymbionts can induce a cytoplasmic incompatibility in haplodiploid thrips populations, which results in male-biased sex ratio (Nguyen et al. 2017, (Fig. 4.1D)).

Thrips possess typical panoistic ovarioles, which are separated into paired ovaries within females. Each ovary harboured four ovarioles that are divided into terminal filament, germarium and vitellarium (Fig. 1.1L). In the germarium, primordial germ cells differentiated into oocytes and follicle cells. During the passage of the vitellarium, the oocytes grow through the incorporation of yolk. The mature eggs are surrounded by a chorion-produced layer of follicular epithelial cells (Tsutsumi 1996). In haplodiploid thrips species, the spermatheca is filled with spermatozoa (Fig. 1.1F), which are transferred as spermatophore (accumulated formation of spermatozoa, Heming 1970) during copulation. The matured egg is transported into the common oviduct, which is connected to the spermatheca and enables the fertilization of the egg. Afterwards, the egg is, depending on the thrips suborder, deposited within (Terebrantia) or on (Tubulifera) the host plant (Bode 1975).

The embryogenesis of thrips is connected to the short/intermediate germ development. During early development, the plasmodial preblastoderm undergoes blastoderm cell differentiation and asynchronous mitotic activities, so that a primordial short germ is formed. This created germ consists of embryonic and extraembryonic (serosa) components. The further development of thrips is characterized by different events: the first invagination of embryonic cells into the yolk, the process of anatrepsis with the result of an S-shaped germ band elongation (Fig. 1.1B) and the subsequent segmentation, which started in the thoracic region. Later, the segmentation progresses in the direction of the cephalad and caudad region. Through regulation procedures during gastrulation, the germ band is organized in two layers, the ectoblastem and hypoblastem (ento- and mesoblastem). The early constructed ectoblastem later forms the nervous system of the organism and has a direct connection to all

other tissue and body parts. A further phase, the katatrepsis, is characterized by prominent dynamic processes and a complete systemic change of the embryo. Afterwards, almost all cells types conceive their physiological work. The organogenesis comprises the complete period from the beginning of the segmentation to the hatching of the first instar larva (Fig. 1.1C).

During the first and second instar stages, the larva exhibit similar morphological characteristics (Fig.1.1G–H). Both larval stages present a similar morphology to the adults, but miss wings, genital appendages, and compound eyes (Moritz 1997). Especially, the basic features of the head structures correspond to the adult shapes and indicate the usage of the same food resources (Moritz 1988). The visual system includes mostly four larval stemmata and no ocelli (Fig. 1.1H). Based on the small head shape and the strong formed cibarial muscles the supraoesophageal ganglion is translocated towards the thoracic area within first and early second instars (Fig. 1.1G) and afterwards is relocated to the initial position (Fig. 1.1H) (Staub 1979, Moritz 1984). Structural construction of the inner organs, like the digestive system, and of the outer appendages, for example the antennae, are largely similar to the adult structures (Fig. 1.1G–H). At the end of the larval development (late second instar), the formation of the outer genital appendages and wings starts.

During the resting stages, a complete metamorphosis takes place, which is associated with a new organization of the external and internal structures. The larval tissue constructions are eliminated, as for example the components of the midgut (Fig. 1.1I–J). The radical remodelling of the gut entails the degeneration of the larval structures and purge of the gut bacteria (Moll et al. 2001). In contrast, within the fore- and hindgut, mitotic cell division takes place. Other precursor organs, like elements of the mouthparts, are degenerated during the pupal stages. Modulations of the nervous system are on one side characterized by the development of the compound eyes and ocelli and the connection to the central brain (Fig. 1.1I). Otherwise, the thoracic ganglia are subdivided (Fig. 1.1D/J). Moreover, the partial replacements of the skeletal musculature and even neoformation of muscles in the area of the pterothorax ensues (Fig. 1.1D/I/J). On the body surface, the formation and growth of the fore- and hind wings, the antennae, and legs, and as well the development of ovipositor (females) occurs (Fig. 1.2C–D) (Moritz 1997).

The adults possess a few time after the hatching external a strong chitinous body surface, sclerotized fringed wings and body (Fig. 1.2E/G antennae, legs) and genital (ovipositor of females, Fig. 1.2F and aedeagus of males Fig. 1.2H) appendages (Moritz 2006). Internally, the adults exhibit an extremely compact nervous system, which is segmented in different brain regions (Fig.1.1D). The nervous system in the head of the adults is subdivided into supraoesophageal ganglion and the suboesophageal ganglion (Fig.1.1D). The supraoesophageal ganglion is differentiated in three categorical brain regions: the proto-,

deuto- and tritocerebrum. While, the suboesophageal ganglion is partitioned into mandibular, maxillar and labial ganglion. This ganglion is connected to the separated prothoracic ganglion, which in turn links with the following thoracic ganglia and finally the abdominal ganglion (Staub 1979, Urbach and Technau 2003, Polilov and Shmakov 2016). The development and the differentiation from the pupal to the adult stages is also associated with the formation of the compound eyes and ocelli (Fig.1.1D). Adult thrips have, like to the larval stages, typical asymmetrical mouthparts that subdivided in mandible, maxilla, labrum and labium (reviewed in Moritz 2006). Most species of Terebrantia are plant sap-sucking insects that puncture and suck the cell sap out of the single plant cell (Moritz 2002). Moreover, the gut can be divided into three regions: foregut, midgut and hindgut (Fig. 1.1J–K). In most insects, the midgut is responsible for digestion and absorption (Engel and Moran 2013). The midgut epithelial cells produce a protective film, the peritrophic membrane that partitioned the midgut into the endo- and ectoperitrophic space, whereby direct contact to microbiota is prevented. A further characteristic of the adult thrips body is the strong developed thoracic musculature (Fig. 1.1K), which are important for the locomotion of thrips. At this, the pterothorax plays a fundamental role as locomotor centre and is associated with the mobility of wings and legs of the insect's body (Moritz 2006). Finally, the adults exhibit complete developed reproductive systems. Females have vast egg tubes, which extend over the entire area of the abdomen (description see above). Male gonads are composed of paired testes (Fig. 1.1E), two lateral ducts (vasa deferentia and seminal vesicles) and a pair (or more) of accessory glands (Fig. 1.1I). The vas deferens (sperm duct) is posteriorly connected with the seminal vesicle (sperm storage organ) and enters the ejaculatory duct. Within the cell wall of the accessory glands are the secreted substances for the sperm transfer, activity and maintenance (Simmons 2001).

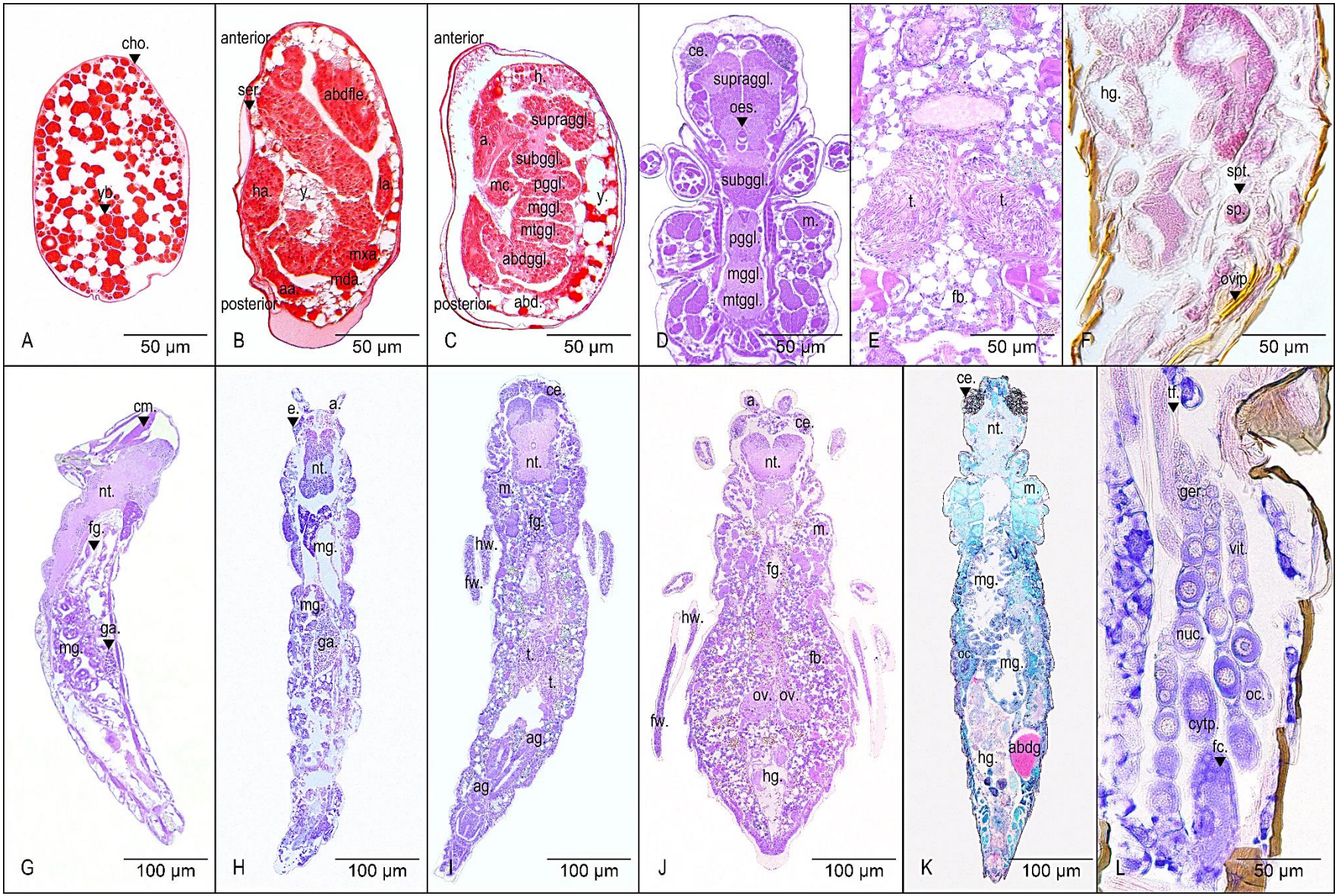

A
cho.
yb.
50 µm
B
anterior
ser.
abdfle.
ha.
y.
la.
mxa.
mda.
aa.
posterior
50 µm
C
anterior
h.
supraggl.
a.
subggl.
mc.
pggl.
mggl.
mtggl.
y.
abdggl.
posterior
abd.
50 µm
D
ce.
supraggl.
oes.
subggl.
pggl.
m.
mggl.
mtggl.
50 µm
E
t.
t.
fb.
50 µm
F
hg.
spt.
sp.
ovip.
50 µm
G
cm.
nt.
fg.
ga.
mg.
100 µm
H
e.
a.
nt.
mg.
mg.
ga.
100 µm
I
ce.
nt.
m.
fg.
hw.
fw.
t.
t.
ag.
ag.
100 µm
J
a.
ce.
nt.
m.
fg.
hw.
fb.
fw.
ov. ov.
hg.
100 µm
K
ce.
nt.
m.
mg.
oc.
mg.
hg.
abdg.
100 µm
L
tf.
ger.
vit.
nuc.
oc.
cytp.
fc.
50 µm

Fig. 1.1. Developmental stages of thrips and their morphological characteristics. (A) *E*. *americanus*, egg 12 h ± 12 h old (semi-thin sections, 1000 nm, safranin stain). (B) *E*. *americanus*, egg 90 h ± 6 h old with germ ligament (sagittal, semi-thin sections, 1750 nm, safranin stain). (C) *E*. *americanus*, egg 126 h ± 6 h old with prolarvae (sagittal, semi-thin sections, 1750 nm, safranin stain). (D) *E*. *americanus*, male, pupa, nervous system segmented in different brain regions (frontal, semi-thin sections, 800 nm, toluidine blue stain). (E) *E*. *americanus*, male, pupa, testes filled with spermatozoa (frontal, semi-thin sections, 800 nm, Macchiavello staining). (F) *E*. *americanus*, female, spermatheca filled with spermatozoa and ovipositor (sagittal, paraffin section, 6 µm, hematoxylin and eosin staining). (G) *E*. *americanus*, male, first instar larva (sagittal, semi-thin section, 800 nm, toluidine blue stain). (H) *E*. *americanus*, male, second instar larva (frontal, semi-thin section, 800 nm, Macchiavello staining). (I) *E*. *americanus*, male, propupa (frontal, semi-thin section, 800 nm, Macchiavello staining). (J) *H*. *femoralis*, female, pupa (frontal, semi-thin section, 800 nm, Macchiavello staining). (K) *E*. *americanus*, female, adult (frontal, semi-thin section, 800 nm, Gimenez staining). (L) *E*. *americanus*, female, adult, ovarioles (frontal, paraffin section, 6 µm, Giemsa staining). (a.– antenna, aa.– antenna anlage, abd.– abdomen, abdfle.– abdominal flexure, abdg.– abdominal gland, abdggl.– abdominal ganglion, ag.– accessory gland, ce.– compound eye, cho.– chorion, cm.– cibarial muscle, cytp.– cytoplasm, e.– eye, fb.– fat body, fc.– follicular epithelial cells, fg.– foregut, fw.– forewing, ga.– gonadal anlage, ger.– germarium, h.– head, ha.– head anlage, hg.– hindgut, hw.– hindwing, la.– leg anlage, m.– muscle, mc.– mouth cone, mda.– mandible anlage, mg.– midgut, mggl.– mesothoracic ganglion, mtggl.– metathoracic ganglion, mxa.– maxilla anlage, nt.– neuronal tissue, nuc.– nucleus, oc.– oocyte, ov.– ovariole, ovip. ovipositor, pggl.– prothoracic ganglion, ser.– serosa, sp.– spermatozoa, spt.– spermathecal, subggl.– suboesophageal ganglion, supraggl.– supraoesophageal ganglion, t.– testis, tf.– terminal filament, vit.– vitellarium, y.– yolk, yb.– protein yolk body).

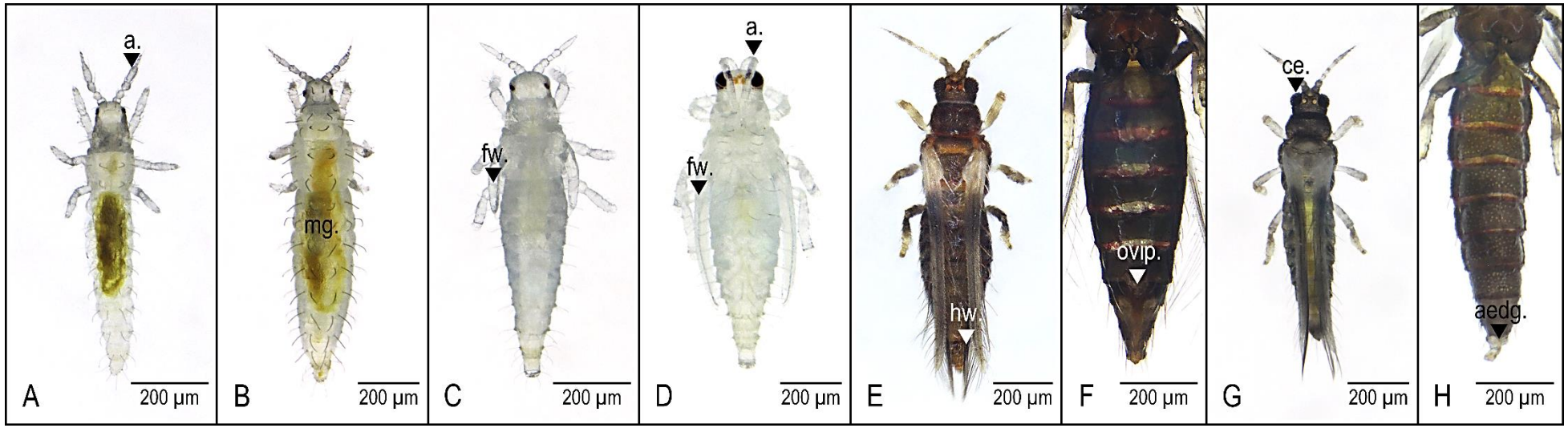


Fig. 1.2. Developmental stages of *E*. *americanus* and their external morphological characteristics. (A) First instar larva, (B) second instar larva, (C) propupa, (D) pupa, (E) adult female. (F) ventral side of the abdomen (adult female), (G) adult male, (H) ventral side of abdomen, (adult male). (a.– antenna, aedg. – aedeagus, ce.– compound eye, fw.– forewing, hw.– hindwing, mg.– midgut, ovip. ovipositor).

Tab. 1.1. Occurrences of facultative symbionts in thrips species and their induced phenomenon. (CI– cytoplasmic incompatibility, PI– thelytokous parthenogenesis, ?– presumed).

Thysanoptera species	Bacteria	Detection	Phenomenon	Reference
Aptinothrips elegans (adult)	*Wolbachia*	DNA	Unknown	van der Kooi and Schwander 2014
Aptinothrips karnyi (adult)	*Wolbachia*	DNA	Unknown	van der Kooi and Schwander 2014
Aptinothrips rufus (adult)	*Wolbachia*	DNA	Thelytoky*?	van der Kooi and Schwander 2014, Fontcuberta García-Cuenca et al. 2016
Aptinothrips stylifer (adult)	*Wolbachia*	DNA	Unknown	van der Kooi and Schwander 2014
Caliothrips fasciatus (adult)	*Wolbachia*	DNA	Unknown	Rugman-Jones et al. 2012
Chaetanaphothrips orchidii	*Wolbachia*	DNA	Unknown	Zchori-Fein & Perlman 2004
Echinothrips americanus	*Wolbachia* (Group A, Subgroup Mel)	DNA	Unknown	Kumm et al. 2006, Dong et al.2012
Frankliniella fusca	*Wolbachia*	DNA	Unknown	Jeyaprakash and Hoy 2000
Frankliniella intonsa	*Wolbachia* (Group B)	DNA	Unknown	Lou et al. 2015
Frankliniella schultzei	*Wolbachia*	DNA	Unknown	Sintupachee et al. 2006
Franklinothrips vespiformis (adult)	*Wolbachia* (Group B)	DNA	PI	Arakaki et al. 2001
Fulmekiola serrata	*Wolbachia*	DNA	Unknown	Buthelezi et al. 2011
Gynaikothrips ficorum	*Wolbachia*	DNA	Unknown	Kumm and Moritz 2008
Heliothrips haemorrhoidalis	*Wolbachia*	DNA	Unknown	Pintureau et al. 1999
Herciothrips femoralis	*Wolbachia*	DNA	PI	Pintureau et al. 1999, Kumm and Moritz 2008
Hoplothrips carpathicus (larva, pupa, adult)	*Wolbachia*	DNA	Unknown	Kaczmarczyk et al. 2018
Hoplothrips pedicularius (larva, pupa, adult)	*Wolbachia*	DNA	Unknown	Kaczmarczyk et al. 2017
Leptothrips mali (adult)	*Wolbachia* (strain *w*Lmal, Subgroup Con/Rug, Group B)	DNA	Unknown	Nirgianaki et al. 2003
Parthenothrips dracaenae	*Wolbachia*	DNA	Unknown	Kumm and Moritz 2008
Pezothrips kellyanus	*Wolbachia* (strain *w*Kelly), *Cardinium* (strain *c*Pkel1)	DNA	CI	Nguyen et al. 2015, Nguyen et al. 2017, Stouthamer et al. 2019
Plicothrips apicalis	*Wolbachia*	DNA, Abdomen	Unknown	Ambika and Rajagopal 2018

Tab. 1.1. – Continued

Thysanoptera species	Bacteria	Detection	Phenomenon	Reference
Sciothrips cardamom (larva, adult)	*Wolbachia* (*w*Scar, subgroup Con, Group B)	DNA	Unknown	Jacob et al. 2015
Suocerathrips linguis	*Wolbachia*	DNA	Unknown	Kumm and Moritz 2008
Thrips palmi	*Wolbachia* (Group B)	Abdomen	Unknown	Saurav et al. 2016
Thrips tabaci (adult)	*Wolbachia*	DNA	Unknown	Gawande et al. 2019

* in the original literature (van der Kooi and Schwander 2014, Fontcuberta García-Cuenca et al. 2016) referred to as asexuality

1.3.2 Distribution and importance of facultative symbionts and their effects on thrips

At first, Arakaki et al. (2001) discovered *Wolbachia*-induced thelytokous parthenogenesis in *Franklinothrips vespiformis*. The bacteria are able to duplicate the chromosomes during the cell division so that a diploid organism develops from unfertilized eggs (Fig. 4.1C) (Stouthamer and Kazmer 1994, Pannebakker et al. 2004). Further *Wolbachia*-mediated parthenogenesis was detected by a tetracycline-based antibiotic treatment in *Hercinothrips femoralis* (Kumm et al. 2006, Kumm and Moritz 2008). In addition, asexual (thelytokous) *Aptinothrips rufus* lineages can also be infected with the *Wolbachia* symbiont (van der Kooi and Schwander 2014), whereby Fontcuberta García-Cuenca et al. (2016) indicated that the asexuality (thelytoky) in this thrips species is possibly not the result of the known *Wolbachia* induced gamete duplication (homozygosity). The findings of this study ascertained heterozygous asexual *A. rufus* females (Fontcuberta García-Cuenca et al. 2016). A few years ago, a second endosymbiont-induced reproductive strategy, the cytoplasmic incompatibility, could be observed in *Pezothrips kellyanus* (Nguyen et al. 2017). *Wolbachia* and *Cardinium* cause in this haplodiploid thrips species independent of one other a complete reproductive incompatibility with different expression levels. While *Cardinium* induced cytoplasmic incompatibility with male development and embryonic mortality of fertilized eggs, the *Wolbachia* symbiont causes embryonic female mortality together with postembryonic mortality (Nguyen et al. 2017). Other endosymbiont-induced effects, which affect the reproductive mode, are currently unknown in thrips.

Many studies detected facultative symbionts, prevalent of the *Wolbachia* genus, in the total DNA of different thrips species (Tab. 1.1). Several thrips species, like *Echinothrips americanus* or *Suocerathrips linguis*, show also a haplodiploid reproductive mode, despite the proven *Wolbachia* infection (Kumm et al. 2006, Kumm and Moritz 2008). The bacteria are mainly maternally transmitted in thrips species (Saurav et al. 2016, Nguyen et al. 2017, Ambika and Rajagopal 2018). DNA analysis detected the *Wolbachia* symbiont (based on specific genes of the symbiont) in different developmental stages of the thrips species (Kaczmarczyk et al. 2017, Kaczmarczyk et al. 2018). So far, the *Wolbachia* bacterium could be localized only in the abdominal body areas of larval and adult stages in various thrips (Saurav et al. 2016, Ambika and Rajagopal 2018). The bacterial distribution patterns during development and the specific localization in the tissues of the body are largely unknown today.

1.3.3 Endosymbiotic presence in *Echinothrips americanus* MORGAN, 1913

E. americanus has its original distribution area in the Eastern parts of the USA (Mound and Marullo 1996, Vierbergen 1998). The thrips was first observed on the greenhouse plant *Euphorbia pulcherrima* (Oetting 1987), why the species is also termed poinsettia thrips. Systematically, within the order of Thysanoptera, the thrips belongs to the suborder of

Terebrantia and family of Thripidae. In Europe, the thrips species was first mentioned 1989 in a British glasshouse (Collins 1998). Conditioned by the trade of ornamental plants this thrips occurs today throughout the room of the North-Eastern USA, in areas of Canada, in many European countries and some areas of South-East Asia (reviewed in Krueger et al. 2015, Krueger 2016). The plant sap-feeding thrips possess a host-plant range of about 24 plant families (Vierbergen 1998).

E. *americanus* exhibits arrhenotokous partheno-genesis, which is characterized by a female-biased sex ratio (1 female : 0.3 male) under normal breeding conditions (Kumm 2002, Fig. 1.3 *E*. *americanus* laboratory culture). The developmental time of that thrips species from the oviposition to the adult hatching comprises ~16 days (Kumm 2002, breeding parameters: 23 °C, RH 75%, L:D 16 : 8).

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Thysanoptera
Suborder: Terebrantia
Family: Thripidae
Genus: Echinothrips
Species: *Echinothrips americanus*

Fig. 1.3. *E*. *americanus* on Gossypium sp. in the larboratory culture and scientific classification of this species.

Various studies detected the presence of the *Wolbachia* symbiont in this thrips (Kumm et al. 2006, Kumm and Moritz 2008, Dong 2012). For *E*. *americanus* Dong et al. (2012) ascertained the *Wolbachia* group A and Mel subgroup. Based on the occurrence of this endosymbiont and the low level of males within the host population of the laboratory, supposed already Kumm and Moritz 2012 a cytoplasmic incompatibility, which, however, has not yet been confirmed.

1.4 Scope of this thesis

This thesis addresses the distribution and importance of endosymbionts in economically relevant thrips species. In the following five chapters, the thrips-symbiont association is investigated as related to specific parameters, like the bacterial distribution during development, the importance on the sex ratio or the protection against entomopathogenic bacteria. The current work has a special focus on the thrips species *E*. *americanus* and its present endosymbiotic infection.

Distribution patterns of the *Wolbachia* endosymbiont during development

The *Wolbachia*-depending distribution during the development of thrips is actual largely unknown. In chapter 2, the distribution patterns of the *Wolbachia* endosymbiont is

demonstrated during the development of the thripine *E*. *americanus*. Through *in situ* technology, the presence of *Wolbachia* is documented at a definite time in the larval, pupal, and adult development of both sexes. The investigations enable the localization of the endosymbiont within the somatic and germline tissues of the thrips. Furthermore, the examinations determine differences of the *Wolbachia* distribution between the various developmental stages and allow conclusions to possible persistence sites during metamorphosis. In addition, the localization technique offers the opportunity to detect sex-dependent distribution patterns, which can be potentially indicative of specific behaviour or metabolic impact. *Wolbachia* symbionts are known to manipulate the reproductive system or benefit the host metabolism (Duron et al. 2008). Consequently, the results of this chapter contribute fundamentally to the determination of potential impacts of the *Wolbachia* symbiont in the thrips species.

Coinfection

The used *E*. *americanus* population possesses a *Wolbachia* infection, which does not result in thelytokous parthenogenesis (Kumm et al. 2006). Numerous studies describe the existence of coinfection with *Wolbachia* and *Cardinium* in several hosts (e.g. Weeks et al. 2003, Zchori-Fein and Perlman 2004). Nguyen et al. 2015 discovered the presence of coinfection in arrhenotokous thrips species with the *Wolbachia* and *Cardinium* endosymbionts. Mostly coinfections with both symbionts lead to the reproductive incompatibility in haplodiploid insect species (e.g. Perlman et al. 2006, Nguyen et al. 2017). The *E*. *americanus* laboratory population shows a low male sex ratio of 0.3, so that a cytoplasmic incompatibility was already assumed in this species (Kumm and Moritz 2012). By use of molecular characterization (*wsp* gene, *ftsZ* gene, 16S rDNA) the existence of a coinfection through the additional presence of the *Cardinium* symbiont was tested (Chapter 3). Additionally, the maternal transmission of the bacteria and the successful transfer to the next host generation is also examined by *in situ* hybridization technology.

Impact of the *Cardinium* endosymbiont on the sex ratio

The determination of a possible *Cardinium* induced reproductive incompatibility in the naturally co-infected *E*. *americanus* is investigated by crossing experiments. In the scope of chapter 4, the *Cardinium* impact in co-infected individuals is tested as compared to the single *Wolbachia* infected crossing partner. The investigations shall show if the bacterium influences the sex ratio and is potentially responsible for the initiation of cytoplasmic incompatibility. Moreover, sequence analyses are used to detect homologies for possible *Wolbachia* and *Cardinium* strains in the total DNA of *E*. *americanus*. For example, the *Cardinium* symbiont can have beneficial effects on the male amount in multiple infected individuals (White et al. 2009).

Otherwise, *Wolbachia* is known to induce cytoplasmic incompatibility with diverse expression levels, depending on the present strain-specific characteristics and host insect (White et al. 2013). Furthermore, previous investigations in thrips observed different expression level for both endosymbionts independent of each other (Nguyen et al. 2017). Therefore, the outcomes of this chapter show the impact of the *Cardinium* bacterium on the sex ratio and provides information about a potential influence in *E*. *americanus*.

Distribution patterns of endosymbionts during oogenesis and embryogenesis

The occurrence of endosymbionts during the female progenesis and embryonic development in different thrips species is described in chapter 5. *E*. *americanus* and *Hercinothrips femoralis* are standing in the centre of these investigations, which are demonstrably infected with endosymbionts. *H*. *femoralis* possesses an infection with the *Wolbachia*-symbiont that results in thelytokous parthenogenesis (Kumm and Moritz 2008). *E*. *americanus* appears a coinfection with *Wolbachia* and *Cardinium*, whereby the consequences are unknown today. In contrast to other insects, like *Drosophila* or mosquito lines, the dispersion of symbionts during the oogenesis and early development of thrips is mostly unknown. Within the chapter 5, the distribution patterns of the *Wolbachia* and *Cardinium* bacterium is documented at the beginning of the development of *E*. *americanus* and *H*. *femoralis*. Moreover, the chapter described similarities and differences between the single symbionts and thrips species and give the facility to create a bacterial distribution model during the oogenesis and embryonic development for thrips.

The toxic impact of *Photorhabdus luminescens* on thrips

Entomopathogenic bacteria, like *P*. *luminescens*, own toxin complexes, which have an oral and injectable toxic activity and function as a class of insecticidal toxins (Blackburn et al. 2005). In chapter 6, the toxic impact of the bacterium *P*. *luminescens* on the survival rate is tested on the thrips species: *E*. *americanus*, *F*. *occidentalis*, *H*. *femoralis* and *T*. *tabaci*. Firstly, the oral ingestion of *P*. *luminescens* is investigated through a molecular characterization of the 16S rDNA and final RFLP of the total thrips DNA in comparison with the *P*. *luminescens* DNA (of the bacteria liquid culture). The toxicity of *P*. *luminescens* is determinate after a 24 h application time of this bacterium on the different thrips species through a daily examination of the viability. At this, an important investigative approach is the determination of protective effects in symbiont-infected thrips and exist differences between single and double infected individuals. Furthermore, by means of *in situ* hybridization, the localization of bacterial DNA within the thrips body is determined. *P*. *luminescens* can overcome the cellular and humoral immune response of the target insect (Vallet-Gely et al. 2008, Nielsen-LeRoux et al. 2012).

Consequently, the investigations explain bacterial distribution inside the thrips body, and ascertain the possibility to overcome the barriers within the digestive system.

In the following chapters and the subsequent general discussion, the importance and distribution of endosymbionts and other bacteria, mainly for the thripine *E*. *americanus*, but also for other thrips species are described.

Chapters 2 to 6 possess additional information, which is shown in separate parts under supplementary material on the attached CD-ROM.

1.5 References

Albertson, R., C. Casper-Lindley, J. Cao, U. Tram, and W. Sullivan. 2009. Symmetric and asymmetric mitotic segregation patterns influence *Wolbachia* distribution in host somatic tissue. J Cell Sci 122: 4570–4583.

Ambika, S., and R. Rajagopal. 2018. Lumen anatomy and localization of *Wolbachia* sp. in the thrips, *Plicothrips apicalis* (Bagnall). Curr Sci 115: 1297–1304.

Andersson, J.O. 2005 Lateral gene transfer in eukaryotes. Cell Mol Life Sci 62: 1182–1197.

Arakaki, N., T. Miyoshi, and H. Noda. 2001. *Wolbachia*-mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (Thysanoptera: Insecta). Proc R Soc Lond B 268: 1011–1016.

Baldo, L., and J.H. Werren. 2007. Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. Curr Microbiol 55: 81–87.

Baldo, L., J.C. Dunning Hotopp, K.A. Jolley, S.R. Bordenstein, S.A. Biber, and et al. 2006. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Appl Environ Microbiol 72: 7098–7110.

Ballinger, M.J., and S.J. Perlman. 2019. The defensive *Spiroplasma*. Curr Opin Insect Sci 32: 36–41.

Baumann, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol 59: 155–189.

Bigliardi, E., L. Sacchi, M. Genchi M, A. Alma, M. Pajoro, and et al. 2006. Ultrastructure of a novel *Cardinium* sp. symbiont in *Scaphoideus titanus* (Hemiptera: Cicadellidae). Tissue Cell 38: 257–261.

Blackburn, M.B., J.M. Domek, D.B. Gelman, and J.S. Hu. 2005. The broadly insecticidal *Photorhabdus luminescens* toxin complex a (Tca): Activity against the Colorado potato beetle, *Leptinotarsa decemlineata*, and sweet potato whitefly, *Bemisia tabaci*. J Insect Sci 5: 32.

Bode, W. 1975. Der Ovipositor und die weiblichen Geschlechtswege der Thripiden (Thysanoptera, Terebrantia. Z Morph Tiere 81: 1–53.

Bonte, D., T. Hovestadt, and H.J. Poethke. 2008. Male-killing endosymbionts: influence of environmental conditions on persistence of host metapopulation. BMC Evol Biol 8: 243.

Bouchon, D., T. Rigaud, and P. Juchault. 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. Proc Biol Sci 265: 1081–1090.

Bourtzis, K., H.R. Braig, and T.L. Karr. 2003. Cytoplasmic incompatibility, pp. 217–246. In K. Bourtzis and T.A. Miller (eds.), Insect symbiosis. CRC Press, Boca Raton, London, New York, Washington D.C.

Böck, D., J.M. Medeiros, H.-F. Tsao, T. Penz, G.L. Weiss, and et al. 2017. In situ architecture, function, and evolution of a contractile injection system. Science 357: 713–717.

Bordenstein, S. 2003. Symbiosis and the origin of species, pp 283–304. In K. Bourtzis, and T. Miller (eds.), Insect symbiosis. CRC, Boca Raton.

Bordenstein, S.R., M.L. Marshall, A.J. Fry, U. Kim, and J.J. Wernegreen. 2006. The tripartite associations between Bacteriophage, *Wolbachia* and Arthropods. PLoS Pathog 2: 384–393.

Braig, H.R., W. Zhou, S.L. Dobson, and S.L. O'Neill. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. J Bacteriol 180: 2373–2378.

Breeuwer, J.A., and J.H. Werren. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature 346: 558–560.

Brown, A.M.V., S.K. Wasala, D.K. Howe, A.B. Peetz, I.A. Zasada, and D.R. Denver. 2018. Comparative genomics of *Wolbachia-Cardinium* dual endosymbiosis in a plant-parasitic nematode. Front Microbiol 9: 2482.

Brownlie, J.C., and S.L. O'Neill. 2006. *Wolbachia* genomics: accelerating our understanding of a pervasive symbiosis, pp. 175–186. In K. Bourtzis, and T.A. Miller (eds.) Insect symbiosis. CRC, Baco Raton.

Brownlie, J.C., and K.N. Johnson. 2009. Symbiont-mediated protection in insect hosts. Trends Microbiol 17: 348–354.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Rev. Eng. ed. [Translated by Bertha Mueller, with the collaboration of Francis H. Fockier]. ed. Interscience Publishers.

Buthelezi, G.K., L.A. Martin, D.L. Sweby, M.J. Way, and R.S. Rutherford. 2011. Incidence of *Wolbachia* in arthropod pests of sugarcane in South Africa and other countries. Proc S Afr Sug Technol Ass 84: 267–270.

Clark, M.E., and T.L. Karr. 2002. Distribution of *Wolbachia* within *Drosophila* reproductive tissue: implications for the expression of cytoplasmic incompatibility. Integr Comp Biol 42: 332–339.

Collins, D.W. 1998. Recent interceptions of *Echinothrips americanus* Morgan (Thysanoptera: Thripidae) imported into England. Entomol Mon Mag 134: 1–4.

Cordaux, R., D. Bouchon, and P. Grève. 2011. The impact of endosymbionts on the evolution of host sex-determination mechanisms. Trends Genet 27: 332–341.

Dai, K., and J.F. Lutkenhaus. 1991. *ftsZ* Is an essential cell division gene in *Escherichia coli*. J Bacteriol 173: 3500–3506.

Dobson, S.L., K. Bourtzis, H.R. Braig, B.F. Jones, W. Zhou and et al. 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem Mol Bio 29: 153–160.

Dong, J., X.W. Li, X.C. Zhang, and J.N. Feng. 2012. Sequencing and phylogenetic analysis of the *wsp* gene of *Wolbachia* in *Echinothrips americanus* Morgan. Journal of Northwest A & F University 3: 131–134.

Douglas, A.E. 2015. Multiorganismal insects: diversity and function of resident microorganisms. Annu Rev Entomol 60: 17–34.

Douglas, A.E., and W.A. Prosser. 1992. Synthesis of the essential amino acid tryptophan in the pea aphid (*Acyrthosiphon pisum*) symbiosis. J Insect Physiol 38: 565–568.

Duron, O., D. Bouchon, S. Boutin, L. Bellamy, L. Zhou, and et al. 2008. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biology 6: 27.

Engel, P., and N. Moran. 2013. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev 37: 699–735.

Erickson, H.P. 1995. FtsZ, a prokaryotic homolog of tubulin? Cell 80: 367–370.

Evans, J.D., D.C.A. Shearman, and B.P. Oldroyd. 2004. Molecular basis of sex determination in haplodiploids. Trends Ecol Evol 19: 1–3.

Feng, H.L., N. Edwards, C.M.H. Anderson, M. Althaus, R.P. Duncan, and et al. 2019. Trading amino acids at the aphid-Buchnera symbiotic interface. Proc Natl Acad Sci USA. 116: 16003–16011.

Feldhaar, H. 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol Entomol 36: 533–543.

Ferree, P.M., H.M. Frydman, J.M. Li, J. Cao, E. Wieschaus, and W. Sullivan. 2005. *Wolbachia* utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte. PLoS Pathog 1: e14.

Fontcuberta García-Cuenca, A., Z. Dumas, and T. Schwander. 2016. Extreme genetic diversity in asexual grass thrips populations. J Evol Biol 29:887–899.

Frank, S.A. 1998. Dynamics of cytoplasmic incompatability with multiple *Wolbachia* infections. J Theor Biol 192: 213–218.

French, D.M., W.C. Brown, and G.H. Palmer. 1999. Emergence of *Anaplasma marginale* antigenic variants during persistent rickettsemia. Infect Immun 67: 5834–5840.

Frydman, H.M., J.M. Li, D.N. Robson, and E. Wieschaus. 2006. Somatic stem cell niche tropism in *Wolbachia*. Nature 441: 509–512.

Gawande, S.J., S. Anandhan, A. Ingle, P. Roylawar, K. Khandagale, and et al. 2019. Microbiome profiling of the onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae). PLoS ONE 14: e0223281.

Gerth, M. 2016. Classification of *Wolbachia* (Alphaproteobacteria, Rickettsiales): No evidence for a distinct supergroup in cave spiders. Infect Genet Evol 43: 378–380.

Gotoh, T., H. Noda, and S. Ito. 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. Heredity. 98: 13–20.

Groot, T.V., and J.A. Breeuwer. 2006. *Cardinium* symbionts induce haploid thelytoky in most clones of three closely related *Brevipalpus* species. Exp Appl Acarol 39: 257–271.

Hansen, A.K., and N.A. Moran. 2014. The impact of microbial symbionts on host plant utilization by herbivorous insects. Mol Ecol 23: 1473–1496.

Hedges, L.M., J.C. Brownlie, S.L. O'Neill, and K.N. Johnson. 2008. *Wolbachia* and virus protection in insects. Science 322: 702.

Heming, B.S. 1970. Postembryonic development of the female reproductive system in *Frankliniella fusca* (Thripidae) and *Haplothrips verbasci* (Phlaeothripidae) (Thysanoptera). Misc Publs Entomol Soc Amer 7: 197–234.

Hertig, M. 1936. The rickettsia, *Wolbachia pipientis* (gen. et sp. n.) and associated inclusions of the mosquito *Culex pipiens*. Parasitology 28: 453–486.

Hertig, M., and S.B. Wolbach. 1924. Studies on rickettsia-like microorganisms in insects. J Med Res 44: 329–374.

Hilgenboecker, K., P. Hammerstein, P. Schlattmann, A. Telschow, and J.H. Werren. 2008. How many species are infected with *Wolbachia*? — a statistical analysis of current data. FEMS Microbiol. Lett. 281: 215–220.

Hotopp, J.C.D., M.E. Clark, D.C.S.G. Oliveira, J.M. Foster, P. Fischer, and et al. 2007. Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. Science 317: 1753–1756.

Hughes, G.L., and J.L. Rasgon. 2012. *Wolbachia* infections in arthropod hosts, pp. 351–366. In E.V. Fernando and K.K. Harry (eds.), Insect pathology, 2nd edition, Academic press, Elsevier, St. Louis, MO, USA.

Hunter, M.S., S.J. Perlman, and S.E. Kelly. 2003. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. Proc R Soc Lond Ser B. 270: 2185–2190.

Hurst, G.D.D., F.M. Jiggens, and M.E.N. Majerus. 2003. Inherited microorganisms that selectively kill male hosts: the hidden players of insect evolution, pp 177–197. In K. Bourtzis, and T.A. Millller (eds.), Insect symbiosis. CRC, Boca Raton.

Ishikawa, H. 2003. Insect symbiosis: An introduction, pp. 5–6. In K. Bourtzis and T.A. Miller (eds.), Insect symbiosis. CRC Press LLC, USA.

Jacob, T.K., Sharon D′Silva, C.M. Senthil Kumar, S. Devasahayam, V. Rajalakshmi, and et al. 2015. Single strain infection of adult and larval cardamom thrips (*Sciothrips cardamomi*) by *Wolbachia* subgroup *Con* belonging to supergroup B in India. Invertebr Reprod Dev 59: 1–8.

Jeyaprakash, A., and M.A. Hoy. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. Insect Mol Biol 9: 393–405.

Jordan, K. 1888. Anatomie und Biologie der Physapoda. Zeitschr. f. wiss. Zoologie. 47: 542–620.

Kaczmarczyk, A., H. Kucharczyk, M. Kucharczyk, and S. Zielińska. 2017. First report of *Wolbachia* in arrhenotokous populations of mycophagous species *Hoplothrips pedicularius* (Haliday 1836) (Thysanoptera, Phlaeothripidae). 5th Symposium on Palaearctic Thysanoptera, Cracow, Poland, 24.

Kaczmarczyk, A., H. Kucharczyk, M. Kucharczyk, P. Kapusta, J. Sell, and S. Zielińska. 2018. First insight into microbiome profile of fungivorous thrips *Hoplothrips carpathicus* (Insecta: Thysanoptera) at different developmental stages: molecular evidence of *Wolbachia* endosymbiosis. Sci Rep 8: 14376.

Kageyama, D., S. Hoshizaki, and Y. Ishikawa. 1998. Female-biased sex ratio in the Asian corn borer *Ostrinia furnacalis*: evidence for the occurrence of feminizing bacteria in an insect. Heredity 81: 311–316.

Kitajima, E.W., T.V.M. Groot, V.M. Novelli, J. Freitas-Astua, G. Alberti, and G.J. de Moraes. 2007. *In situ* observation of the *Cardinium* symbionts of *Brevipalpus* (Acari: Tenuipalpidae) by electron microscopy. Exp Appl Acarol 42: 263–271.

Klasson, L., T. Walker, M. Sebaihia, M.J. Sanders, M.A. Quail, and et al. 2008. Genome evolution of *Wolbachia* strain *w*Pip from the *Culex pipiens* group. Mol Biol Evol 25: 1877–1887.

Klasson, L., Z. Kambris, P.E. Cook, T. Walker, and S.P. Sinkins 2009. Horizontal gene transfer between *Wolbachia* and the mosquito *Aedes aegypti*. BMC Genomics 10: 33.

Kondo, N., N. Nikoh, N. Ijichi, M. Shimada, and T. Fukatsu. 2002. Genome fragment of *Wolbachia* endosymbiont transferred to X chromosome of host insect. Proc Natl Acad Sci 99: 14280–14285.

Krueger, S. 2016. Reproductive strategies of plant-sap sucking insects with special focus on Thysanoptera. Ph.D. dissertation. Martin-Luther-University Halle-Wittenberg, Halle.

Krueger, S., L.A. Mound, and G.B. Moritz. 2015. Offspring sex ratio and development are determined by copulation activity in *Echinothrips americanus* MORGAN 1913 (Thysanoptera: Thripidae). J Appl Entomol 140: 462–473.

Kumm, S. 2002. Reproduction, progenesis and embryogenesis of thrips (Thysanoptera, Insecta). Ph.D. dissertation. Martin-Luther-University Halle-Wittenberg, Halle.

Kumm, S., and G. Moritz. 2008. First detection of *Wolbachia* in arrhenotokous populations of thrips species (Thysanoptera: Thripidae and Phlaeothripidae) and its role in reproduction. Environ Entomol 37: 1422–1428.

Kumm, S., and G. Moritz. 2012. *Wolbachia* in arrhenotokous thrips species. Julius-Kühn-Archiv 438: 125–126.

Kumm, S., R. Kranz, and G. Moritz. 2006. Mikroorganismen-gesteuerte Parthenogenese bei Thysanopteren. Mitt Dtsch Ges allg angew Ent 15: 153–156.

Kurtti, T.J., U.G. Munderloh, T.G. Andreadis, L.A. Magnarelli, and T.A. Mather. 1996. Tick cell culture isolation of an intracellular prokaryote from the tick *Ixodes scapularis*. J Invertebr Pathol 67: 318–321.

Landmann, F. 2019. The *Wolbachia* endosymbionts. Microbiol Spectrum 7: BAI-0018-2019.

Lo, N., M. Casiraghi, E. Salati, C. Bazzochi, and C. Bandi. 2002. How many *Wolbachia* supergroups exist? Mol Biol Evol 19: 341–346.

Lou, H., Y.-T.Chen, Y.-K. Zhang, X.-M. Yang, J.-T. Sun, and X.-Y. Hong. 2015. *Wolbachia* infection and its relationship with mt DNA diversity in the flower thrips, *Frankliniella intonsa* (Thysanoptera: Thripidae). Acta Entomol Sin 58: 750–760.

Lv, N., J. Peng, X.-Y. Chen, C.-F. Guo, W. Sang, and et al. 2020. Antagonistic interaction between male-killing and cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* in the whitefly, *Bemisia tabaci*. Insect Sci 00: 1–17.

Ma, W.J., F. Vavre, and L.W. Beukeboom. 2014. Manipulation of arthropod sex determination by endosymbionts: diversity and molecular mechanisms. Sex Dev 8: 59–73.

Marzorati, M., A. Alma, L. Sacchi, M. Pajoro, S. Palermo, and et al. 2006. A novel bacteroidetes symbiont is localized in *Scaphoideus titanus*, the insect vector of flavescence dorée in *Vitis vinifera*. Appl Environ Microbiol 72: 1467–1475

Matalon, Y., N. Katzir, Y. Gottlieb, V. Portnoy, and E. Zchori-Fein. 2007. *Cardinium* in *Plagiomerus diaspidis* (Hymenoptera: Encyrtidae). Journal of Invertebrate Pathology 96: 106–108.

Melnikow, E., S. Xu, J. Liu, A.J. Bell, E. Ghedin, and et al. 2013. A potential role for the interaction of *Wolbachia* surface proteins with the *Brugia malayi* glycolytic enzymes and cytoskeleton in maintenance of endosymbiosis. PLoS Negl Trop Dis 7: e2151.

Mohan Nair, M.K., and K. Venkitanarayanan. 2007. Role of bacterial OmpA and host cytoskeleton in theinvasion of human intestinal epithelial cells by *Enterobacter sakazakii*. Pediatr Res 62: 664–669.

Moll, R.M., W.S. Romoser, M.C. Modrakowski, A.C. Monocayo, and K. Lerdthusnee. 2001. Meconial peritrophic membranes and fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. J Med Entomol 38: 29–32.

Moran, N., P. Tran, and N.M. Gerardo. 2005. Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. Appl Environ Microbiol 71: 8802–8810.

Moran, N.A., J.P. McCutcheon, and A. Nakabachi. 2008. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42: 165–190.

Moritz, G. 1984. Differenzierungsformen der imaginalen Bauchganglienkette bei Thysanoptera (Insecta). Entomol Nachr Ber 28: 27–29.

Moritz, G. 1988. Die Ontogenese der Thysanoptera (Insecta) unter besonderer Berücksichtigung des Fransenflüglers *Hercinothrips femoralis* (O.M. Reuter, 1891) (Thys., Thripidae, Panchaetothripinae) 2. Mitt. Erst- und Zweitlarve. Zoologische Jahrbücher, Anatomie 117: 299–351.

Moritz, G. 1997. Structure, growth and development. In T. Lewis (ed.), Thrips as crop pests. CAB International, Oxon and New York, USA.

Moritz, G. 2002. The biology of thrips is not the biology of their adults: a developmental view, pp. 259–267. In R. Marullo, and L. Mound (eds.) Thrips and Tospoviruses: proceedings of the 7th International Symposium on Thysanoptera. Australian National Insect Collection, Canberra.

Moritz, G. 2006. Thripse Fransenflügler, Thysanoptera. Die neue Brehm-Bücherei, Westarp Wissenschaft, Hohenwarsleben.

Mound, L.A. 1996. The Thysanoptera Vector Species of Tospoviruses. Acta Hortic 431: 298–309.

Mound, L.A., and R. Marullo. 1996. The thrips of Central and South America: an introduction. Memoirs Entomol Int 6: 1–488.

Nakamura, Y., S. Kawai, F. Yukuhiro, S. Ito, T. Gotoh, and et al. 2009. Prevalence of *Cardinium* in planthoppers and spider mites and taxonomic revision of "*Candidatus* Cardinium hertigii" based on detection of a new *Cardinium* group from biting midges. App Environ Microbiol 75: 6757–6763.

Newton, I.L.G., O. Savytskyy, and K.B. Sheehan. 2015. *Wolbachia* utilize host actin for efficient maternal transmission in *Drosophila melanogaster*. PLoS Pathog 11: e1004798.

Nguyen, D.T. 2015. Genetic diversity, reproductive systems and endosymbionts of two invasive thrips species. Ph.D. dissertation. University of Western Sydney, Hawkesbury Campus, Sydney.

Nguyen, D.T., J.L. Morrow, R.N. Spooner-Hart, and M. Riegler. 2017. Independent cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* maintains endosymbiont coinfections in haplodiploid thrips populations. Evolution 71: 995–1008.

Nielsen-LeRoux, C., S. Gaudriault, N. Ramarao, D. Lereclus, and A. Givaudan. 2012. How the insect pathogen bacteria *Bacillus thuringiensis* and *Xenorhabdus/Photorhabdus* occupy their hosts. Curr Opin Microbiol 15: 220–231.

Nirgianaki, A., G.K. Banks, D.R. Frohlich, Z. Veneti, H.R. Braig, and et al. 2003. *Wolbachia* infections of the whitefly *Bemisia tabaci*. Curr Microbio 47: 93–101.

Noh, S.M., K.A. Brayton, D.P. Knowles, J.T. Agnes, M.J. Dark, and et al. 2006. Differential expression and sequence conservation of the *Anaplasma marginale* msp2 gene superfamily outer membrane proteins. Infect Immun 74: 3471–3479.

Oetting, R. 1987. Laboratory evaluation of the toxicity and residual activity of abamectin to *Echinothrips americanus*. J Agr Entomol 4: 321–326.

Pannebakker, B.A., L.P. Pijnacker, B.J. Zwaan, and L.W. Beukeboom. 2004. Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). Genome 47: 299–303.

Penz, T., S. Schmitz-Esser, S.E. Kelly, B.N. Cass, A. Müller, and et al. 2012. Comparative genomics suggests an independent origin of cytoplasmic Incompatibility in *Cardinium hertigii*. PLoS Genet 8: e1003012.

Perlman, S.J., S.E. Kelly, E. Zchori-Fein, and M.S. Hunter. 2006. Cytoplasmic incompatibility and multiple symbiont infection in the ash whitefly parasitoid, *Encarsia inaron*. Biol Control 39: 474–480.

Perlmutter, J.I., and S.R. Bordenstein. 2020. Microorganisms in the reproductive tissues of arthropods. Nat Rev Microbiol 18: 97–111.

Pietri, J.E., H. DeBruhl, and W. Sullivan. 2016. The rich somatic life of *Wolbachia*. Microbiologyopen 5: 923–936.

Pintureau, B., F. Lassablière, C. Khatchadourian, and J. Daumal. 1999. Parasitoïdes oophages et symbiotes de deux thrips europeens. Ann Soc Entomol Fr 35: 416–420.

Polilov, A.A., and A.S. Shmakov. 2016. The anatomy of the thrips *Heliothrips haemorrhoidalis* (Thysanoptera, Thripidae) and its specific features caused by miniaturization. Arthropod Struct Dev 45: 496–507.

Provencher, L., G.E. Morse, A.R. Weeks, and B.B. Normark. 2005. Parthenogenesis in the *Aspidiotus nerii* complex (Hemiptera: Diaspididae): a single origin of a worldwide polyphagous lineage asssociated with *Cardinium* bacteria. Ann Entomol Soc Am 98: 629–635.

Reed, K.M., and J.H. Werren. 1995. Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. Mol Reprod Dev 40: 408–418.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault, and M. Solignac. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proc R Soc London Ser B 250: 91–98.

Ros, V.I.D., and J.A.J. Breeuwer. 2009. The effects of, and interactions between, *Cardinium* and *Wolbachia* in the double infected spider mite *Bryobia sarothamni*. Heredity 102: 413–422.

Rugman-Jones, P.F., M.S. Hoddle, R. Amrich, J.M. Heraty, C.E. Stouthamer-Ingel and R. Stouthamer. 2012. Phylogeographic structure, outbreeding depression, and reluctant virgin oviposition in the bean thrips, *Caliothrips fasciatus* (Pergande) (Thysanoptera: Thripidae), in California. Bulletin of Entomological Research 102: 698–709.

Sacchi, L., M. Genchi, E. Clementi, E. Bigliardi, A.M. Avanzati, and et al. 2008. Multiple symbiosis in the leafhopper *Scaphoideus titanus* (Hemiptera: Cicadellidae): Details of transovarial transmission of *Cardinium* sp. and yeast-like endosymbionts. Tissue Cell 40: 231–242.

Santos-Garcia, D., P.A. Rollat-Farnier, F. Beitia, E. Zchori-Fein, F. Vavre, and et al. 2014. The genome of *Cardinium* cBtQ1 provides insights into genome reduction, symbiont motility, and its settlement in *Bemisia tabaci*. Genome Biol Evol 6: 1013–1030.

Saurav, G.K., G. Daimei, V.S. Rana, S. Popli S, and R. Rajagopal. 2016. Detection and Localization of *Wolbachia* in *Thrips palmi* Karny (Thysanoptera: Thripidae). Indian J Microbiol 56: 167–171.

Schulenburg, J.H., G.D. Hurst, T.M. Huigens, M.M. van Meer, F.M. Jiggins, and M.E. Majerus. 2000. Molecular evolution and phylogenetic utility of *Wolbachia ftsZ* and *wsp* gene sequences with special reference to the origin of male-killing. Mol Biol Evol 17: 584–600.

Serbus, L.R., and W. Sullivan. 2007. A cellular basis for *Wolbachia* recruitment to the host germline. PLoS Pathog 3: 1930–1937.

Serbus, L.R., C. Casper-Lindley, F. Landmann, and W. Sullivan. 2008. The genetics and cell biology of *Wolbachia*-host interactions. Annu Rev Genet 42: 683–707.

Showmaker, K.C., K.K.O. Walden, C.J. Fields, K.N. Lambert, and M.E. Hudson. 2018. Genome sequence of the soybean cyst nematode (*Heterodera glycines*) endosymbiont "*Candidatus* Cardinium hertigii" strain cHgTN10. Genome Announc 6: e00624-18.

Simmons, L.W. 2001. Sperm competition and its Evolutionary Consequences in the Insects. Princeton University Press, Princeton, USA.

Sintupachee, S., J. R. Milne, S. Poonchaisri, V. Baimai, and P. Kittayapong. 2006. Closely related *Wolbachia* strains within the pumpkin arthropod community and the potential for horizontal transmission via the plant. Microbial Ecology 51: 294–301.

Siozios, S., J. Pilgrim, A.C. Darby, M. Baylis, and G.D.D Hurst. 2019. The draft genome of strain *c*Cpun from biting midges confirms insect *Cardinium* are not a monophyletic group and reveals a novel gene family expansion in a symbiont. PeerJ 7: e6448.

Staub, P.F. 1979. Die postembryonale Entwicklung des Nervensystems von *Parthenothrips dracaenae* HEEGER (Thysanopt., Terebr.). Revue suisse Zool 86: 367–394.

Stouthamer, C.M., S.E. Kelly, E. Mann, S. Schmitz-Esser, and M.S. Hunter. 2019. Development of a multi-locus sequence typing system helps reveal the evolution of *Cardinium hertigii*, a reproductive manipulator symbiont of insects. BMC Microbiol 19: 266.

Stouthamer, R., and D.J. Kazmer. 1994. Cytogenetics of microbe associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. Heredity 73: 317–327.

Stouthamer, R., R. Luck, and W.D. Hamilton. 1990. Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/ Trichogrammatidae) to revert sex. PNAS 87: 2424–2427.

Stouthamer, R., J.A.J. Breeuwer, and G.D.D. Hurst. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu Rev Microbiol 53: 71–102.

ThripsWiki. 2020. ThripsWiki - providing information on the World's thrips. <https://thrips.info/wiki/> [Retrieved August 31, 2020].

Toomey, M.E., K. Panaram, E.M. Fast, C. Beatty, and H.M. Frydman. 2013. Evolutionarily conserved *Wolbachia*-encoded factors control pattern of stem-cell niche tropism in *Drosophila* ovaries and favor infection. Proc Natl Acad Sci USA 110: 10788–10793.

Tram, U., and W. Sullivan. 2002. Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. Science 296: 1124–1126.

Tsutsumi, T. 1996. Formation of the egg membranes of an idolothripine thrips, *Bactrothrips brevitubus* (Insecta: Thysanoptera). Proc Arthropod Embyol Soc Jpn 31: 9–13.

Urbach, R., and G.M. Technau. 2003. Early steps in the building the insect brain: neuroblast formation and segmental patterning in the developing brain of different insect species. Arthropod Struct Dev 32: 103–123.

Vallet-Gely, I., B. Lemaitre, and F. Boccard. 2008. Bacterial strategies to overcome insect defences. Nat Rev Microbiol 6: 302–313.

van der Kooi, C.J., and T. Schwander. 2014. Evolution of asexuality via different mechanisms in grass thrips (Thysanoptera: *Aptinothrips)*. Evolution. 68: 1883–1893.

Vierbergen, G. 1998. *Echinothrips americanus* Morgan, a new thrips in Dutch greenhouses (Thysanoptera: Thripidae). Proc Sect Exp Appl Entomol Neth Entomol Soc 9: 155–160.

Wang, G.-H., L.-Y. Jia, J.-H. Xiao, and D.-W. Huang. 2016. Discovery of a new Wolbachia supergroup in cave spider species and the lateral transfer of phage WO among distant hosts. Infect Genet Evol 41: 1–7.

Weeks, A.R., and J.A.J Breeuwer. 2003. A new bacterium from the Cytophaga-Flavobacterium-Bacteroides phylum that causes sex ratio distortion, pp. 165–176. In K. Bourtzis, and T.A. Miller (eds.), Insect symbiosis. CRC, Boca Raton.

Weeks, A.R., and R. Stouthamer. 2004. Increased fecundity associated with infection by a Cytophaga-like intracellular bacterium in the predatory mite, *Metaseiulus occidentalis*. Proc Roy Soc B 271: S193–S195.

Weeks, A.R., F. Marec, and J.A.J. Breeuwer. 2001. A mite species that consists entirely of haploid females. Science 292: 2479–2482.

Weeks, A.R., R. Velten, and R. Stouthamer. 2003. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. Proc Biol Sci 270: 1857–1865.

Weinert, L. A., E. V. Araujo-Jnr, M. Z. Ahmed, and J. J. Welch. 2015. The incidence of bacterial endosymbionts in terrestrial arthropods. Proc R Soc Lond B 282: 20150249.

Werren, J.H. 1997. Biology of *Wolbachia*. Annu Rev Entomol 42: 587–609.

Werren, J.H., and S.L. O'Neill. 1997. The evolution of heritable symbionts, pp. 1–41. In J.H. Werren, and S.L. O'Neill (eds.), Influential Passengers: Inherited microorganisms and arthropod reproduction. Oxford University Press, New York, USA.

Werren, J.H., W. Zhang, and L.R. Guo. 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc R Soc Biol Sci 261: 55–63.

Werren, J.H., L. Baldo, and M.E. Clark. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 6: 741–751.

White, J.A., S.E. Kelly, S.J. Perlman, and M.S. Hunter. 2009. Cytoplasmic incompatibility in the parasitic wasp *Encarsia inaron:* disentangling the roles of *Cardinium* and *Wolbachia* symbionts. Heredity 102: 483–489.

White, J.A., S.E. Kelly, S.N. Cockburn, S.J. Perlman, and M.S. Hunter. 2011. Endosymbiont costs and benefits in a parasitoid infected with both *Wolbachia* and *Cardinium*. Heredity 106: 585–591.

White, J.A., M. Giorgini, M.R. Strand, and F. Pennacchio. 2013. Arthropod endosymbiosis and evolution, pp. 441–477. In A. Minelli, G. Boxshall, and G. Fusco (eds.), Arthropod Biology and Evolution. Springer Verlag, Berlin, Heidelberg.

Wilson, A.C.C., and R.P. Duncan. 2015. Signatures of host/symbiont genome coevolution in insect nutritional endosymbioses. Proc Natl Acad Sci USA 112: 10255–10261.

Wu, K., and M.A. Hoy. 2012. *Cardinium* is associated with reproductive incompatibility in the predatory mite Metaseiulus occidentalis (Acari: Phytoseiidae). J Invertebr Pathol 10: 359–365.

Yen, J.H., and A.R. Barr. 1971. New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. Nature 232: 657–658.

Yun, J.-H., S.W. Roh, T.W. Whon, M.-J. Jung, M.-S. Kim, and et al. 2014. Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. Appl Environ Microbiol 80: 5254–5264.

Zchori-Fein, E., and S.J. Perlman. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. Mol Ecol 13: 2009–2016.

Zchori-Fein, E., Y. Gottlieb, S.E. Kelly, J.K. Brown, Wilson JM, and et al. 2001. A newly discovered bacterium associated with parthenogenesis and a change in host selection behaviour in parasitoid wasps. Proc Natl Acad Sci USA 98: 1255–1260.

Zchori-Fein, E., S.J. Perlman, S.E. Kelly, N. Katzir, and M.S. Hunter. 2004. Characterization of a 'Bacteroidetes' symbiont in Encarsia wasps (Hymenoptera: Aphelinidae): proposal of Candidatus *Cardinium hertigii*. Int J Syst Evol Microbiol 54: 961–968.

Zeng, Z., Y.T. Fu, D.Y. Guo, Y.X. Wu, O.E. Ajayi, and Q.F. Wu. 2018. Bacterial endosymbiont *Cardinium c*Sfur genome sequence provides insights for understanding the symbiotic relationship in *Sogatella furcifera* host. BMC Genomics 19: 688.

Zhang, X.F., D.X. Zhao, and X.Y. Hong. 2012. *Cardinium*-the leading factor of cytoplasmic incompatibility in the planthopper *Sogatella furcifera* doubly infected with *Wolbachia* and *Cardinium*. Environ Entomol 41: 833–840.

Zhao, D.X., X.F. Zhang, and X.Y. Hong. 2013. Host-symbiont interactions in spider mite *Tetranychus truncates* doubly infected with *Wolbachia* and *Cardinium*. Environ Entomol 42: 445–452.

CHAPTER 2

Detection of the *Wolbachia* distribution in the developmental stages of *Echinothrips americanus* (Thysanoptera: Thripidae)

2.1 Summary

Wolbachia are gram-negative α-proteobacteria, which exist in many orders of insects and can cause a variety of reproduction alterations in their hosts, including feminization, thelytokous parthenogenesis, male-killing and cytoplasmic incompatibility. The distribution patterns of the intracellular bacteria in the host tissues is enabled through specific factors. So far, *Wolbachia* has been located in the germline and in various parts of the somatic tissue of several insects. In the order Thysanoptera, the distribution patterns of this endosymbiont are largely unknown. For the first time, we present the distribution patterns of *Wolbachia* in the developmental stages (larvae, resting stages and adults of both sexes) of the thripine *E*. *americanus*. *Wolbachia* bacteria were detected by *in situ* hybridization in the nervous system, in parts of the digestive tract, the reproductive organs, the skeletal muscles and several parts of the fat body.

Keywords: thrips, α-proteobacterium, *in situ* hybridization, bacterial localization in tissue types

2.2 Introduction

Many bacterial endosymbionts belong to α-proteobacteria and form one of the important groups in the animal kingdom. One of the best-known bacteria from this group is the genus *Wolbachia*. These microorganisms are intracellular gram-negative bacteria and occur in more than 50% of all insects (Sazama et al. 2019). The primarily vertical transmitted bacteria divide in a variety of different supergroups (Werren et al. 2008), so that the host-bacteria interactions can range from mutualism through commensalism to parasitism. Frequently, reproductive changes occur to modify the sex ratio for the benefit of the female progeny. The known induced manipulation types are the embryonic and larval killing of infected male stages (Hurst et al. 1999), the development of phenotypic and functional females from genotypic males (Rousset et al. 1992), the induction of thelytokous parthenogenesis (Stouthamer et al. 1990) and reproductive incompatibility (Yen and Barr 1971, Hoffmann et al. 1986). Furthermore, it is known that *Wolbachia* can influence the immunity (e.g. McMeniman et al. 2009), metabolism (Kremer et al. 2009), sex determination (Vala et al. 2004, Negri et al. 2009) and host speciation (Miller et al. 2010) of their hosts. The enormous host diversity of *Wolbachia* supports a crucial role in the evolution of the sex-determining systems and reproductive strategies of insects (Cordaux et al. 2011, Awrahman et al. 2014, Ma et al. 2014). Several species of Thysanoptera are provably influenced through the presence of *Wolbachia.* Most species of thrips reproduce by haplodiploid reproduction (Evans et al. 2004), whereby females develop from fertilized eggs and males from unfertilized eggs (arrhenotoky). Arakaki et al. (2001) first detected an infection of *Wolbachia* in *Franklinothrips vespiformis*, which caused thelytokous parthenogenesis. In addition, the initiation of reproductive incompatibility is also known in thrips (Nguyen et al. 2017). The cytoplasmic incompatibility appears when an uninfected female mate with an infected male, or when both hosts are infected with different *Wolbachia* strains (White et al. 2013). In haplodiploids the elimination of the paternal chromosomes results in a male-based progeny production or leads to embryonic mortality (Reed and Werren 1995). The study by Nguyen et al. (2017) demonstrated that the reproductive incompatibility in *Pezothrips kellyanus* is caused by a coinfection induced by *Cardinium* and *Wolbachia*. Currently, other *Wolbachia* induced reproduction modes are unknown in thrips.

Compared to the known *Wolbachia* effects in thrips, bacterial transmission paths and tissue-dependent distribution are largely unknown. As in other insects, the microorganisms present a mainly maternal transmission in thrips species (Nguyen et al. 2017). Furthermore, the *Wolbachia* could be detected in the total DNA of various developmental stages of these holometamorphic insects (Kaczmarczyk et al. 2017, Kaczmarczyk et al. 2018). The intracellular bacteria were localized only in the abdominal region of larvae and adults of different thrips species (Saurav et al. 2016, Ambika and Rajagopal 2018). In contrast to the initial assumption of germline-based *Wolbachia* distribution, various studies emphasise that

these endosymbionts can be localized in almost all tissue types of insects (e.g. Dobson et al. 1999, Clark et al. 2005, Albertson et al. 2009, Osborne et al. 2009, Zouache et al. 2009, Hughes et al. 2011, Albertson et al. 2013). So far, the bacteria could be detected in the head, especially in different parts of the nervous system, gland structures, various parts of the digestive and reproductive system, but also in body appendages like the extremities, wings, and antennae (reviewed in Pietri et al. 2016). Commonly, vertical transmission of *Wolbachia* can happen by a germline stem cell division or by a transfer from a soma cell to the germline (Russel et al. 2019). The later distribution in the germline and somatic tissues depends on the *Wolbachia* strain and its specific localization during egg maturation and early embryonic development (Ferree et al. 2005, Frydman et al. 2006). Various studies indicate that the distribution patterns of *Wolbachia* are connected to the regulation processes of microtubules and motor proteins (e.g. Kose and Karr 1995, Ferree et al. 2005, Albertson et al. 2009). Here, the later *Wolbachia* dispersion in the developing somatic tissue is dependent on symmetric and asymmetric mitotic cell segregations (Albertson et al. 2009). Besides the early embryogenesis, a second event during the development of insects, the metamorphosis, affords another opportunity to distribute bacteria inside the host cell types.

The purpose of this study is the identification of the *Wolbachia*-dependent distribution patterns during the development of Thysanoptera. Therefore, we investigated the developmental stages of *E*. *americanus* Morgan 1913, an increasingly serious pest in European greenhouses with a food spectrum of over 24 plant families (Vierbergen 1998). In the lab culture could be proved a *Wolbachia* infection, although this thrips species shows an arrhenotokous reproduction mode (Kumm & Moritz 2008, Chapter 3). *E*. *americanus* possesses a female-biased sex ratio under normal breeding conditions (1 : 0.3, Kumm 2002), which could be an indication of a partial interference *Wolbachia* induced cytoplasmic incompatibility. In the study, a first discovery of the *Wolbachia*-dependent distribution patterns in the developmental stages of thrips is realized, which can possibly suggest potential effects between the insect and host.

2.3 Materials and methods

2.3.1 Rearing of the developmental stages

We focused on the following developmental stages of *E*. *americanus*: the early first instar larva immediate after hatching, because at this stage the embryonal structures still exist; the early propupa immediate after hatching as this involves the remaining larval structures as well as newly developed adult structures; and the adult stages with completed tissues. For rearing of the selected developmental stages of both sexes, we used *E*. *americanus* and *Frankliniella occidentalis* from the laboratory culture at the University of Halle-Wittenberg (at 23 ± 1 °C, RH 50 ± 10%, and a 16 : 8 h L:D photoperiod, with lights on 6:00 a.m. CET). *E. americanus* for the experiment was reared in the laboratory on *Gossypium* sp. and *Hibiscus* sp., and *F. occidentalis* on *Phaseolus vulgaris* and *Chrysanthemum* sp.. *F*. *occidentalis* was used as a control because this thrips species is not infected with *Wolbachia* (Kumm and Moritz 2008, Chapter 3).

The specific stages of both thrips species were bred on prepared 12-well Greiner plates with 2 ml of 1.4% (*w*/*v*) agar (agar-agar, danish, Carl Roth GmbH + Co. KG, Germany) and a leaf disc of *P*. *vulgaris* on the surface of the agar. For the male stages, we placed one virgin female, and for the female stages one mated female in each well of the 12-well Greiner plates. The plates were sealed with a glass lid and Parafilm® M (Pechiney Plastic Packaging, Chicago, IL, USA) and placed in the climate chamber for 24 h (under the same conditions as the laboratory culture) before the females were removed. Afterwards, the Greiner plates (with the deposited eggs) were closed and placed back in the climate chamber for the rearing of the developmental stages. For the examinations we used following developmental stages of *E*. *americanus* and *F*. *occidentalis*: first instar larva, second instar larva, propupa, pupa and adults of both sexes. The rearing time for the corresponding developmental stage of each thrips species is present in table 2.1.

Tab. 2.1. Rearing times for the developmental stages of *E*. *americanus* and *F*. *occidentalis*. The stated developmental time refers to the time point after oviposition.

Developmental stage	***E*. *americanus* developmental time**	***F*. *occidentalis* developmental time**
First instar larva	10 d ± 12 h	6 d ± 12 h
Second instar larva	11 d ± 12 h	8 d ± 12 h
Propupa	13 d ± 12 h	10 d ± 12 h
Pupa	14 d ± 12 h	12 d ± 12 h
Adult	16 d ± 12 h	14 d ± 12 h

2.3.2 *In situ* hybridization

The aim of these investigations was the detection of the *Wolbachia* distribution patterns during the development of *E*. *americanus*. Preliminary examinations indicated the *Wolbachia* symbiont in the somatic and reproductive tissue parts of the adults (PCR analyses of prepared tissue and body parts, S2 Tab. 1). For this study, we modified an *in situ* hybridization method after Morel et al. (2001) for paraffin sections. The localization of the *Wolbachia* DNA was realized using the *ftsZ* gene (primers 494F/1262R, Holden et al. 1993). Table 2.2 shows the preparation procedure of the samples (30 larvae, resting stages and adults of both sexes) for the *in situ* hybridization.

Tab. 2.2. Preparation procedure of specimens for *in situ* hybridization. (Preparation information of the used primary fixation mixture and the Sorensen's phosphate buffer is shown in S2 Tab. 2).

Procedure	Chemicals	Conditions
Primary fixation	4% paraformaldehyde and 0.25% glutaraldehyde	3 h at 4 °C
Wash	0.1 M Sorensen's phosphate buffer, pH 7.2	4 x 20 min at 4 °C,
	0.1 M Sorensen's phosphate buffer, pH 7.2	Overnight at 4 °C
Dehydration	10%, 30%, 50% ethanol	Each 15 min
	70%, 80%, 90%, 100% ethanol	Each 1 h
Transition solvent	100% isopropyl	2 h, overnight, 1 d
Infiltration	50 : 50, isopropyl : paraffin	1 d
	Pure paraffin	3 x 1d
Embedding	Pure paraffin	Room temperature

The embedding specimens were sliced in 6 µm thick sections and fixed onto Polysine® coated microscope slides (Thermo Scientific, USA). After drying, the sections were deparaffinized (S2 Tab. 3) and pre-treated through a proteolysis with Proteinase K (Carl Roth GmbH + Co. KG, Germany) (Tab. 2.3).

Tab. 2.3. Proteolysis with Proteinase K. (Preparation information of the used Sorensen's phosphate buffer, the Proteinase K, the TRIS-HCl/$CaCl_2$ buffer and the 4% paraformaldehyde is shown in S2 Tab. 2).

Procedure	Chemicals	Conditions
Wash	9% sodium chloride	5 min
	0.1 M Sorensen's phosphate buffer, pH 7.2	5 min
Proteolyse	2.5 µg/ml Proteinase K	15 min at 37 °C
Wash	TRIS-HCl/$CaCl_2$ buffer, pH 7.6	2 min
	0.1 M Sorensen's phosphate buffer, pH 7.2	5 min
Post fixation	4% paraformaldehyde	5 min at 4 °C
Wash	0.1 M Sorensen's phosphate buffer, pH 7.2	5 min
	9% sodium chloride	2 min
Dehydration	30%, 50%, 75%, 85%, 90%, 100% ethanol	Each 2 min

We used *in situ* hybridization technology based on an immunohistochemistry-like method, which detects digoxigenin-(DIG)-labelled probes. For the *in situ* PCR, we used the

DIG-11-dUTPs (Roche Diagnostics, Germany) and the *Wolbachia* primers for the *ftsZ* gene (Holden et al. 1993). The slides were prepared with Gene Frames® (Thermo Scientific, USA). Each affixed frame was filled with a reaction mixture composed of: 7.5 µl 10x reaction buffer, 6 µl $MgCl_2$ (25 mM), 0.045 µl DIG-11-dUTPs (10 mM, Roche Diagnostics, Germany), 0.855 µl dTTPs (10 mM), 0.9 µl dATPs, dGTPs and dCTPs (10 mM) (dNTP Set, VWR Großenhain), 1.5 µl of each primer (20 pmol/µl) and 0.3 µl *Taq* DNA polymerase (Thermo Scientific, Maxima hot start *Taq* DNA polymerase) for a total volume of 45 µl. The frames were closed with coverslips (Thermo Scientific, USA) and the microscope slides transferred to an *in situ* adapter (Eppendorf AG, Germany) of the mastercycler. For the DNA amplification, we used the following program conditions: initial denaturation for 4 min at 95 °C, denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C and extension for 30 s at 72 °C for 25 cycles with a final extension for 1 min at 72 °C. After the *in situ* PCR the coverslips were removed and the sections were washed with 0.1 M Sorensen's phosphate buffer for 5 min. Subsequently, the samples were post fixed with 4% paraformaldehyde for 10 min at 4 °C. Following this, the slides were rinsed in 0.1 M Sorensen's phosphate buffer for 5 min and in 9% sodium chloride for 2 min. Finally, the slides were transferred into 95% and 100% ethanol for 2 min and afterwards dried. The integrated DIG marked dUTPs were detected by an anti-digoxigenin antibody (Roche Diagnostics, Germany) and visualized through Fab fragments from an anti-digoxigenin antibody, conjugated with alkaline phosphatase (Roche Diagnostics, Germany) (Tab. 2.4).

Tab. 2.4. Immunological detection procedure. (Preparation information of the used TRIS-HCl/NaCl buffer, the blocking buffer, the anti-digoxigenin antibody, the anti-digoxigenin-AP Fab fragments and the substrate solution is shown in S2 Tab. 2).

Procedure	Chemicals	Conditions
Wash	TRIS-HCl/NaCl buffer, pH 7,6	10 min
Blocking step	Blocking buffer	30 min
Wash	TRIS-HCl/NaCl buffer, pH 7,6	3 x 10 min
Antibody	Anti-digoxigenin antibody	75 min (moist chamber)
Wash	TRIS-HCl/NaCl buffer, pH 7,6	3 x 10 min
Antibody	Anti-digoxigenin-AP Fab fragments	75 min (moist chamber)
Wash	TRIS-HCl/NaCl buffer, pH 7,6	3 x 10 min
Visualization	Substrate solution	2 h at 37 °C in the dark
Wash	Double-distilled water	5 min

Finally, the specimens were mounted in Mowiol (Carl Roth GmbH + Co. KG, Germany) and examined with a light microscope (Leitz DMRBE, Leica, Germany). Pictures were taken with a Leica DFC450 C digital microscope camera and processed with Photoshop CS6 (Adobe, Ireland). For the interpretation of data and the creation of the image plate fig. 2.2, also histological sections of Fahlteich 2018 were used.

2.4 Results

Our main purpose was the detection of *Wolbachia* during the development of *E*. *americanus*. The localization of *Wolbachia* was realized by the detection of the *ftsZ* gene (for the *Wolbachia* symbiont, Holden et al. 1993) through *in situ* hybridization. This method has a strong impact on the quality of the tissue, which means that the histological sections lose contrast and sharpness of the cellular structures.

A general overview of the *Wolbachia* distribution patterns in the developmental stages of *E*. *americanus* is shown in figure 1. The bacteria were localized in the yolk mass of eggs (Fig. 2.1A); in the nervous system and intestinal tract of the embryonal stages (Fig. 2.1A); in the nervous system, salivary glands, midgut epithelium, extremities and fat body of the first (Fig. 2.1B) and second (Fig. 2.1C) instar larvae; in the antennae, nervous system, wings and fat body of the propupae (Fig. 2.1D) and pupae (Fig. 2.1E); and in the nervous system, muscles, midgut epithelium, fat body and reproductive organs of both adult sexes (Fig. 2.1F). In the following, we focused on three selected developmental stages: the first instar larva, the propupa and the adult of both sexes.

In male and female first instar larvae we located a considerable amount of *Wolbachia* in all tested samples in the supraoesophageal ganglion of the nervous system, which occurred as a great number of single occurrences (Fig. 2.1B, Fig. 2.2A). Furthermore, the microorganism could be detected in the lobular salivary gland of some specimens (Fig. 2.1B, Fig. 2.2B) and intestinal epithelium of the midgut in all investigated larvae (Fig. 2.2C) of both sexes as correlated clusters. Only a few bacteria could be found in the fat body of the abdomen of all examined males and females (Fig. 2.2D, S2 Tab. 4). In contrast, *Wolbachia* appeared as accumulations in the extremities of the most first instar larvae in both sexes (Fig. 2.1B, Fig. 2.2E). In the control organism, *F*. *occidentalis*, no *Wolbachia* could be detected in the comparable tissues of the male and female first instar larvae (S2 Fig. 1A–C).

The *Wolbachia* bacteria were found in the propupae in the supraoesophageal ganglion (Fig. 2.2F) and in the metathoracic ganglion (S2 Tab. 4) of the nervous system in both sexes of all tested organisms. In the supraoesophageal ganglion, we localized the bacteria in all three great brain regions, the proto-, deuto- and tritocerebrum, as strong accumulations (Fig. 2.1D). In both sexes, we detected *Wolbachia* in the mushroom bodies of the protocerebrum, the tritocerebrum and metathoracic ganglion (S2 Tab. 4). Some distribution patterns were sex-specific. A sex-specific distribution was found in the female protocerebrum. In addition to the occurrence in the mushroom bodies, females had clusters of *Wolbachia* in the central body (S2 Tab. 4). In contrast to that, males showed accumulations of the bacteria in the antennal lobe of the deutocerebrum (S2 Tab. 4). Further sex-specific distribution patterns of *Wolbachia*

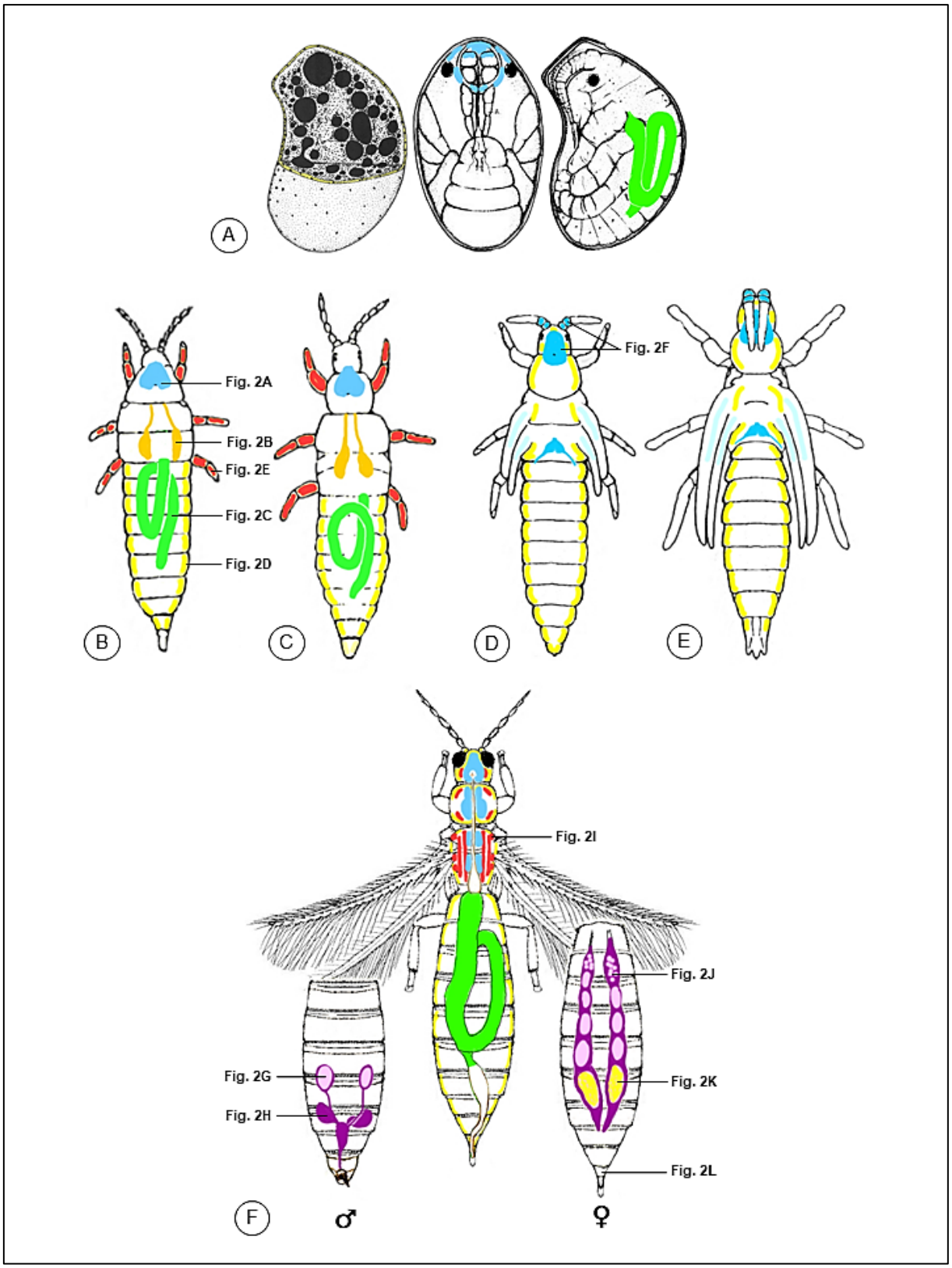


Fig. 2.1. Overview of the evidence for *Wolbachia* in the developmental stages of *E. americanus* by use of *in situ* hybridization. Illustrated developmental stages: (A) egg and embryonal stage, (B) first instar larva, (C) second instar larva, (D) propupa, (E) pupa and (F) adult. (yellow – yolk or fat body, blue – neuronal tissue, green – midgut epithelium, orange – salivary gland, red – muscles or extremity, cyan – wings, purple – reproductive organs).

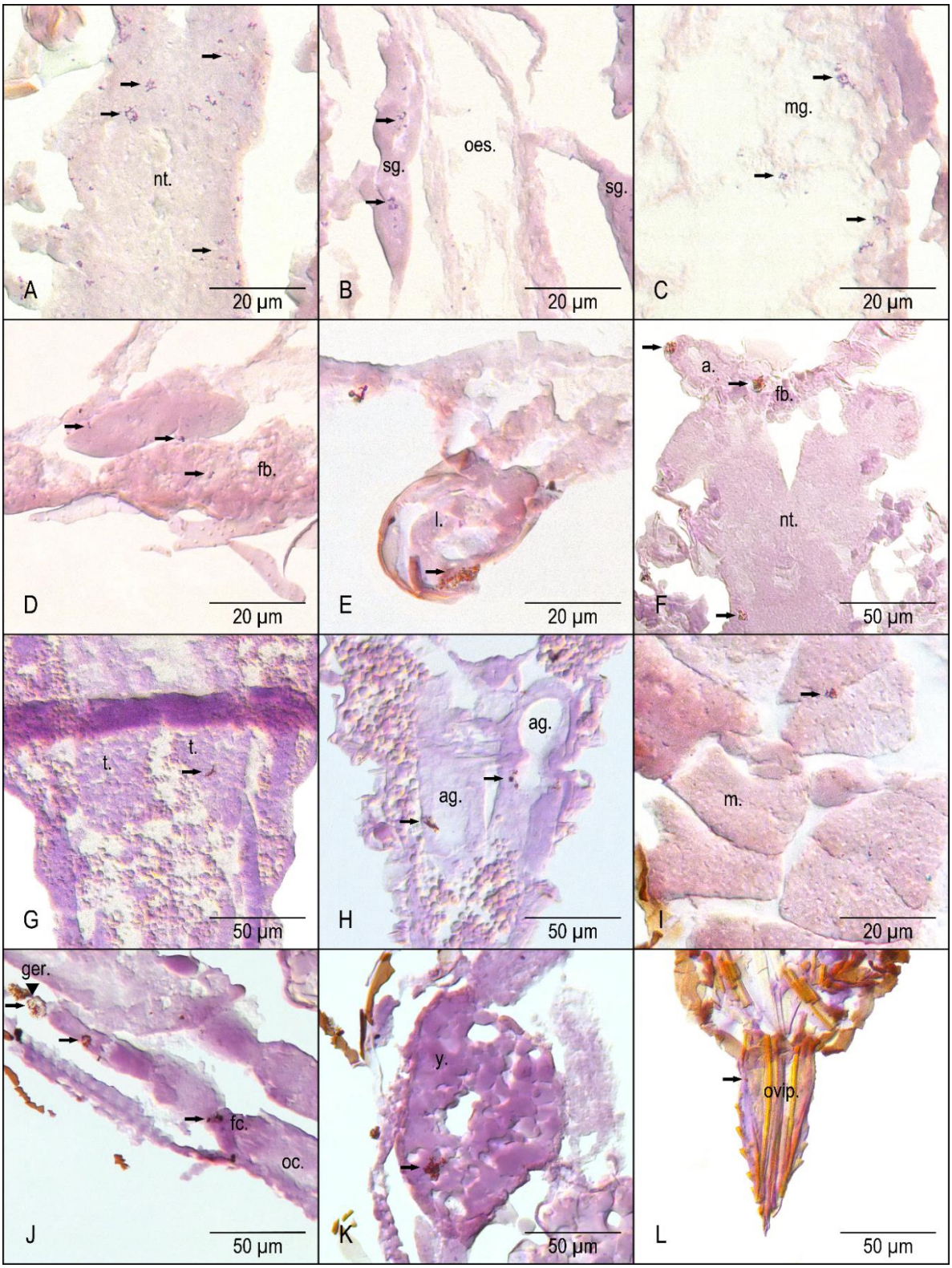


Fig. 2.2. Detection of *Wolbachia* clusters in selected developmental stages (frontal sections of first instar larva, propupa and adult stages) of *E. americanus* by use of *in situ* hybridization. The amplified *Wolbachia* DNA is clearly recognizable as red-brown signals (arrows). Fig. 2.2A–E first instar larva: (A) neuronal tissue (female), (B) second salivary gland and areas of the oesophagus (female), (C) loop of midgut (female), (D) fat body (male), (E) femur-tibia area of a mesothoracic leg (male). Fig. 2.2F–H propupa: (F) scape, pedicel and parts of protocerebrum (female), (G) testis (male), (H) accessory glands (male). Fig. 2.2I–L adult: (I) oscillatoric flight muscles, pterothorax (male), (J) germarium and vitellarium (female), (K) vitellogenic egg (female), (L) basal structures of ovipositor valves (female). (a.– antenna, ag.– accessory gland, fb.– fat body, fc.– follicular epithelial cells , ger.– germarium, l.– leg/extremity m.– muscles, mg.– midgut, nt.– neuronal tissue, oc.– oocyte, oes.– oesophagus, ovip.– ovipositor, sg.– salivary gland, t.– testis, y.– yolk).

were localized in the head of the female propupae. We found the bacteria on the base of the antennae, in the area of the vertex and clypeofrons, and even in the antennae especially in the scape pedicel area (Fig. 2.1D, Fig. 2.2F). Likewise, male propupae exhibited a sex-specific distribution of the bacteria outside the nervous system. We detected *Wolbachia* in clusters in the reproductive organs only in the testes (Fig. 2.2G), and in the accessory glands (Fig. 2.2H) in all investigated samples. However, we observed *Wolbachia* throughout in the fat body (head, thorax, and abdomen) and sometimes in the wings of both sexes (S2 Tab. 4). In the control animal, *F. occidentalis*, no *Wolbachia* were found in the comparable tissues of the propupae in both sexes (S2 Fig. 1D–F).

As in the other developmental stages, we localized *Wolbachia* in adult males and females in the nervous system in the same regions as in the propupae. In addition, the bacteria were also found in the intestinal epithelium of the midgut and in the fat body of the different body parts (Fig. 2.1F). For the first time, we detected *Wolbachia* in the thoracic muscles of both sexes in all tested specimens (Fig. 2.1F, Fig. 2.2I). Additionally, the bacteria were also present mostly in the muscles of the ovipositor (Fig. 2.2L). Another first evidence of the bacteria was in the reproductive organs of females (Fig. 2.1F) especially in the ovaries. The bacteria already appeared in the germarium in some thrips (Fig. 2.2J) and as accumulations in the follicular epithelium of developing oocytes (Fig. 2.2J) as well as in the egg yolk before oviposition of the most examined females (Fig. 2.2K). In contrast, the distribution patterns of *Wolbachia* in the reproductive tissues of adult males showed no difference from the male propupae (S2 Tab. 4). Furthermore, *F. occidentalis* showed no bacteria in the comparable tissues of the adults in both sexes (S2 Fig. 1G–I).

2.5 Discussion

The current study is the first to show the distribution pattern of the *ftsZ* gene of *Wolbachia* during the development of Thysanoptera. This gene encodes for the FtsZ protein, which plays an important role during the cell division, by recruiting more proteins to form the septum of the new cell (Begg and Donachie 1985). We used *in situ* hybridization technology for the localization of the bacteria in three selected developmental stages (first instar larva, early propupa, adult) of both sexes. Similar developmental stages of *F. occidentalis* were used as a control because this thrips species previously tested negative for *Wolbachia* (Kumm and Moritz 2008, Chapter 3). The *ftsZ* gene of *Wolbachia* was present during all developmental stages (S2 Tab. 4). The bacteria were present in some tissues throughout the complete development, such as in the nervous system and the fat body, but in other tissues only in specific developmental stages. In the following discussion, we focus on the different tissues in which *Wolbachia* was present during development.

2.5.1 Head and thorax (nervous system, antennae, salivary gland, muscles)

In the early first instar larvae (females and males) the bacteria were distributed in all parts of the supraoesophageal ganglion (Fig. 2.2A), whereas in the propupa the bacteria were localized in only a few specific brain regions (Fig. 2.2F). Generally, we detected *Wolbachia* in the propupae of both sexes as accumulations in the supraoesophageal ganglion, particularly in regions of the differentiated protocerebrum (comprises the central body and mushroom bodies), deutocerebrum (only in males) and tritocerebrum, and also in the metathoracic ganglion (S2 Tab. 4). A number of observations in *Drosophila* showed that *Wolbachia* was present in the symmetric as well as asymmetric cell division of stem cells during early development (O'Neill and Karr 1990, Callaini et al. 1994, Kose and Karr 1995). *Wolbachia* was evenly distributed between the cells in the early embryonic symmetric and asymmetric segregation of neuroblasts, whereas the bacteria were predominantly in the asymmetric division of neuroblasts in the later embryogenesis and larval stages (Albertson et al. 2009). During the early larval development stages, asymmetric divisions generated self-renewing daughter neuroblasts, which specialize in a variety of neural cells (Ceron et al. 2001). The findings of this study suggest that the definitive localization of *Wolbachia* in the neuronal tissue in *E*. *americanus* takes place in the later larval stages because the *Wolbachia* are distributed throughout in the complete supraoesophageal ganglion of the early first instar larva (Fig. 2.2A). Our investigations showed a sex-dependent distribution of *Wolbachia* in the protocerebrum and deutocerebrum of *E*. *americanus* propupal and adult stages. Both sexes exhibited clusters of this endosymbiont in the mushroom bodies (S2 Tab. 4). Additionally, we localized *Wolbachia* accumulations superior to the oesophagus only in females. This part of the protocerebrum (central body) is associated with the governance of flight, visual memory, and courtship (Hanesch et al. 1989, Zars et al. 2000). Moreover, we detected *Wolbachia* in the basal segments of the female antennae in the scape pedicel area (Fig. 2.2F), so that the bacteria might have a potential effect on the movements of the female antennae, and therewith maybe on the courtship of the thrips species. This suggests that *Wolbachia* could be involved in signal perception and interpretation in this species. As a consequence, it is conceivable that *Wolbachia* has an impact on sexual behaviour. This possibility is supported by the detection of *Wolbachia* in the deutocerebrum of males. The area is associated via antennal lobes with the scape muscles (movements of the antennae) and receives signals from a range of antennal sensilla (including the perception of pheromones). In the antennal lobes, *Wolbachia* occurred in clusters only in the males. This finding points to the possibility that bacteria are involved in the perception of sex-specific pheromones in *E*. *americanus* males. For example, observations in *Drosophila* lines demonstrated that the presence of *Wolbachia* in specific areas of the protocerebrum are associated with various consequences for the host (Albertson et al. 2009, Strunov et al. 2017). In *Drosophila paulistorum,* an infection of the Kenyon cells of the

mushroom bodies and local interneurons of perioesophageal neuropils results in a change in sexual behaviour (Strunov et al. 2017). Another study illustrated that elimination of the *Wolbachia* strain *w*Pau in the brain of fruit fly females leads to an alteration of the primitive mating behaviour (Miller et al. 2010). Furthermore, investigations of *Drosophila* demonstrated that mating behaviour is triggered by specific pheromone profiles (Kim et al. 2004, Chao et al. 2010).

Reproductive manipulators can influence insect-host pheromones and result in a modulation of host mating signals (Engl and Kaltenpoth 2018). *E*. *americanus* males exhibit pheromonal substances in the head-thorax complex, which have an antiaphrodisiac and male recognition impact (Krueger et al. 2016), influence the longevity of mated females and affects the oviposition rate, total fecundity, and developmental time of offspring (Krueger et al. 2020). Furthermore, Kirk et al. (2017) assumed a function as mating disruption. In this context, it is interesting that in *E*. *americanus* no gland could be observed in the head-thorax complex, which produced the pheromones (Krueger et al. 2016). Krueger et al. (2016) supposed that the substances were produced in the fat body or oenocytes in this area. A number of pheromone derivatives are commonly located in organs housing symbionts (Sudakaran et al. 2017). This aspect might explain the clusters of *Wolbachia* in the fat body of the head, especially in the area of the clypeofrons and vertex (S2 Tab. 4). In *Drosophila paulistorum*, the endosymbionts affect a change in the cuticular hydrocarbon profile, and function as anti-aphrodisiacs. The result of this *Wolbachia*-induced modification in sexual species is an assortative mating of males (Kim et al. 2004, Chao et al. 2010, Miller et al. 2010). The modification of the cuticular hydrocarbon profiles through symbionts can have particular repercussions on mating and courtship (Engl et al. 2018).

In contrast to the sex-dependent distribution of *Wolbachia* in the proto- and deutocerebrum of *E*. *americanus*, we detected the microorganisms in the tritocerebrum (Fig. 2.2F) and in the metathoracic ganglion (S2 Tab. 4) in the propupae and adults of both sexes. An interesting aspect concerning this is the distribution of *Wolbachia* in other organs. The tritocerebrum controls the sensory and motor functions of the labrum and is connected to the stomatogastric nervous system. The stomatogastric system innervates the salivary glands and the pharynx musculature (Milde 2003). We detected *Wolbachia* in the salivary gland of the first instar larva (Fig. 2.2B). *E*. *americanus* is a phytophagous thrips species. During the feeding process, saliva finds its way into the plant cells to digest their ingredients (Miles 1968, Gray and Banerjee 1999). An infection of the salivary gland seems beneficial for the horizontal transmission pathway of bacteria. Investigations on different insects suggest that microorganisms in the saliva can be transmitted to plant tissues (e.g. Sintupachee et al. 2006). A plant-mediated horizontal transfer of *Wolbachia* was detected in 2017 in the whitefly *Bemisia tabaci* (Li et al. 2017). The experiments of that study showed that *Wolbachia*-free

insects were infected after feeding on infected plants and transmitted the infection vertically to the next generation (Li et al. 2017). Based on the findings so far, the opportunity for horizontal transmission in *E*. *americanus* exists and therewith a potential infection pathway from the tritocerebrum. However, molecular analyses of the plant material (on which *E*. *americanus* lives) so far show no indications of the *Wolbachia* symbiont and thereby for a potential horizontal transfer (data not shown).

Since the bacteria were found in the metathoracic ganglion (S2 Tab. 4), wings (S2 Tab. 4) and limbs (Fig. 2.2E), the locomotion of the thrips could be affected. From the metathoracic ganglion, nerves lead to the limbs and the wings, particularly the hindwings, and the ganglion thus contributes directly to locomotion in thrips. The presence of *Wolbachia* in this area of the neuronal system could have a direct effect on the activity of the organism. In *Drosophila* lines, Peng et al. (2008) observed increased or decreased locomotor activity, depending on the host species and *Wolbachia* strain. It is possible that *Wolbachia* has an effect on the cell and tissue function of the metathoracic ganglion and therefore, is able to alter the energy demands of the thrips.

This supposition is also supported by the discovery of the bacteria in the muscles of *E*. *americanus* (Fig. 2.1F, Fig. 2.2I). The thoracic muscles are important for the flight process and the locomotion of the thrips. In this study, we could not identify in which type of musculature, *Wolbachia* was present. But it is striking that the distribution patterns alter from the larval to the adult stages. In the first instar larva, the bacteria could localize only in the muscles of the extremities (Fig. 2.1B/C, Fig. 2.2E), whereas in the adults *Wolbachia* is present in the thoracic muscles (Fig. 2.2I). The infection density increased with the host's age. This phenomenon was also noticed in the study of Andersen et al. (2012). In the leaf-cutting ant *Acromyrmex octospinosus* the *Wolbachia* was localized in the muscle fibres only in adult workers (Andersen et al. 2012). These findings suggest that bacteria can invade new tissue types during metamorphosis (Andersen et al. 2012). During the metamorphosis process, a vast alteration in the muscles system takes place, with the flight musculature developing directly after the propupal moult (Moritz 1997). Thus, the metamorphosis process offers an opportunity for the bacteria to transfer to new tissues, and, in the case of our study, to the thoracic muscles of both adult stages.

2.5.2 Abdomen (digestive system, fat body, reproductive system)

We localized *Wolbachia* in three large areas of the abdomen: the intestinal tract, the fat body and the reproductive system of both sexes (S2 Tab. 4). The morphological appearance of the digestive system of insects depends on their feeding modes. Generally, the gut can be divided into three regions: foregut, midgut, and hindgut. In most insects, the midgut is responsible for digestion and absorption (Engel and Moran 2013). Hence, the presence of *Wolbachia* in the

digestive tract might provide a beneficial aspect on the host physiology, life history and pathogen resistance (Pietri et al. 2016). Our investigations show that the bacteria occur in the larval and also, the adult stages (Fig. 2.1) of both sexes in *E*. *americanus* so that a direct effect is possible. However, vector competence and/or the ability of virus transmission has not yet been detected in *E*. *americanus*. An interesting feature in our study is the presence of *Wolbachia* in the first instar larva, especially in the salivary gland (Fig. 2.2B) and in the anterior part of the midgut (Fig. 2.2C). *Wolbachia* are able to interact with pathogens because they occur in a variety of tissues and cell types within insects (Dobson et al. 1999). For example, *Wolbachia*-infected flies and mosquitoes are able to generate resistance against a wide range of viruses, bacteria, and fungi (Eleftherianos et al. 2013). This is related to the benefit to host fitness and therewith the transmission of endosymbionts to the next generation (Brownlie and Johnson 2009). A number of observations have shown that *Wolbachia* can cause a decreasing of the pathogen level, protection (resistance) or a combination of both processes (Hedges et al. 2008, Teixeira et al. 2008).

Another important metabolism-regulated organ is the fat body of insects. This organ possesses a high capability for biosynthetic and metabolic activities (Law and Wells 1989), and fat body cells possess a synthesis, storage and utilization feature for several processes (Arrese and Soulages 2010). We detected *Wolbachia* in the fat body of all developmental stages of both sexes, although the distribution patterns changed between the larval and subsequent developmental stages (Fig. 2.1). The bacterium was detected in the abdomen in all developmental stages (S2 Tab. 4). In contrast, we localized the endosymbiont in the fat body of the head and thorax, especially in the early propupae and adult stages (S2 Tab. 4). The detection of *Wolbachia* in the head (in the area of the clypeofrons and vertex) and also in the thorax might be related to the occurrence of oenocytes (see above discussion deutocerebrum and antennae). *Wolbachia* is primarily distributed in the fat body layer directly under the cuticular surface of the thrips (Fig. 2.1, Fig. 2.2D). In the different developmental stages, the role of the fat body can vary drastically (Anand and Lorenz 2008). *Wolbachia* bacteria, housed in the fat body cells, might use the vitellogenin mechanism to move inside the oocyte. Herren et al. (2013) investigated the distribution patterns of the endosymbiont *Spiroplasma* during egg maturation in *Drosophila melanogaster* and proposed a model for a path of infection during the vitellogenic stages of oogenesis. The transmission route from the fat body to the oocytes is a powerful strategy for endosymbiont colonization of the germline and thus for maternal transmission. The presence of *Wolbachia* in the fat body and the possible associated germline infection is directly related to the localization of these bacteria in the reproductive system of adult females (Fig. 2.2J/K).

In the reproductive tissues, we localized the bacteria in the germarium and also in the follicle cells and oocytes in the vitellarium (Fig. 2.2J/K). The occurrence of *Wolbachia* was

conspicuous as strong accumulations in the eggs before oviposition (Fig. 2.2K). The model of Herren et al. (2013), above mentioned, could explain the *Wolbachia* distribution patterns in ovarioles and fat body of adult females of *E*. *americanus*. If a portion of the *Wolbachia* clusters in the fat body, transfected during the process of vitellogenesis in the developing oocytes, this could result in the colonization of the oocytes by *Wolbachia* (Fig. 2.2K). This main infection route of *Wolbachia* is the vertical transmission through the germline by transfer from the somatic cells to the germ stem cells at the germarium (Werren et al. 2008, Correa and Ballard 2016). But a more probable infection route is a continuous association of the bacterium with the germline, resulting in a symbiont-host association throughout the complete egg development. In this case, an immediate transfer of the endosymbiont takes place through the progenitor germline stem cell. Ultimately, the *Wolbachia*-infected oocyte developed through a proliferation of a primordial germline stem cell (Serbus et al. 2008). Many experiments document that female germline infection is an essential component for the transfer of the endosymbionts to the next generation (Werren et al. 2008). Furthermore, this route of infection could be responsible for the different distribution patterns of the bacterium within the larval stage.

The localization of *Wolbachia* in the reproductive system of the males is interesting. Our investigations showed that this endosymbiont occurs during the process of metamorphosis in the developing testes and accessory glands of the early propupae (Fig. 2.2G/H) and later, in adult males (S2 Tab. 4). The *Wolbachia* are present as accumulations within the testes (Fig. 2G) and especially in the wall of the accessory glands (Fig. 2.2H). The accessory glands have a two-layered wall, which is composed of a thin cell layer outside and secretory epithelium inside (Heming 1975). An essential element of the seminal fluid, stored within the accessory glands, are the accessory gland proteins (Acps). These proteins can have a direct effect on the female physiology and behaviour (Ram and Wolfner 2007, Findlay et al. 2008). Conditioned by the mating process, the Acps are transferred into the *Drosophila* female genital tract and generate, for example, an enhancement of ovulation and oviposition, and a diminution of attractiveness for further copulations or support of sperm storage (Chen et al. 1988, Neubaum and Wolfner 1999). Krueger et al. (2017) observed an extremely low re-mating frequency in females of our *E*. *americanus* laboratory culture. This behaviour could be explained through the presence of a special sex peptide, produced in the accessory glands, causing a decrease in further mating events in females. The localized accumulations of *Wolbachia*, mainly in the wall of the accessory glands, and the documented reproductive behaviour might indicate an endosymbiont regulated production of the sex peptide. Likewise, it is also possible that there is preferential synthesis of other Acps through the presence of bacteria, which stimulate the physiology of the females.

The current study demonstrated clear evidence of *Wolbachia* in the reproductive system of the males and females of *E*. *americanus*. These results might point towards a cytoplasmic incompatibility (CI) present in our experimental animal. This supposition is underpinned through the female-biased sex ratio in *E*. *americanus*, in contrast to other arrhenotokous species of Thysanoptera (Kumm 2002). Sex ratio distorters, such as *Wolbachia*, are able to control the sex determination of their hosts through the maternal microorganism transfer to the offspring. The mainly vertical transmission causes an increased production of female progeny and reduces the amount of male progeny (Sabelis et al. 2002).

2.6 References

Albertson, R., C. Casper-Lindley, J. Cao, U. Tram, and W. Sullivan. 2009. Symmetric and asymmetric mitotic segregation patterns influence *Wolbachia* distribution in host somatic tissue. J Cell Sci 122: 4570–4583.

Albertson, R., V. Tan, R.R. Leads, M. Reyes, W. Sullivan, and C. Casper-Lindley. 2013. Mapping *Wolbachia* distributions in the adult *Drosophila* brain. Cell Microbiol 15: 1527–1544.

Ambika, S., and R. Rajagopal. 2018. Lumen anatomy and localization of *Wolbachia* sp. in the thrips, *Plicothrips apicalis* (Bagnall). Curr Sci 115: 1297–1304.

Anand, A.N., and M.W. Lorenz. 2008. Age-dependent changes of fat body stores and the regulation of fat body lipid synthesis and mobilisation by adipokinetic hormone in the last larval instar of the cricket, *Gryllus bimaculatus*. J Insect Physiol 54: 1404–1412.

Andersen, S.B., M. Boye, D.R. Nash, and J.J. Boomsma. 2012. Dynamic *Wolbachia* prevalence in *Acromyrmex* leaf-cutting ants: potential for nutritional symbiosis. J Evol Biol 25: 1340–1350.

Arakaki, N., T. Miyoshi, and H. Noda. 2001. *Wolbachia*-mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (Thysanoptera: Insecta). Proc R Soc Lond B 268: 1011–1016.

Arrese, E.L., and J.L. Soulages. 2010. Insect fat body: Energy, metabolism and regulation. Annu Rev Entomol 55: 207–255.

Awrahman, Z.A., F. Championde Crespigny, and N. Wedell. 2014. The impact of *Wolbachia*, male age and mating history on cytoplasmic incompatibility and sperm transfer in *Drosophila simulans*. J Evol Biol 27: 1–10.

Begg, K., and W.D. Donachie. 1985. Cell shape and division in *Escherichia coli*: experiments with shape and division mutations. J Bacteriol 174: 63–70.

Brownlie, J.C., and K.N. Johnson. 2009. Symbiont-mediated protection in insect hosts. Trends Microbiol 17: 348–354.

Callaini, G., M.G. Riparbelli, and R. Dallai. 1994. The distribution of cytoplasmic bacteria in the early Drosophila embryo is mediated by astral microtubules. J Cell Sci 107: 673–682.

Ceron, J., C. Gonzalez, and F.J. Tejedor. 2001. Patterns of cell division and expression of asymmetric cell fate determinants in postembryonic neuroblast lineages of *Drosophila*. Dev Biol 230: 125–138.

Chao, T.H., L. Ehrman, A. Permaul, R. Vincent, L. Sattaur, and D. Brandt. 2010. Male-specific cuticular compounds of six *Drosophila paulistorum* semispecies: structural identification and mating effect. J Chem Ecol 36: 933–942.

Chen, P.S., E. Stummzollinger, T. Aigaki, J. Balmer, M. Bienz, and P. Bohlen. 1988. A male accessory-gland peptide that regulates reproductive behavior of female *Drosophila melanogaster*. Cell 54: 291–298.

Clark, M.E., C.L. Anderson, J. Cande, and T.L. Karr. 2005. Widespread prevalence of *Wolbachia* in laboratory stocks and the implications for *Drosophila* research. Genetics 170: 1667–1675.

Cordaux, R., D. Bouchon, and P. Grève. 2011. The impact of endosymbionts on the evolution of host sex-determination mechanisms. Trends Genet 27: 332–341.

Correa, C.C., and J.W.O. Ballard. 2016. *Wolbachia* associations with insects: winning or losing against a master manipulator. Front Ecol Evol 3: 153.

Dobson, S.L., K. Bourtzis, H.R. Braig, B.F. Jones, W. Zhou and et al. 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem Mol Bio 29: 153–160.

Eleftherianos, I., J. Atri, J. Accetta, and J.C. Castillo. 2013. Endosymbiotic bacteria in insects: guardians of the immune system? Front Physiol 4: 46.

Engel, P., and N. Moran. 2013. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev 37: 699–735.

Engl, T, and M. Kaltenpoth. 2018. Influence of microbial symbionts on insect pheromones. Nat Prod Rep 35: 386–397.

Engl, T., N. Eberl, C. Gorse, T. Krueger, T.H.P. Schmidt, R. Plarre, C. Adler, and M. Kaltenpoth. 2018. Ancient symbiosis confers desiccation resistance to stored grain pest beetles. Mol Ecol 27: 2095–2108.

Evans, J.D., D.C.A. Shearman, and B.P. Oldroyd. 2004. Molecular basis of sex determination in haplodiploids. Trends Ecol Evol 19: 1–3.

Fahlteich, F. 2017. Histological and molecular detection and distribution of *Wolbachia* infections in both sexes of *Echinothrips americanus* Morgan, 1913 (Thysanoptera: Thripidae). Master thesis. Martin-Luther-University Halle-Wittenberg, Halle.

Ferree, P.M., H.M. Frydman, J.M. Li, J. Cao, E. Wieschaus, and W. Sullivan. 2005. *Wolbachia* utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte. PLoS Pathog 1: e14.

Findlay, G.D., X.H. Yi, M.J. MacCoss, and W.J. Swanson. 2008. Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating. PLoS Biol 6: 1417–1426.

Frydman, H.M., J.M. Li, D.N. Robson, and E. Wieschaus. 2006. Somatic stem cell niche tropism in *Wolbachia*. Nature 441: 509–512.

Gray, S.M., and N. Banerjee. 1999. Mechanisms of arthropod transmission of plant and animal viruses. Microbio Mol Bio Rev 63: 128–148.

Hanesch, U., K.F. Fischbach, and M. Heisenberg. 1989. Neuronal architecture of the central complex in *Drosophila melanogaster*. Cell Tissue Res 257: 343–366.

Hedges, L.M., J.C. Brownlie, S.L. O'Neill, and K.N. Johnson. 2008. *Wolbachia* and virus protection in insects. Science 322: 702.

Heming, B.S. 1975. Antennal structure and metamorphosis in *Frankliniella fusca* (Hinds) (Thripidae) and *Haplothrips verbasci* (Osborn) (Phlaeothripidae) (Thysanoptera). Quaest Entomol 11: 25–68.

Herren, J.K., J.C. Paredes, F. Schupfer, and B. Lemaitre. 2013. Vertical transmission of a *Drosophila* endosymbiont via cooption of the yolk transport and internalization machinery. mBio 4: 1–8.

Hoffmann, A.A., M. Turelli, and G.M. Simmons. 1986. Unidirectional incompatibility between populations of *Drosophila simulans*. Evolution 40: 692–701.

Holden, P.R., J.F.Y. Brookfield, and P. Jones. 1993. Cloning and characterization of an *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. Mol Gen Genet 240: 213–220.

Hughes, G.L., X. Ren, J.L. Ramirez, J.M. Sakamoto, J.A. Bailey, and et al. 2011. *Wolbachia* infections in *Anopheles gambiae* cells: Transcriptomic characterization of a novel host-symbiont interaction. PLoS Pathog. 7: e1001296.

Hurst, G.D.D., J.H. Graf von der Schulenburg, T.M. Majerus, D. Bertrand, I.A. Zakharov, and et al. 1999. Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. Insect Mol Biol 8: 133–139.

Kaczmarczyk, A., H. Kucharczyk, M. Kucharczyk, and S. Zielińska. 2017. First report of *Wolbachia* in arrhenotokous populations of mycophagous species *Hoplothrips pedicularius* (Haliday 1836) (Thysanoptera, Phlaeothripidae). 5th Symposium on Palaearctic Thysanoptera, Cracow, Poland, 24.

Kaczmarczyk, A., H. Kucharczyk, M. Kucharczyk, P. Kapusta, J. Sell, and S. Zielińska. 2018. First insight into microbiome profile of fungivorous thrips *Hoplothrips carpathicus* (Insecta: Thysanoptera) at different developmental stages: molecular evidence of *Wolbachia* endosymbiosis. Sci Rep 8:14376.

Kim, Y.K., D.R. Phillips, T. Chao, and L. Ehrman. 2004. Developmental isolation and subsequent adult behaviour of *Drosophila paulistorum*. VI. Quantitative variation in cuticular hydrocarbons. Behav Genet 34: 385–394.

Kirk, W.D.J. 2017. The aggregation pheromones of thrips (Thysanoptera) and their potential for pest management. J Trop Insect Sci 37: 41– 49.

Kose, H., and T.L. Karr. 1995. Organization of *Wolbachia pipientis* in the *Drosophila* fertilized egg and embryo revealed by an anti-*Wolbachia* monoclonal antibody. Mech Dev 51: 275–288.

Kremer, N., D. Voronin, D. Charif, P. Mavingui, B. Mollereau, and F. Vavre. 2009. *Wolbachia* interferes with ferritin expression and iron metabolism in insects. PLoS Pathog 5: e1000630.

Krueger, S., G. Moritz, P. Lindemann, D. Radisch, and G. Tschuch. 2016. Male pheromones influence the mating behavior of *Echinothrips americanus*. J Chem Ecol 42: 294–299.

Krueger, S., M. Jilge, L. Mound, and G.B. Moritz. 2017. Reproductive behavior of *Echinothrips americanus* (Thysanoptera: Thripidae). J Insect Sci 17: 1–9.

Krueger, S., B. Müller, and G. Moritz. 2020. Olfactory and physical manipulation by males on life-history traits in *Echinothrips americanus* MORGAN 1913 (Thysanoptera: Thripidae). J Appl Entomol 144: 64–73.

Kumm, S. 2002. Reproduction, progenesis and embryogenesis of thrips (Thysanoptera, Insecta). Ph.D. dissertation. Martin-Luther-University Halle-Wittenberg, Halle.

Kumm, S., and G. Moritz. 2008. First detection of *Wolbachia* in arrhenotokous populations of thrips species (Thysanoptera: Thripidae and Phlaeothripidae) and its role in reproduction. Environ Entomol 37: 1422–1428.

Law, J.H., and M.A. Wells. 1989. Insects as biochemical models. J Biol Chem 264: 16335–16338.

Li, S.J., M.Z. Ahmed, N. Lv, P.Q. Shi, X.M. Wang, J.L. Huang, and B.L. Qiu. 2017. Plant-mediated horizontal transmission of *Wolbachia* between whiteflies. ISME J 11: 1019–1028.

Ma, W.J., F. Vavre, and L.W. Beukeboom. 2014. Manipulation of arthropod sex determination by endosymbionts: diversity and molecular mechanisms. Sex Dev 8: 59–73.

McMeniman, C.J., R.V. Lane, B.N. Cass, A.W. Fong, M. Sidhu, and et al. 2009. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. Science 323: 141–144.

Milde, J. 2003. Nervensystem, pp 205–227. In K. Dettner and W. Peters (eds.), Lehrbuch der Entomologie, vol. 2. Elsevier, Heidelberg, Germany.

Miles, P.W. 1968. Insect secretions in plants. Annu. Rev. Phytopathol 6: 137–164.

Miller, W.J., L. Ehrman, and D. Schneider. 2010. Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. PLoS Pathog 6e1001214.

Morel, G., A. Cavalier, and L. Williams. 2001. *In situ* hybridization in electron microscopy. CRC Press, USA.

Moritz, G. 1997. Structure, growth and development. In T. Lewis (ed.), Thrips as crop pests. CAB International, Oxon and New York, USA.

Negri, I., A. Franchini, E. Gonella, D. Daffonchio, P.J. Mazzoglio, and et al. 2009. Unravelling the *Wolbachia* evolutionary role: the reprogramming of the host genomic imprinting. Proc Bio Sci 276: 2485–2491.

Neubaum, D.M., and M.F. Wolfner. 1999. Mated *Drosophila melanogaster* females require a seminal fluid protein, Acp36DE, to store sperm efficiently. Genetics 153: 845–857.

Nguyen, D.T., J.L. Morrow, R.N. Spooner-Hart, and M. Riegler. 2017. Independent cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* maintains endosymbiont coinfections in haplodiploid thrips populations. Evolution 71: 995–1008.

O'Neill, S.L., and T.L. Karr. 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature 348: 178–180.

Osborne, S.E., Y.S. Leong, S.L. O'Neill, and K. Johnson. 2009. Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. PLoS Pathog 5: e1000656.

Peng, Y., J.E. Nielsen, J.P. Cunningham, and E.A. McGraw. 2008. *Wolbachia* infection alters olfactory-cued locomotion in *Drosophila* spp. Appl Environ Microbiol 74: 3943–3948.

Pietri, J.E., H. DeBruhl, and W. Sullivan. 2016. The rich somatic life of *Wolbachia*. Microbiologyopen 5: 923–936.

Ram, K.R., and M.F. Wolfner. 2007. Seminal influences: *Drosophila* Acps and the molecular interplay between males and females during reproduction. Integr Comp Biol 47: 427–445.

Reed, K.M., and J.H. Werren. 1995. Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. Mol Reprod Dev 40: 408–418.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault, and M. Solignac. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proc R Soc Lond B 250: 91–98.

Russell, S., L. Chappell, and W. Sullivan. 2019. A Symbiont's Guide to the Germline. Preprints 2019010149.

Sabelis, M.W., C.J. Nagelkerke, and J.A.J. Breeuwer. 2002. Sex ratio control in arrhenotokous and pseudo-arrhenotokous mites, pp. 246-248. In I.C.W. Hardy (ed.), Sex ratios concepts and research methods, vol. 11, Cambridge University Press, United Kingdom.

Saurav, G.K., G. Daimei, V.S. Rana, S. Popli S, and R. Rajagopal. 2016. Detection and Localization of *Wolbachia* in *Thrips palmi* Karny (Thysanoptera: Thripidae). Indian J Microbiol 56: 167–171.

Sazama, E.J., S.P. Ouellette, and J.S. Wesner. 2019. Bacterial endosymbionts are common among, but not necessarily within, insect species. Environ Entomol 48: 127–133.

Serbus, L.R., C. Casper-Lindley, F. Landmann, and W. Sullivan. 2008. The genetics and cell biology of *Wolbachia*-host interactions. Annu Rev Genet 42: 683–707.

Sintupachee, S., J.R. Milne, S. Poonchaisri, V. Baimai, and P. Kittayapong. 2006. Closely related *Wolbachia* strains within the pumpkin arthropod community and the potential for horizontal transmission via the plant. Microb Ecol 51: 294–301.

Stouthamer, R., R.F. Luck, and W.D. Hamilton. 1990. Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/ Trichogrammatidae) to revert to sex. PNAS 87: 2424–2427.

Strunov, A., D.I. Schneider, R. Albertson, and W.J. Miller. 2017. Restricted distribution and lateralization of mutualistic *Wolbachia* in the *Drosophila* brain. Cell Microbiol 19: e12639.

Sudakaran, S., C. Kost, and M. Kaltenpoth. 2017. Symbiont acquisition and replacement as a source of ecological innovation. Trends Microbiol 25: 375–390.

Teixeira, L., A. Ferreira, and M. Ashburner. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol 6: e2.

Vala, F., M. Egas, J.A.J. Breeuwer, and M.W. Sabelis. 2004. *Wolbachia* affects oviposition and mating behaviour of its spider mite host. J Evol Biol 17: 692–700.

Vierbergen, G. 1998. *Echinothrips americanus* Morgan, a new thrips in Dutch greenhouses (Thysanoptera: Thripidae). Proc Exp Appl Entomol 9: 155–160.

Werren, J.H., L. Baldo, and M.E. Clark. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 6: 741–751.

White, J.A., M. Giorgini, M.R. Strand, and F. Pennacchio. 2013. Arthropod endosymbiosis and evolution, pp. 441–447. In A. Minelli, G. Boxshall, and G. Fusco (eds.), Arthropod Biology and Evolution. Springer-Verlag, Berlin and Heidelberg, Germany.

Yen, J.H., and A.R. Barr. 1971. New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. Nature 232: 657–658.

Zars, T., M. Fischer, R. Schulz, and M. Heisenberg. 2000. Localization of a short-term memory in *Drosophila*. Science 288: 672–675.

Zouache, K., D. Voronin, V. Tran-Van, L. Mousson, A-B. Failloux, and P. Mavingui. 2009. Persistent *Wolbachia* and cultivable bacteria infection in the reproductive and somatic tissues of the mosquito vector *Aedes albopictus*. PLoS ONE 4: e6388.

CHAPTER 3

Evidence of a coinfection with *Cardinium* and *Wolbachia* in the arrhenotokous thrips *Echinothrips americanus* (Thysanoptera: Thripidae)

3.1 Summary

The sex determination of many insects can be manipulated through diverse endosymbionts, which can have remarkable consequences for the sexual phenotypes. *Wolbachia*, one of the most widespread gram-negative bacteria, is known to induce parthenogenesis (bacteria induced thelytoky or pseudo-thelytoky) in Thysanoptera. Most Thysanoptera reproduce via arrhenotoky, so as *Echinothrips americanus*. This thrips is infected with *Wolbachia,* but no thelytoky is induced by these intracellular bacteria (infection status of our laboratory culture). We investigated the possible existence of a double infection in *Echinothrips americanus*, through molecular characterization (*wsp* gene, *ftsZ* gene, 16S rDNA), and detected a coinfection with *Wolbachia* and *Cardinium* in all developmental stages of both sexes. We showed, by use of *in situ* hybridization, that both endosymbionts are mainly maternally transmitted.

Keywords: double infection, thrips, developmental stages, maternal transmission

3.2 Introduction

Many insects live in close and mutually beneficial relationships with microorganisms. The existence of these bacterial symbioses is important for host development (McFall-Ngai et al. 2012), immunity (Oliver et al. 2003), metabolism (Grill et al. 2014), behaviour (Kenyon and Hunter 2007), nutrition (Fierer et al. 2009), reproduction (Leonardo and Mondor 2006) and specification (Brucker and Bordenstein 2013). These, and many other processes are essential for the evolution and diversification success of insects. Microbes in and on insects are regulated through diverse types of symbiosis, whereby the host-symbiont interactions can range from mutualism, commensalism to parasitism. It is estimated that more than 70% of all insects exhibit an endosymbiosis with intracellular bacteria (also called endosymbionts) (Ishikawa 2003). The mainly maternally transmitted bacteria are categorized into two types: primary and secondary symbionts, of which the obligatory and mutualistic primary bacteria possess a special function with beneficial effects for the host. These endosymbionts play an essential role in insect nutrition (Feldhaar 2011). Primary symbionts characteristically occur in specialized discrete organs, known as mycetocytes or bacteriocytes (Buchner 1965). Microbes of the facultative or secondary symbionts invade various cells and tissues of their hosts. They can have diverse effects on the host fitness. Several microorganisms impact defence against stress or natural enemies, and others improve host metabolism or influence behaviour. In contrast to the mutualistic impacts, secondary symbionts can function as reproductive manipulators. These effectively parasitic bacteria have an impact on host sexuality and reproduction (Breeuwer and Werren 1990). Endosymbionts are able to alter the reproduction in different ways to the benefit of the female progeny (Rousset et al. 1992, Werren et al. 1995), and this ensures the bacteria a high transfer rate to the next generation (Werren et al. 2008, White et al. 2013). The most common form of endosymbiont-induced phenotype is cytoplasmic incompatibility (CI). The simplest form, the unidirectional CI, appears when an infected male mates with an uninfected female (Somerson et al. 1984). Alternatively, CI can occur if the organisms exhibit different strains of the symbiont (bidirectional CI) (Sinkins et al. 1995). The repercussions of this manipulation type are different. In haplodiploid insect species the endosymbionts cause an elimination of the paternal chromosomes, all progeny are males or infection leads to embryonic mortality (Reed and Werren 1995). Another bacteria-induced phenotype is male-killing infected males die during embryonic or larval development (Hurst and Jiggins 2000). Furthermore, reproductive manipulators affect the development of genetic males through feminization and genotypic males turn into phenotypic and functional females (Bandi et al. 2001). In addition, endosymbionts can cause parthenogenesis (thelytoky), by which virgins produce only female offspring (Stouthamer et al. 1990). The most common facultative symbionts, which exist in many orders of insects and can cause a variety of reproductive alterations on insect hosts are *Wolbachia* (alpha-Proteobacteria) and Candidatus

Cardinium (Bacteroidetes) (Duron et al. 2008). Many studies show that horizontal transfer of bacterial symbionts within, and between, species takes place, which enable coinfections of the same host cell with various symbionts (e.g. Weinert et al. 2007, Duron et al. 2008, Skaljac et al. 2010). Variances of the population structure are consequences of lateral transfers. Particularly, the sex ratio of hosts can interfere through the presence of specific microbes and result in drastic shifts in the population dynamic. Haplodiploids show a number of sex allocations caused by the prevalence and diversity of different bacteria types, mitochondrial variants, or supernumerary B chromosomes (Beukeboom and Perrin 2014). A common effect of sex ratio distorters in haplodiploid organisms is cytoplasmic incompatibility, which is mostly characterized through a double infection of two endosymbionts from the same genus (Raychoudhury and Werren 2012) or from various groups (Zhu et al. 2012). Otherwise, extreme sex ratios in haplodiploid (arrhenotokous) insects could also be based on different natural phenomena (Hamilton 1967).

The most species of Thysanoptera reproducing by haplodiploid reproduction (arrhenotoky), which means males develop from unfertilized (haploid) and females from fertilized (diploid) eggs. *Echinothrips americanus*, the thrips in our study, present a male-female ratio based on an arrhenotokous reproduction mode, although this thrips species is provable infected with *Wolbachia* (Kumm and Moritz 2008). Commonly, in species with haplodiploid development, like hymenoptera, mites and thrips, *Wolbachia* induce thelytokous parthenogenesis (Werren et al. 2008). In most cases, an elimination of the endosymbiont (antibiotic treatment) result in an arrhenotoky. Another interesting aspect of our *E*. *americanus* population is the low level of males (1 female : 0.3 male) (Kumm 2002). For instance, thrips species, like *Frankliniella occidentalis*, show a sex ratio of 1 : 0.9 (female : male) (Kumm 2002). Other observations on haplodiploid insects identify the increased number of females as a result of a coinfection (Perlman et al. 2006). In the arrhenotokous wasp, *Encarsia inaron*, double infection with *Wolbachia* and *Cardinium*, induced an extreme sex ratio with a low level of males (Perlman et al. 2006). With the scope of this study, we investigated all developmental stages (egg, first instar larva, second instar larva, propupa, pupa, adult) of both sexes on the presence of *Cardinium*. Thereby we identify a potential coinfection with *Wolbachia* and *Cardinium* in *Echinothrips americanus*, which is possibly the reason for the conspicuous sex ratio.

3.3 Materials and methods

3.3.1 Rearing of thrips stages

For the breeding of the developmental stages we used *E*. *americanus* Morgan 1913 and *F*. *occidentalis* (Pergande, 1895) from the laboratory culture at the University of Halle-Wittenberg (at 23 ± 1 °C, RH 50 ± 10%, and a 16 : 8 h L:D photoperiod, with lights on 6:00 a.m. CET). These thrips species were reared on *Gossypium* sp. and *Hibiscus* sp. (*E. americanus*) as well as *Phaseolus vulgaris* and *Chrysanthemum* sp. (*F. occidentalis*). *Frankliniella occidentalis* is a *Wolbachia* (Kumm and Moritz 2008) and *Cardinium* uninfected species and conduces as control in our study.

To obtain the developmental stages we placed one virgin female (to obtain male progeny) or mated female (for female offspring) in each well of a 12-well Greiner plate, which was filled with 2 ml of 1.4% (*w*/*v*) agar (agar-agar, danish, Carl Roth GmbH + Co. KG, Germany) and a leaf disc of *P. vulgaris* on the surface of agar. The plates were sealed with a glass lid and Parafilm® M (Pechiney Plastic Packaging, Chicago, IL, USA) and placed in the climate chamber for 24 h (under the same conditions as the laboratory culture), before the females were removed from the Greiner plates. Subsequently, the Greiner plates with the deposited eggs were placed back in the climate chamber until use. For the investigations we used all developmental stages of *E*. *americanus* and *F*. *occidentalis*: egg (7 d ± 12 h old, 4 d ± 12 h old), first instar larva (10 d ± 12 h old, 6 d ± 12 h old), second instar larva (11 d ± 12 h old, 8 d ± 12 h old), propupa (13 d ± 12 h old, 10 d ± 12 h old), pupa (14 d ± 12 h old, 12 d ± 12 h old) and adults (16 d ± 12 h old, 14 d ± 12 h old) of both sexes. The stated developmental time refers to the time point after oviposition.

3.3.2 DNA extraction, gene amplification, agarose gel electrophoresis

In this study, we used a modified variety of the “100 fly method” after Roberts (1998) (described in Kumm et al. 2006). Each developmental stage was crushed in liquid nitrogen, homogenized in 100 µl extraction buffer (0.2 M Sucrose, 0.1 M TRIS, 0.1 M NaCl, 0.05 M EDTA, 0.5% SDS, pH 9.2) and then incubated for 15 min at 70 °C. Following this, 15 µl of ice-cold 8 M potassium acetate was added, mixed, and incubated for 15 min on ice. After the centrifugation (15 min at 13200 rpm), the supernatant (90 µl) was transferred to a new tube with 2.5 vol. ethanol and 0.1 vol. 3 M sodium acetate (4 °C) and vigorously mixed. Subsequently, the DNA was precipitated at -80 °C for 45 min. After this step, the DNA was sedimented by centrifugation at 13200 rpm (15 min) and the supernatant was discarded. The sediment was washed with 70% ethanol by centrifugation (13200 rpm) for 5 min. Afterwards, the supernatant was discarded again and the sediment dried at 45 °C for 5 min. Finally, the sediment was dissolved in 16 µl ddH_2O for 10 min at 45 °C.

For *Wolbachia* detection, we used specific primers for the 16S rDNA (99F/994R, O'Neill et al. 1992), *ftsZ* gene (494F/1262R, Holden et al. 1993) and *wsp* gene (81F/691R, Zhou et al. 1998) (S3 Tab. 1). Otherwise, the *Cardinium* was proven through primers for the 16S rDNA (ChF/ChR, Zchori-Fein et al. 2004 and CLOf1/CLOr1, Gotoh et al. 2007) (S3 Tab. 1). The PCR reaction mixture comprised 5 µl 10x reaction buffer, 4 µl $MgCl_2$ (25 mM), 0.4 µl dNTPs (25 mM), 1.5 µl of each primer (20 pmol/µl for the *Wolbachia* primers and 10pmol/µl for the *Cardinium* primers), 0.2 µl *Taq* polymerase and 2 µl of the template DNA in a total volume of 50 µl. Depending on the specific primers, the following programs were used for the PCR: for the detection of *Wolbachia* we used the program conditions of Kumm and Moritz 2008 and for the characterization of *Cardinum*, the PCR thermo-cycling conditions of Nguyen et al. 2017. The PCR products were verified through a 2% agarose gel, visualized with the gel documentation system DIAS-III from SERVA and documented with GelScan V6.0.

3.3.3 *In situ* hybridization

For this investigation, we modified an *in situ* hybridization method of Morel et al. 2001. This used *in situ* hybridization technology, based on an immunohistochemistry-like method, in which the detection is incorporated with labelled specimens. To determine the maternal transmission of *Cardinium* and *Wolbachia*, the presence of both symbionts was investigated in the vitellogenic eggs of the female adults and first instar larvae of *E. americanus*. After fixation (mixture: 4% paraformaldehyde and 0.25% glutaraldehyde), embedding (dehydration with ethanol, embedding in paraffin) and pre-treatment (proteolysis with Proteinase K) of the samples (20 female adults and 20 first instar larva) the *in situ* hybridization took place. With the scope of the *in situ* PCR, the digoxigenin-labelled dUTPs (digoxigenin-11-dUTPs, Roche Diagnostics) were integrated with the new synthesized DNA section. For amplification of the *Cardinium* DNA, we used the CLOf1/CLOr1 primers (Gotoh et al. 2007) and for the *Wolbachia* DNA 494F/1262R primers (Holden et al. 1993). In the immunohistochemical detection, the digoxigenin-marked dUTPs were localized through an antibody (anti-digoxigenin antibody, Roche Diagnostics) and visualized through a colour reaction of the reporter enzymes (5-bromo-4-chloro-3-indolyl-phosphate, nitro blue tetrazolium). The complete method was realized after the instruction in chapter 2.

3.4 Results

3.4.1 Molecular characterization by means of gene detection

In our investigations, we tested several primers for specific endosymbionts and found double infection with *Wolbachia* and *Cardinium* in *E*. *americanus*. The *Wolbachia* was proofed with primers for the 16S rDNA, the *ftsZ* gene and *wsp* gene, whereas the *Cardinium* was detected with two different primers for the 16S rDNA (S3 Tab. 1). The results of our investigations are present in the figures 3.1A and B.

Figure 3.1A depicts the results for the molecular characterization of the *ftsZ* gene. All developmental stages, from egg to adult, of both sexes are infected with *Wolbachia* (Fig. 3.1A, S3 Tab. 2). The results for the *wsp* gene and 16S rDNA provide the same infection status for *Wolbachia* in both sexes (S3 Tab. 2), as for the *ftsZ* gene (Fig. 3.1A). For the *ftsZ* gene, the infection level of each developmental stage is presented in S3 Tab. 3 and amounts to ~90% in both sexes.

Figure 3.1B shows the results for the primers CLOf1 and CLOr1. The *Cardinium* infection is also present in all developmental stages of males and females (Fig. 3.1B). But the ChF and ChR primers detected the *Cardinium* bacterium only in very few stages (S3 Tab. 2). The mean differences are summarized in S3 Tab. 2. For the 16S rDNA (primers CLOf1/CLOr1) the infection levels between the female and male developmental stages comprise ~80% to 85% (S3 Tab. 3).

Frankliniella occidentalis, the control organism, showed no infection with *Wolbachia* nor *Cardinium* in the tested comparable developmental stages (S3 Tab. 2).

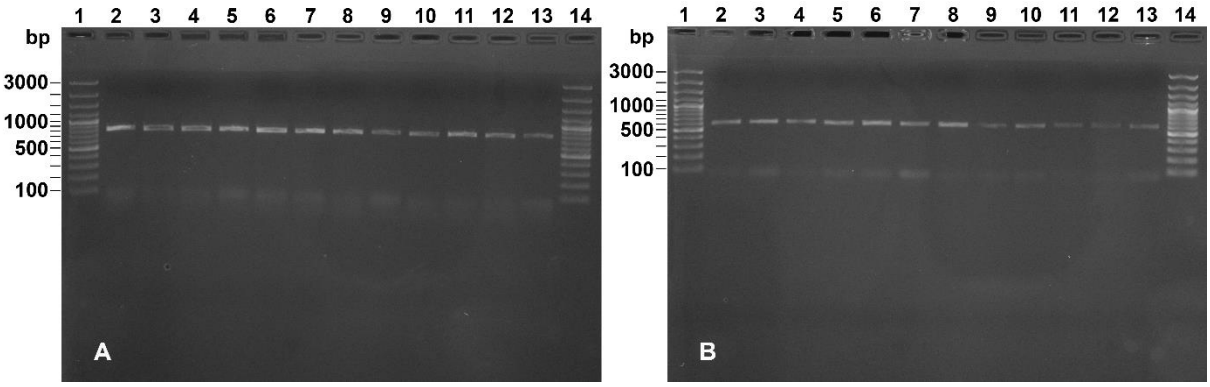


Fig. 3.1. Detection of *Wolbachia* (fragment length: 730 bp) and *Cardinium* (fragment length: 468 bp) in the developmental stages of both sexes in *E. americanus*. Fig. 3.1A *Wolbachia* detection (*ftsZ* gene, 494F/1262R): lanes 1 and 14 – marker (Gene Ruler^{TM} 100 bp (Thermo Scientific, Waltham)), 2 – adult (female), 3 – pupa (female), 4 – propupa (female), 5 – second instar larva (female), 6 – first instar larva (female), 7 – egg (female), 8 – adult (male), 9 – pupa (male), 10 – propupa (male), 11 – second instar larva (male), 12 – first instar larva (male), 13 – egg (male). Fig. 3.1B *Cardinium* detection (16S rDNA gene, CLO-f1/CLO-r1): lanes 1 and 14 – marker, 2 – adult (female), 3 – pupa (female), 4 – propupa (female), 5 – second instar larva (female), 6 – first instar larva (female), 7 – egg (female), 8 – adult (male), 9 – pupa (male), 10 – propupa (male), 11 – second instar larva (male), 12 – first instar larva (male), 13 – egg (male).

3.4.2 Molecular characterization by means of *in situ* hybridization

The early developmental stages (eggs during vitellogenesis and first instar larva) of *E*. *americanus* were investigated to verify the possibility of maternal transmission of the identified endosymbionts. By use of *in situ* hybridization, we could detect a potential transmission path via the egg system to the following developmental stages of the bacteria (Fig. 3.2). Both *Wolbachia* (Fig. 3.2A and B) and *Cardinium* (Chapter 5) occurred in all investigated samples as accumulations in the yolk mass (protein yolk bodies) of the oocytes just before oviposition. Additionally, the microorganisms were localized in all tested specimens in diverse tissues of the first instar larvae (for *Wolbachia* detection see Chapter 2), especially the *Cardinium* bacterium was detected in the supraoesophageal ganglion (Fig. 3.2A and B).

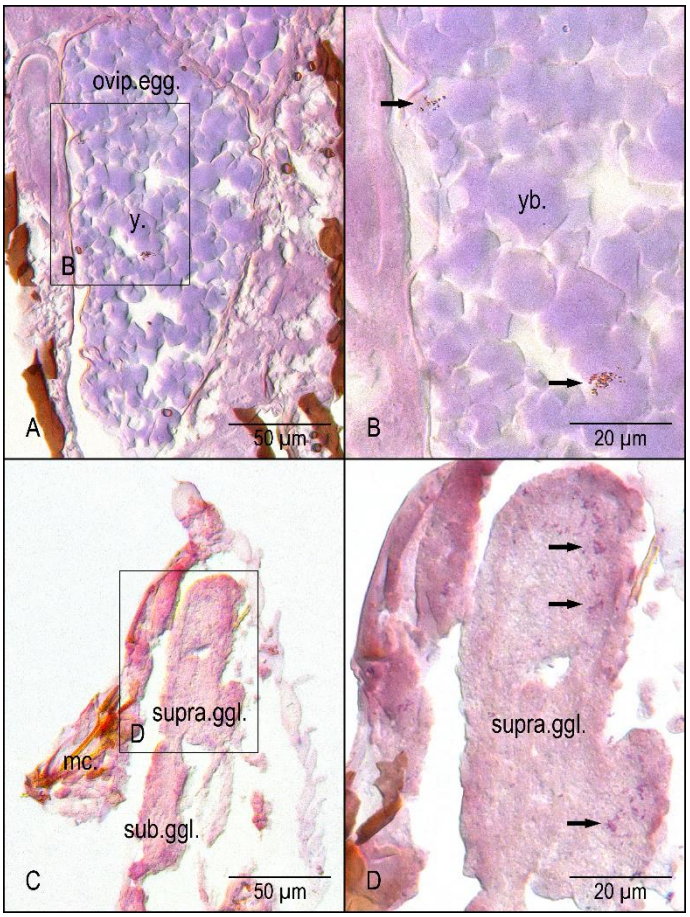


Fig. 3.2. Detection of *Wolbachia* and *Cardinium* in different tissues of the developmental stages of *E*. *americanus* by use of the *in situ* hybridization. Fig. 3.2A–B *Wolbachia* occurrences in a vitellogenic egg (frontal section, female, adult): (A) overview of the egg, (B) detail view of the egg with the proofs of *Wolbachia* (arrows). Fig. 3.2C–D *Cardinium* occurrences in the neuronal tissue (frontal sections, female, first instar larva): (C) overview of the head, (D) detail view of the supraoesophageal ganglion with the proofs of *Cardinium* (arrows). (mc.– mouth cone, ovip.egg– vitellogenic egg before oviposition, subggl.– suboesophageal ganglion, supraggl.– supraoesophageal ganglion, y.– yolk, yb.– protein yolk body).

3.5 Discussion

Previous investigations demonstrated that *E*. *americanus* is infected with *Wolbachia*, although it clearly exhibits a haplodiploid reproduction mode (Kumm and Moritz 2008, Dong et al. 2012). In haplodiploids, like thrips, endosymbionts are able to duplicate the chromosomes of their host eggs. The bacterial caused thelytokous parthenogenesis effected that females transmit only maternal genes, so all offspring are females. First of all, Arakaki et al. (2001) detected the *Wolbachia*-induced parthenogenesis in thrips. Furthermore, Nguyen et al. (2017) evidenced an independent reproductive incompatibility by *Cardinium* and *Wolbachia* in thrips.

As a result of this, we tested in our study the possibility of double infection with *Wolbachia* and *Cardinium*, which is maybe the cause of the observed arrhenotoky. The findings showed that all developmental stages (egg, first and second instar larva, propupa, pupa, adult) of both sexes are infected with both bacteria (S3 Tab. 2). A second major finding was the evidence of a potential transmission way (Fig. 3.2). We localized both *Wolbachia* (Fig. 3.2A and B) and *Cardinium* (data not shown) through *in situ* hybridizations in the yolk mass of the oocytes before oviposition. Additionally, we detected the *Cardinium* in different somatic tissues of the first instar larva (data not shown), but mainly in the supraoesophageal ganglion (Fig. 3.2C and D). Several studies showed that infections with endosymbionts are primarily vertically transmitted to embryos via the maternal cytoplasm (Chen et al. 2000, Pontes and Dale 2006). Previously, we localized *Wolbachia* during oogenesis of *E*. *americanus* in the germarium, in the follicular epithelium of the developing oocytes and in the yolk mass of eggs before oviposition (Chapter 2). Albertson et al. localized 2009 *Wolbachia* in the apical cell cortex during the symmetric and asymmetric divisions of embryonic neuroblasts, whereby the bacteria are evenly distributed between the cells. It is conceivable that the segregation patterns of the neuronal stem cells in Thysanoptera are also associated with the distribution patterns of *Cardinium*. Consequently, *Cardinium* could distribute in the entire supraoesophageal ganglion. Taken as a whole, both endosymbionts exhibited similar distribution patterns in the early development phase of *E*. *americanus*.

A number of studies discovered a horizontal transfer within or between maternal lines (O'Neill et al. 1992, Sandström et al. 2001). Especially coinfections develop through horizontal transfers among different hosts and often result in manipulations of the population structure and genome dynamics (Moran et al. 2008). *Wolbachia* and *Cardinium* are able to induce cytoplasmic incompatibility, the most common strategy of reproductive manipulation. In *Pezothrips kellyanus*, the endosymbionts induced independently of each other a reproductive incompatibility with different characteristics, whereby both bacteria can cause an embryonic mortality (Nguyen et al 2017). However, we could not observe embryonic mortality in our laboratory culture (Krueger et al. 2016, Chapter 4 for the *Cardinium* symbiont). Otherwise, Li et al. (2012) observed in their *E*. *americanus* population an extremely low hatchability of

eggs. Bourtzis et al. (1996) determined the level of embryonic mortality in different *Wolbachia* infected *Drosophila melanogaster* lines and, based on the genetic background found that various *D. melanogaster* strains had different egg mortality rates, despite the same *Wolbachia* infection. For instance, Perlman et al. (2006) found in the haplodiploid wasp, *Encarsia inaron*, double infection with *Wolbachia* and *Cardinium*, but these infections caused no embryonic mortality. Instead, the bacteria impair the sex ratio of this insect. Antibiotic treatment could establish that the low level of males materialized only through the coinfection with *Wolbachia* and *Cardinium* in *Encarsia inaron* (Perlman et al. 2006).

However, it is still unknown how the coinfection of *Wolbachia* and *Cardinium* impacts *Echinothrips americanus*. It is conceivable that the double infection induced a reproductive incompatibility, the sex ratio shifted for the benefit of the female progeny. Further investigations will show if the bacteria cause this phenotype in our laboratory culture, independent of each other with different expression, or if only one endosymbiont is the determining factor, or both together.

3.6 References

Albertson, R., C. Casper-Lindley, J. Cao, U. Tram, and W. Sullivan. 2009. Symmetric and asymmetric mitotic segregation patterns influence *Wolbachia* distribution in host somatic tissue. J Cell Sci 122: 4570–4583.

Arakaki, N., T. Miyoshi, and H. Noda. 2001. *Wolbachia*-mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (Thysanoptera: Insecta). Proc R Soc Lond B 268: 1011–1016.

Bandi, C. 2001. Inherited microorganisms, sex specific virulence and reproductive parasitism. Trends Parasitol 17: 88–94.

Beukeboom, L.W., and N. Perrin. 2014. Evolutionary correlates of sex-determination systems, pp. 115–132. In L.W Beukeboom and N. Perrin (eds.), The evolution of sex determination. Oxford University Press, Oxford.

Bourtzis, K., A. Nirgianaki, G. Markakis, and C. Savakis. 1996. *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. Genetics 144: 1063–1073.

Breeuwer, J.A., and J.H. Werren. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature 346: 558–560.

Brucker, R.M., and S.R. Bordenstein. 2013. The hologenomic basis of specification: gut bacteria cause hybrid lethality in the genus *Nasonia*. Science 341: 667–669.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience publishers, New York.

Chen, D.Q., C.B. Montllor, and A.H. Purcell. 2000. Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. Entomol Exp Appl 95: 315–323.

Dong, J., X.W. Li, X.C. Zhang, and J.N. Feng. 2012. Sequencing and phylogenetic analysis of the *wsp* gene of *Wolbachia* in *Echinothrips americanus* Morgan. Journal of Northwest A & F University 3: 131–134.

Duron, O., D. Bouchon, S. Boutin, L. Bellamy, L. Zhou, and et al. 2008. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biology 6: 27.

Feldhaar, H. 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol Entomol 36: 533–543.

Fierer, N., M.S. Strickland, D. Liptzin, M.A. Bradford, and C.C. Cleveland. 2009. Global patterns in belowground communities. Ecol Lett 12: 1238–1249.

Gill, A.C., A.C. Darby, and B.L. Makepeace. 2014. Iron necessity: The secret of *Wolbachia´*s success? PLOS Negl Trop Dis 8: e3224.

Gotoh, T., H. Noda, and S. Ito. 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. Heredity 98: 13–20.

Hamilton, W.D. 1967. Extraordinary sex ratio. Science. 156: 477–488.

Holden, P.R., J.F.Y. Brookfield, and P. Jones. 1993. Cloning and characterization of an *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. Mol Gen Genet 240: 213–220.

Hurst, G.D., and F.M. Jiggins. 2000. Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerg Infect Dis 6: 229–336.

Ishikawa, H. 2003. Insect symbiosis: An introduction, pp. 5–6. In K. Bourtzis and T.A. Miller (eds.), Insect symbiosis. CRC Press LLC, USA.

Kenyon, S.G., and M.S. Hunter. 2007. Manipulation of oviposition choice of the parasitoid wasp, *Encarsia pergandiella*, by the endosymbiotic bacterium *Cardinium*. J Evol Biol 20: 707–716.

Krueger, S., L.A. Mound, and G.B. Moritz. 2016. Offspring sex ratio and development are determined by copulation activity in *Echinothrips americanus* MORGAN 1913 (Thysanoptera: Thripidae). J Appl Entomol 140: 462–473.

Kumm, S. 2002. Reproduction, progenesis and embryogenesis of thrips (Thysanoptera, Insecta). PhD thesis. Martin-Luther-University Halle-Wittenberg, Halle, Germany.

Kumm, S., and G. Moritz. 2008. First detection of *Wolbachia* in arrhenotokous populations of thrips species (Thysanoptera: Thripidae and Phlaeothripidae) and its role in reproduction. Environ Entomol 37: 1422–1428.

Kumm, S., R. Kranz, and G. Moritz. 2006. Mikroorganismen-gesteuerte Parthenogenese bei Thysanopteren. Mitt Dtsch Ges allg angew Ent 15: 153–156.

Leonardo, T.E., and E.B. Mondor. 2006. Symbiont modifies host life-history traits that affect gene flow. Proc Biol Sci 273: 1079–1084.

Li, X.-W., X.-C. Zhang, H.-X. Jiang, and J.-N. Feng. 2012. Comparisons of developmental and reproductive biology between parthenogenetic and sexual *Echinothrips americanus* (Thysanoptera: Thripidae). Environ Entomol 41: 706–713.

McFall-Ngai, M., E.A.C. Heath-Heckman, A.A Gillette, S.M. Peyer, and E.A. Harvie. 2012. The select languages of coevolved symbiosies: insights from the *Euprymna scolopes-Vibrio fischeri* symbiosis. Semin Immunol 24: 3–8.

Moran, N.A., J.P. McCutcheon, and A. Nakabachi. 2008. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42: 165–190.

Morel, G., A. Cavalier, and L. Williams. 2001. *In situ* hybridization in electron microscopy. CRC Press, USA.

Nguyen, D.T., J.L. Morrow, R.N. Spooner-Hart, and M. Riegler. 2017. Independent cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* maintains endosymbiont coinfections in haplodiploid thrips populations. Evolution 71: 995–1008.

Oliver, K.M., J.A. Russell, N.A. Moran, and M.S. Hunter. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci USA 100: 1803–1807.

O'Neill, S.L., R. Giordano, A.M.E Colbert, T.L. Karr, and H.M. Robertson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc Natl Acad Sci USA 89: 2699–2702.

Perlman, S.J., S.E. Kelly, E. Zchori-Fein, and M.S. Hunter. 2006. Cytoplasmic incompatibility and multiple symbiont infection in the ash whitefly parasitoid, *Encarsia inaron*. Biol Control 39: 474–480.

Pontes, M.H., and C. Dale. 2006. Culture and manipulation of insect facultative symbionts. Trends Microbiol 14: 406–412.

Raychoudhury, R., and J.H. Werren. 2012. Host genotype changes bidirectional to unidirectional cytoplasmic incompatibility in *Nasonia longicornis*. Heredity 108: 105–114.

Reed, K.M., and J.H. Werren. 1995. Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. Mol Reprod Dev 40: 408–418.

Roberts, D.B. 1998. *Drosophila*, a practical approach, pp. 399. 2rd edn. Oxford University Press, Oxford, New York, Tokyo.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault, and M. Solignac. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proc R Soc London Ser B 250: 91–98.

Sandström, J.P., J.A. Russell, J.P. White, and N.A. Moran. 2001. Independent origins and horizontal transfer of bacterial symbionts of aphids. Mol Eco 10: 217–228.

Skaljac, M., K. Zanic, S.G. Ban, S. Kontsedalov, and M. Ghanim. 2010. Co-infection and localization of secondary symbionts in two whitefly species. BMC Microbiology 10: 142.

Sinkins, S.P., H.R. Braig, and S.L. O'Neill. 1995. *Wolbachia* superinfections and the expression of cytoplasmic incompatibility. Proc R Soc Lond B 261: 325–330.

Somerson, N.L., L. Ehrman, J.P Kocka, and F.J. Gottlieb. 1884. Streptococcal L-forms isolated from *Drosophila paulistorum* semispecies cause sterility in male progeny. Proc Natl Acad Sci USA 81: 282–285.

Stouthamer, R., R. Luck, and W.D. Hamilton. 1990. Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/ Trichogrammatidae) to revert sex. PNAS 87: 2424–2427.

Weinert, L.A., M.C. Tinsley, M. Temperley, and F.M. Jiggins. 2007. Are we underestimating the diversity and incidence of insect bacterial symbionts? A case study in ladybird beetles. Biol Lett 3: 678–681.

Werren, J.H., W. Zhang, and L.R. Guo. 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc R Soc Biol Sci 261: 55–63.

Werren, J.H., L. Baldo, and M.E. Clark. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741–751.

White, J.A., M. Giorgini, M.R. Strand, and F. Pennacchio. 2013. Arthropod endosymbiosis and evolution, pp. 441–477. In A. Minelli, G. Boxshall, and G. Fusco (eds.), Arthropod Biology and Evolution. Springer Verlag, Berlin, Heidelberg.

Zchori-Fein, E., and S.J. Perlman. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. Mol Ecol 13: 2009–2016.

Zhou, W., F. Rousset, and S. O'Neill. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc Lond B 265: 509–515.

Zhu, L.-Y., K.-J. Zhang, Y.-K. Zhang, C. Ge, T. Gotoh, and X.-Y. Hong. 2012. *Wolbachia* strengthens *Cardinium*-induced cytoplasmic incompatibility in spider mite *Tetranychus piercei* McGregor. Curr Microbiol 65: 516–523.

CHAPTER 4

Are *Wolbachia* and/or *Cardinium* infections responsible for a cytoplasmic incompatibility in *Echinothrips americanus*? Investigation to the impact of the endosymbiont *Cardinium* on the male sex ratio

4.1 Summary

Many endosymbionts of insects have the ability to cause a variety of reproductive alterations in their hosts for their own benefit. The most common manipulation strategy is cytoplasmic incompatibility (CI), which occurs when an infected male mates with an uninfected female or when the organisms exhibit multiple lineages of symbionts. In haplodiploid insects, like thrips, the CI can lead to an elimination of the paternal chromosome, whereby all progeny are males, or results in an embryonic mortality of fertilized eggs. The thrips in our study, *E*. *americanus*, possesses a coinfection with *Wolbachia* and *Cardinium*. Both endosymbionts are known as initiators of reproductive incompatibility, together or independent of each other. In this work, the impact of *Cardinium* was determined in the double infected *E*. *americanus* population. A beneficial effect of *Cardinium* was proven as related to the male production. The number of males increased in crossing combination, where only one crossing partner was infected with the *Cardinium* bacterium (I_W x I_{WC}, I_{WC} x I_W). An embryonic mortality of fertilized eggs couldn't be detected in all tested crossing combinations. Also, the female production was unaffected through the symbiont induced effect. The current study indicated that the presence of *Cardinium* in both crossing partners supressed the beneficial effect and indicates to a rescue function of the present *Cardinium* strain. Additionally, sequence analysis show an identity homology to the *W*. *pipientis* strain *w*Mel and *Wolbachia* sp. *w*Ri for both females and males of *E*. *americanus*.

Keywords: symbionts, beneficial effect, increasing number of males, potential bacterial strains

4.2 Introduction

Thysanoptera are serious pests for many ornamental plants and economically important crops worldwide (Mound 2005). Especially, some representatives of the family Thripidae are known as pest species with a high agricultural damage (Lewis 1997). A relevant parameter for the pest potential of this thrips species is their rapid reproduction capability. Most thrips reproduce by haplodiploid parthenogenesis (arrhenotoky), which means males develop from unfertilized (haploid) and females from fertilized (diploid) eggs (Evans et al. 2004) (Fig. 4.1A). In addition, several thrips possess a diploid parthenogenesis (thelytoky) (Jordan 1888) (Fig. 4.1B). Females produce diploid eggs (all offspring are females) by fusion of egg prenucleus and one of the polar bodies during meiosis. Conversion of arrhenotokous haplodiploidy to thelytoky can be caused by endosymbionts. During early embryonic development, the bacteria engage in the cell division by reduplication of chromosomes, so that a diploid organism develops from unfertilized eggs (Stouthamer and Kazmer 1994, Pannebakker et al. 2004) (Fig. 4.1C). First, Arakaki et al. (2001) detected the *Wolbachia*-induced thelytoky in thrips, by elimination of this endosymbiont. Recently, the commonest form of endosymbiont caused modifications, the cytoplasmic incompatibility (CI), was also proven in thrips (Nguyen et al. 2017). Commonly, the CI appears if an infected male mates with an uninfected female (Yen & Barr 1971) or when the organisms possess multiple strains or lineages of symbionts (e.g. Zchori-Fein and Perlman 2004, Chiel et al. 2007, Weinert et al. 2007). The repercussions of this manipulation type are different. In haplodiploid insects, the CI results in male-biased sex ratio (Fig. 4.1D). On one side, the bacteria induced a complete haploidization of fertilized eggs in incompatible crosses (Fig. 4.1D). Consequently, the elimination of the paternally chromosomes result in an increased male amount (F_1) in incompatible matings (Breeuwer and Werren 1990). Alternatively, the symbiont caused an increased embryonic mortality of fertilized eggs (Fig. 4.1D). Hence, the incomplete haploidization leads to a decreased number of females (F_1), wherefore the male amount increased in the population (Vavre et al. 2000). Currently, the molecular mechanisms of this effect are largely unknown.

The *Cardinium* symbiont belong to the phylum Bacteroidetes and have the ability to manipulate insects with the same suite of mechanisms as *Wolbachia*. Antibiotic studies indicated three reproductive alterations in insects: the parthenogenesis (Zchori-Fein et al. 2001), the feminization (Giorgini et al. 2009) and cytoplasmic incompatibility (Hunter et al. 2003). In *Pezothrips kellyanus*, a coinfection of *Wolbachia* and *Cardinium* effected a reproductive incompatibility (Nguyen et al. 2017). At this, both bacteria induced this phenomenon independently of each other. The *Cardinium* endosymbiont caused a male production and an embryonic female mortality of fertilized eggs. Crossing experiments indicated that the

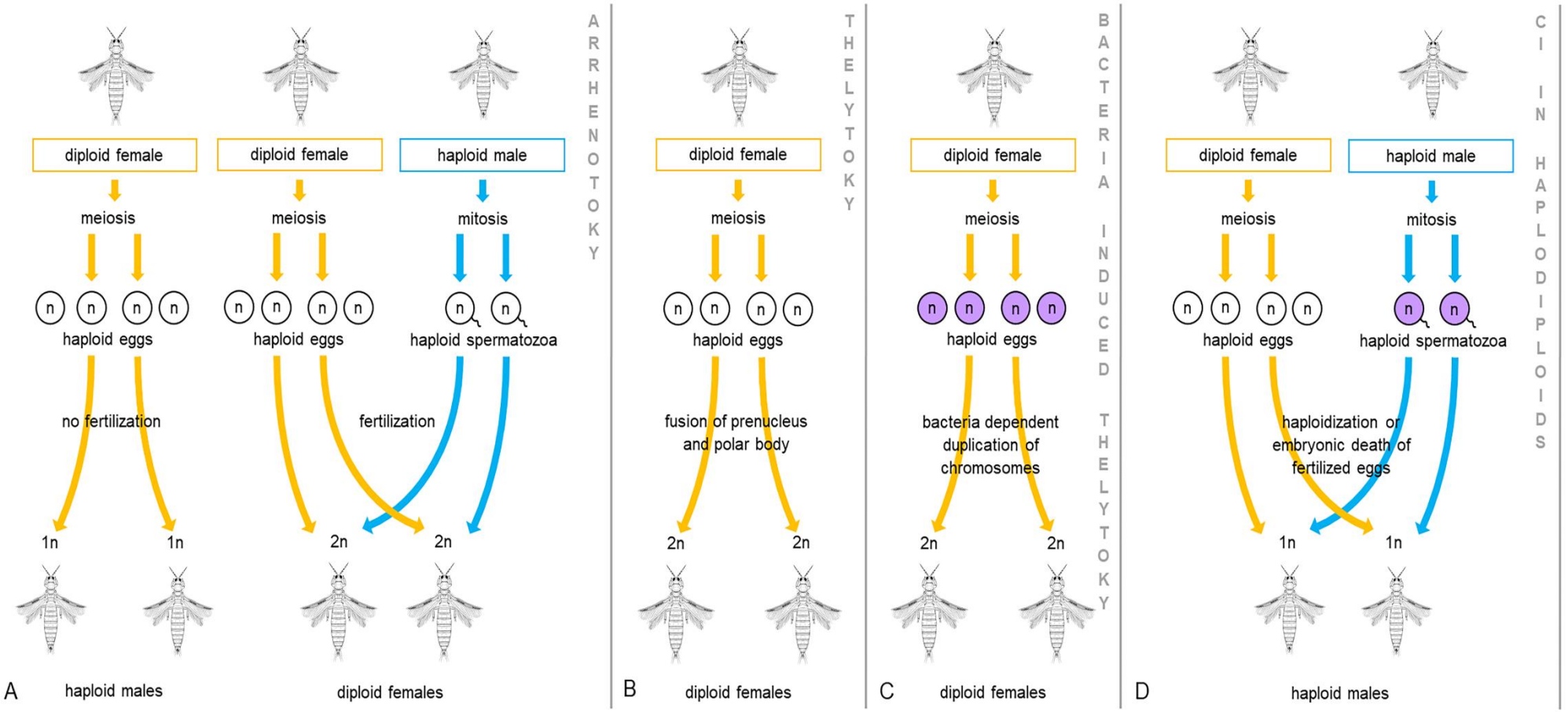


Fig. 4.1. Parthenogenesis types of thrips and bacteria induced strategies to alter the reproduction and/or sex ratio. (A) Haplodiploid parthenogenesis (arrhenotoky), males develop from unfertilized eggs and females develop from fertilized eggs. (B) Diploid parthenogenesis (thelytoky), all offspring are females, which develop by fusion of egg prenucleus and one of the polar bodies during meiosis. (C) Bacteria induced parthenogenesis (induced thelytoky), all offspring are females, which develop by bacteria induced duplication of chromosomes. (D) Bacteria induced cytoplasmic incompatibility (CI) in haplodiploids leads to haploidization or embryonic death of fertilized eggs, wherefore the number of males increases in populations. (n– haploid chromosome set, 2n– diploid chromosome set, purple highlighted eggs or spermatozoa are infected with endosymbionts/bacteria).

Wolbachia bacterium induced CI, which resulted in a female mortality with postembryonic mortality (Nguyen et al. 2017). Numerous observations show that hosts are infected with *Wolbachia* and *Cardinium* (eg. Weeks et al. 2003, Zchori-Fein and Perlman 2004). Both symbionts together or independent of each other can function as manipulator of the host reproduction (e.g. Perlman et al. 2006, Gotoh et al. 2007, White et al. 2009). Multiple infections of host species are often characterized by different distribution patterns and/or interaction intensity of each symbiont (Mouton et al. 2004, Kondo et al. 2005). For example, the parasitoid wasp *Encarsia inaron* is naturally infected with *Wolbachia* and *Cardinium*, whereby the reproductive incompatibility is induced through double infected organisms (Perlman et al. 2006). Crossing experiments identified that the *Wolbachia* symbiont was responsible for the CI (White et al. 2009) and the *Cardinium* bacterium is able to benefit the survivorship of males and enhancement of the male production (White et al. 2011) in this wasp. Present, the interaction pattern of each bacterium is sparse investigated in double infected organisms differ to single infections.

In this study, we focus on the interaction pattern of the *Cardinium* endosymbiont in the double infected *E. americanus* population (*Wolbachia* and *Cardinium* infection, see chapter 3). The prior purpose of this work is to identify the *Cardinium* impact on the sex ratio of this thrips species. Furthermore, the identification of the female and male *Wolbachia* and *Cardinium* strains take place by sequencing. The findings of this study can play a part in contributing to understand the specific reproductive interaction pattern of the *Cardinium* endosymbiont.

4.3 Materials and methods

4.3.1 Rearing of *Echinothrips americanus*

To obtain cohorts of individuals with similar age, breeding of *Echinothrips americanus* took place in rearing boxes (Take-away rect. food cont. 1000 ml, L-011201-4, 175.5 x 119 x 82 mm, Dai Dong Tien Corporation, Vietnam). Every rearing box was equipped with two plastic Petri dishes, filled with 25 ml of 1.4% agar (*w*/*v*) (agar-agar, danish, Carl Roth GmbH + Co. KG, Germany). Each Petri dish was stocked with a leaf of French bean (*Phaseolus vulgaris*). Additionally, a moist paper towel was placed on the bottom of each box. For the breeding, 20 adult females from the laboratory culture of the Martin-Luther-University Halle-Wittenberg (at 23 ± 1 °C, RH 50 ± 10%, and a 16 : 8 h L:D photoperiod, with lights on 6:00 a.m. CET) were transferred to each prepared box for 72 h. Afterwards the females were removed. Boxes were incubated in a climatic chamber (conditions as in the laboratory culture), until the first instar larvae hatched (8 d ± 24 h after egg deposition).

4.3.2 Crossing experiments

4.3.2.1 Application of antibiotics

First instar larvae (1 d ± 24 h old) were starved for 24 h. Then, the first instar larvae were treated for 72 h with 50 mg/ml tetracycline (tetracycline hydrochlorid, Sigma Aldrich, USA) in a special application system. Larvae (~50 first instar larvae) were transferred to a plastic container (O'canny shot glasses from plastic, 0.2 cl, produced for MÄC GEIZ Handelsgesellschaft mbH, Germany) with a gauze on one side (S4 Fig. 1A). The plastic container was closed with Parafilm® M (Pechiney Plastic Packaging, Chicago, IL, USA) and on the surface of foil the antibiotic mixture was applied (S4 Fig. 1B). Subsequently, the antibiotic mixture was enclosed with a second layer of parafilm. The antibiotic mixture composed of 100 µl of the dissolved antibiotic agent (S4 Tab. 1) and 100 µl of artificial diet (containing patent blue V (Sigma-Aldrich, USA)) after Jilge 2016 (S4 Tab. 2). Depending on the later desired infection status, the larvae were treated with the antibiotic mixture (uninfected) or only with the artificial diet (infected, artificial diet application solution composed of 100 µl artificial diet and 100 µl ddH_2O). Finally, the application container was placed on a second plastic container with a layer of 1.4% agar (*w*/*v*) on the bottom (S4 Fig. 1C). The complete application system was transferred in a humid chamber and kept under light exclusion 72 h in the climatic chamber (under the same conditions as above). After the duration of treatment, only thrips with proven uptake of the substances (visible blue gut, based on patent blue V in offered solution), were relocated in a new application system for 72 h again. In the next period, the larvae were treated with 50 mg/ml ampicillin (Carl Roth GmbH + Co. KG, Germany), in the same way as mentioned above. Following this, the treated larvae were placed in a rearing box. The used boxes (Take-away round food cont. 450 ml, L621-3, Ø 120 x 66.5 mm, Dai Dong Tien Corporation, Vietnam) comprised only one 1.4% agar (*w*/*v*) filled Petri dish with a leaf. After moulting, the propupae and pupae were transferred to Greiner plates (CELLSTAR® cell culture multiwell plates, 12 wells, Greiner Bio-One GmbH, Austria). The Greiner plates were prepared with 1.4% (*w*/*v*) agar and a leaf disc of *Phaseolus vulgaris* on the agar surface. Adult thrips (~24 h after hatching) were treated again with an antibiotic agent (uninfected) or only with the artificial diet (infected). For this purpose, the virgin females and males were placed in a separate application system (described above). After a 24 h starve time, the adult thrips were treated with 50 mg/ml tetracycline for 72 h and then with 15 mg/ml rifampicin (Carl Roth GmbH + Co. KG, Germany) for the same duration. Afterwards, the treated adults were used for the mating-experiments.

4.3.2.2 Mating conditions and breeding of the offspring of each pair

To determine CI-effects, cross-mating experiments with obtained infection lines were conducted. The fecundity, hatching rate and sex ratio of the offspring was analysed. For mating, one female and one male were placed in a well of a Greiner plate (see section above) and observed until mating occurred. Afterwards, each pair was transferred for 8 d in a rearing box for oviposition (description above). Subsequently, parents were investigated on the presence of endosymbionts by molecular investigation (see section below). The hatched propupae and pupae in the rearing boxes were individually placed in prepared Greiner plates (description above), so that later the determination of progenies sex ratio of each pair can took place. For ascertainment of the hatching rate, the French bean leaves from the rearing boxes were transferred in Petri dishes, filled with 100% ethanol (~20 days after parent relocation). After the elimination of the chlorophyll, the non-hatched eggs were counted under the stereomicroscope (Leica EZ4, Microsystems, Germany).

4.3.2.3 Statistical analyses

The statistical evaluation of the *Cardinium* effect in the double infected *E*. *americanus* line was determined by Winstat© for Excel (R. Fitch Software, Bad Krozingen, Germany). First, the data were tested on normal distribution by Shapiro-Wilk test ($p > 0.05$) and on homogeneity of group variances by Bartlett's test ($p < 0.05$). Square roots were extracted from the values to obtain a normality and homogeneity of group variances. One-way analysis followed by Tukey post hoc tests ($p < 0.05$) was realised for the total number of offspring, female and male amount of offspring, sex ratio of male, and total number of eggs. The nonparametric test Kruskal-Wallis was used for not normal distributed and inhomogeneous variances of group data, even after transformation (number of unhatched eggs) ($p < 0.05$).

4.3.3 Molecular investigations

4.3.3.1 DNA extraction, gene amplification, agarose gel electrophoresis

The thrips DNA was extracted by a modified protocol of the “100 fly method” after Roberts (1998) (described in Kumm et al. 2006). For the evidence of *Wolbachia* utilized primers for the *Wolbachia* surface protein (*wsp*) gene (81F/691R, Zhou et al. 1998) and *Cardinium*-specific primers were used for the detection of the 16S rDNA (CLOf1/CLOr1, Gotoh et al. 2007). For *Wolbachia*, the program condition after Kumm and Moritz 2008 and for *Cardinium* the instructions after Nguyen et al. 2017 were used. A detailed description of the used extraction method, polymerase chain reaction (PCR) and agarose gel electrophoresis is demonstrated in chapter 3.

4.3.3.2 DNA sequencing

The sequencing was generated by the DNA (female and male) polymerase chain reaction (PCR) products of *E*. *americanus*. The following primers were used for the detection of the *Wolbachia* strain: 81F/691R for the *wsp* gene (Zhou et al. 1998), 494F/1262R for the *ftsZ* gene (Holden et al. 1993) and 99F/994R for the 16S rDNA (O'Neill et al. 1992). Furthermore, the *Cardinium* strain was determined by primers for the 16S rDNA after Gotoh et al. 2007 (CLO-f2/rP2). The sequencing was realized by Eurofins Genomics Germany GmbH, Anzinger Str. 7a, 85560 Ebersberg. For that, the samples with the double-stranded DNA were cleaned up and afterwards sequenced after Sanger method. The results of the tested *E*. *americanus* females and males are documented in Supp. S4 Tab. 3. Through a database search in the National Center of Biotechnology Information (NCBI, Bethesda MD, USA, www.ncbi.nlm.nih.gov, accessed on 17th April 2020) the bacteria strain for the PCR products was determined with the BLASTN program (Altschul et al. 1990).

4.4 Results

4.4.1 *Cardinium* effect in the co-infected *E. americanus*

Significant differences were found between the investigated crossing combinations for the sex ratio of males (ANOVA, $p = 0.005$) (Tab. 4.1). Here, the control group, the double infected line ♀I_{wc} x ♂I_{wc} differs from ♀I_{w} x ♂I_{wc} (female *Wolbachia* infected, male double infected) and ♀I_{wc} x ♂I_{w} (female double infected, male *Wolbachia* infected) lines (Tab. 4.1). It is independent which crossing partner (female or male) is double infected with *Wolbachia* and *Cardinium* or single infected with *Wolbachia* (Tab. 4.1). The control group (♀I_{wc} x ♂I_{wc}) differs significantly with a male amount of 27% compared to the crossing combination ♀I_{wc} x ♂I_{w}, with a 41% male rate (ANOVA, $p = 0.002$) (Tab. 4.1). In terms of the control group, the number of males increased by ~14% in the infection line ♀I_{wc} x ♂I_{w} (Tab. 4.1). Likewise, crosses between ♀I_{wc} x ♂I_{wc} (27% male amount) show a significant distinction to crosses with ♀I_{w} x ♂I_{wc}, which generate a 39% male amount (ANOVA, $p = 0.003$) (Tab. 4.1). The male sex ratio increased by ~11% in crossing combination ♀I_{w} x ♂I_{wc}, referring to the control group.

In contrast, other investigated parameters: number of eggs (ANOVA, $p = 0.13$), unhatched eggs (Kruskal-Wallis, $p = 0.25$), male offspring (ANOVA, $p = 0.34$), female offspring (ANOVA, $p = 0.09$) and total offspring (ANOVA, $p = 0.36$) showed no significant variances between the different crossing combinations (Tab. 4.1).

Tab. 4.1. Results for the *Cardinium* effect in the co-infected *E*. *americanus* for the crossing combinations over an 8 d period. Data for each trait are mean ± SE. Percentage dates for the unhatched eggs and sex ratio male were calculated from means values. Different letters indicate the significant differences (One-way ANOVA and Tukey's post hoc test ($p < 0.05$): number of eggs, sex ratio males, male offspring, female offspring, total offspring. Kruskal-Wallis ($p < 0.05$): unhatched eggs). (I_W – *Wolbachia* infected line, I_{WC} – *Wolbachia* and *Cardinium* co-infected line).

Crosses f x m	N	Number of eggs	Unhatched eggs in %	Sex ratio male in %	Male offspring	Female offspring	Total offspring
I_W x I_W	38	23.58 ± 1.16	2.57 ± 0.67	33.73 ± 2.31^{ab}	6.97 ± 0.55	14.29 ± 1.05	21.26 ± 1.26
I_W x I_{WC}	12	22.33 ± 2.36	5.22 ± 1.54	38.78 ± 3.09^{b}	7.67 ± 0.74	13.00 ± 1.90	20.67 ± 2.29
I_{WC} x I_W	13	20.77 ± 2.80	2.22 ± 1.29	40.79 ± 4.44^{b}	6.69 ± 1.28	11.46 ± 2.25	18.15 ± 2.99
I_{WC} x I_{WC}	33	26.30 ± 1.61	2.88 ± 1.10	27.33 ± 1.85^{a}	5.88 ± 0.54	16.97 ± 1.58	22.85 ± 1.87
		$p = 0.13$	$p = 0.25$	$p = 0.005$	$p = 0.34$	$p = 0.09$	$p = 0.36$

4.4.2 Detection of potential *Wolbachia* strains in *E*. *americanus*

By means of sequencing, three products could be realized for the 16S rDNA. The female sample for the 99F primer, with a product length of 834 bp, showed a number of homologous sequences with a 99.76% identity. Highest identity possessed the sequencing product to the *W*. *pipientis* strain *w*Irr chromosome, complete genome (S4 Tab. 5, Accession no. CP037426.1). Further homologous alignments could be found to the *W*. *pipientis* strains *w*Mel (S4 Tab. 5, Accession no. CP042445.1) and hr1/dsz (S4 Tab. 5, Accession no. AJ306308.1), but also to *W*. *pipientis* partial sequences of 16S ribosomal RNA (S4 Tab. 5). Moreover, the 99F female sequencing result had a high identity to a range of *Wolbachia* endosymbionts in different host organisms with specific strains and/or partial sequences of the 16S rDNA (S4 Tab. 5). Finally, the sequence showed an affinity to the *Wolbachia* strain *w*Ri (S4 Tab. 5, Accession no. CP001391.1). The further investigated sequencing products, with a length of 837 bp for the male 99F sample and 839 bp for the male 994R sample, obtained identical homologous alignments (S4 Tab. 5).

For the *wsp* gene, four products could be generated by sequencing procedure. All investigated samples possessed an over 96% homology to the *Wolbachia* endosymbiont of *E*. *americanus* strain Morgan outer surface protein precursor (*wsp*) gene, partial cds (S4 Tab. 5, Accession no. JN315668.1). Moreover, the sequence for the 81F female sample (product length 519 bp) had several 99.42% identities to the *Wolbachia* endosymbiont of different *Anastrepha* species for the outer surface protein precursor (*wsp*) gene, partial cds (S4 Tab. 5, Accession no. EU116316.1). The 81F male product, with a length of 590 bp, had a 99.49% homology to the *Wolbachia* endosymbiont of *Drosophila incompta w*Inc_Cu genome (S4 Tab. 5, Accession no. CP011148.1). In addition, the sequences of the outer surface proteins of the *Wolbachia* endosymbiont of *Ostrinia furnacalis* strain *w*Fur7 (Accession no. GU166594.1), *E*. *americanus* strain Morgan (Accession no. JN315668.1) and *Drosophila septentriosaltans* strain *w*Spt (Accession no. AY620209.1) owned the same affinity to the 81F male sample (S4 Tab. 5). The

sequencing product for 691R female specimen (product length 518 bp) had a 99.80% homology to a number of sequences. Aside from the 99.80% homology to the *Wolbachia* endosymbiont of *E*. *americanus*, the most homological alignments were found for the outer surface protein (*wsp*) gene of *Wolbachia* endosymbionts of different *Drosophila* lines (9 hits) and other insects (S4 Tab. 5). Similar findings were ascertained for the 691R male sample (percent identity 96.84%), with a product length of 764 bp (S4 Tab. 5).

For the *ftsZ* gene, four sequencing results were obtained for both female and male of *E*. *americanus*. The 494F female sample, with a product length of 700 bp, have 100% identity to the *Wolbachia* endosymbiont of *Drosophila ananassae* strain W2.1 (Accession no. CP042904.1) and *Carposina sasakii* (*w*CauA) (Accession no. CP041215.1), but also to *Wolbachia* sp. *w*Ri (S4 Tab. 5, Accession no. CP001391.1). Similar is shown for the male products: 494F (length 708 bp) and 1262R (length 674 bp) primers (S4 Tab. 5). Additionally, the 1262R male and female sequencing result had a 100% sequence homology to the *w*Ri strain cell division protein (*ftsZ* gene) of *D*. *ananassae* and *D*. *simulans* (S4 Tab. 5). Moreover, the 1262R female sample (product length 704 bp) showed a 100% homology to the *Wolbachia* endosymbiont of *D*. *simulans w*Au genome assembly, chromosome: 1 (Accession no. LK055284.1) and *D*. *simulans w*Ha, complete genome (S4 Tab. 5, Accession no. CP003884.1). Furthermore, the sequencing product owned a 99.86% affinity to sequences of the *Wolbachia* sp. *w*Ri, complete genome (S4 Tab. 5, Accession no. CP001391.1).

4.4.3 Detection of potential *Cardinium* strains in *E. americanus*

For the endosymbiont *Cardinium*, only two sequencing products could be realized for the 16S rDNA and tested afterwards on homologous sequences by BLAST® in NCBI data base. The CLO-f2 sample showed the highest e-value ($6e^{-66}$) to a 16S rDNA ribosomal RNA gene of the *Cardinium* endosymbiont of *Aphytis* sp. (S4 Tab. 5, Accession no. AY327473.1).

For the rP2 sequencing product, the best e-value ($1e^{-156}$) could detect to the *Cardinium* endosymbiont of *Bemisia tabaci* (partial 16S rDNA gene, clone 29_2_3) (S4 Tab. 5, Accession no. LN829689.2).

4.5 Discussion

Host-symbiont systems can range from interactions between one microorganism and one insect, to simultaneously or mixed infections with multiple strains or symbionts (e.g. Weeks et al. 2003, Werren et al. 2008, Weinert et al. 2015). Especially, mixed infections pose challenges for host organisms by the complex interactions between symbionts and host (Cox 2001). The microbial agents may cause different levels of stress to the host by different expression levels. Consequently, mixed infection possesses the ability for dramatic changes in population dynamics in insect-symbiont interaction.

The main purpose of this study was to determine the impact of *Cardinium* in the (natural) double infected thrips species *E*. *americanus*. Particularly, the influence of the endosymbiont *Cardinium* was ascertained by comparison with co-infected lines. The findings exhibit significant differences in male sex ratio between crossing combinations, in which only one crossing partner owns a *Cardinium* infection (I_{WC} x I_W, I_W x I_{WC}), and the consistently co-infected line (I_{WC} x I_{WC}) (Tab. 4.1). Furthermore, this study couldn't determine a rise of the embryonal mortality rate (Tab. 4.1). Based on the supposition that *E*. *americanus* possess a reproductive incompatibility, the documented data showed that the *Cardinium* effect cannot be rescued by individuals with a single infection of *Wolbachia* (I_W x I_{WC}) nor double infected females (I_{WC} x I_W). Otherwise, the present outcomes indicate a clear impact of the *Cardinium* symbiont on the *E*. *americanus* male sex ratio, which results in a rise of the male amount, without female embryonic mortality. In contrast, other investigations on thrips document high embryonal mortality, lower total number of progeny and no female progeny in incompatible crosses with *Wolbachia* and *Cardinium* infected males (U x I_{WC}) (Nguyen et al. 2017). For the symbiont *Wolbachia*, the cytoplasmic incompatibility can be best described with the “modification/ rescue” model (Werren 1997). During spermatogenesis, the *Wolbachia* bacterium secreted proteins, which modify the paternal chromosomes or eliminate required host proteins for a normal paternal chromosome condensation/decondensation (Bourtzis et al. 2003). In the early mitotic divisions, the asynchronous development of the pronuclei takes place, so that the nuclear envelope breakdown (Tram and Sullivan 2002). Finally, the paternal chromosomes condense before or during zygote formation. Otherwise, infected females are able to reverse the sperm modification by producing a “rescue factor”, so that successful fertilization takes place (Hughes and Rasgon 2012). Likewise, *Wolbachia* strains can recruit host molecules, which are necessary for the normal sperm-egg interaction (Bourtzis et al. 2003). The characteristics of the modification-rescue mechanisms are dependent on the respective *Wolbachia* strain (Werren 1997). Ros and Breeuwer (2009) observed that the *Cardinium* endosymbiont used the same modification-rescue model to induce a CI as *Wolbachia*. Otherwise, the study of White et al. (2009) identified that the *Cardinium* bacterium can have an impact on the fitness cost and benefit for *Encarsia inaron*. The symbiont decreased the

fecundity of infected wasps in comparison to the cured organisms. *Cardinium* increased the survivorship of males and resulted in an enhancement of the male production, whereby the increased male amount did not affect female production. Furthermore, the study showed that the additional presence of the *Wolbachia* symbiont in *Cardinium* infected organisms resulted in an absence of this benefit (White et al. 2009). The results of the current investigation suggest that the *Cardinium* symbiont could benefit the male production of our *E*. *americanus* population. Crosses in which only one crossing partner has a *Cardinium* infection, (and the other organism possesses a *Wolbachia* single infection) resulted in an enhancement of male production. The ascertained number of unhatched eggs showed that this effect processes without a female embryonic mortality. Furthermore, all investigated crossing combinations showed no differences in the number of females. Otherwise, this work indicated that the presence of *Cardinium* in both crossing partners suppressed the beneficial effect. In relation to this, it is important to note that only the symbiont *Cardinium* was possible to eliminate in the thrips species continuously. The additional ascertained results of our investigations point to the possibility that the endosymbiont *Wolbachia* has a symbiotic effect for *E*. *americanus* (S4 Tab. 4). The few observed uninfected lines (n = 3) presented a low number of total offspring and more than 50% of unhatched eggs (S4 Tab. 4). Beneficial effects on the host fitness are associated with the genetic background of each *Wolbachia* strain (Bordenstein and Werren 2000). For example, Dobson et al. (2002) observed in *Wolbachia*-free *Aedes albopictus* populations a decreased fecundity of females, in combination with a reduced number of offspring and longevity of female and males. Additionally, Sinkins et al. (1995) showed that all natural *Wolbachia*-infected organisms were infected with more than one *Wolbachia* strain. Cytoplasmic incompatibility could be caused by beneficial *Wolbachia* strains, and result in both mutualism and reproductive effects in one organism (Dedeine et al. 2003).

Based on this fact, we investigated specific regions (16S rRNA gene, *ftsZ* gene, *wsp* gene) of the female and male total DNA of *E*. *americanus* on potential *Wolbachia* and *Cardinium* strains by sequencing. The findings of all tested sequencing products indicated a clear preference of the endosymbiont *W*. *pipientis* strain *w*Mel and the *Wolbachia* sp. *w*Ri, complete genome (S4 Tab. 5). Furthermore, the results point to a tendency that both female and male of *E*. *americanus* exhibit the same *Wolbachia* strain (S4. Tab. 5). For the sequencing products of the *wsp* gene the *Wolbachia* endosymbiont of *E*. *americanus* strain Morgan outer surface protein precursor (*wsp*) gene, partial cds (S4 Tab. 5, Accession no. JN315668.1) was identified in both female and male samples. The *Wolbachia* symbiont has a high genetic variety in the occurrence of specific nucleotides, including housekeeping (Baldo et al. 2006) and prophage genes (Bordenstein and Wernegreen 2004), and up to 43% of *Wolbachia* surface proteins (Baldo et al. 2005). Based on the remarkable recombination of nucleotides between the single *Wolbachia* strains, the *Wolbachia* surface proteins is used as a marker for the genetic diversity.

Dong et al. (2012) previously reported the presence of a *Wolbachia* endosymbiont of the group A and Mel subgroup in *E*. *americanus*. The natural originated *D*. *melanogaster* strain can cause diverse levels of CI dependent on the bacteria and insect genetic background and male age (Hoffmann 1988, McGraw et al. 2001, Reynolds and Hoffmann 2002, Riegler et al. 2005). In crossing experiments with *D*. *simulans*, the CI couldn't be induced, when both crossing partners have an infection with *w*Mel (Zabalou et al. 2008). Also, infection with the *w*Ri strain of *D*. *simulans* female and male results in an absence or low level of CI (Zabalou et al. 2008). In naturally infected host species, the *w*Ri strain (originated from *D*. *simulans*) can affect both modification and rescue function (Hoffmann et al. 1986, O'Neill and Karr 1990, Merçot et al. 1995). Finally, both detected *Wolbachia* strains have the ability to rescue the induced CI. This fact could explain the low level of embryonic mortality in crossing combination, especially in which the crossing partners are only infected with *Wolbachia* (I_W x I_W) (Tab. 4.1). Moreover, the results suggest the possibility of a *Wolbachia* induced rescue function in a double infected individual (I_{WC} x I_{WC}) (Tab. 4.1). Therefore, *Wolbachia* single infected lines and *Wolbachia* and *Cardinium* double infected lines do not differ in the investigated parameters (Tab. 4.1).

Contrary to the *Wolbachia* determination of the sequencing products, the *Cardinium* classification was very difficult. In most cases, no product could be realized by sequencing. Finally, only two sequencing products could be tested on their homology to other sequences. For the 16S rDNA, the CLO-f2 primer identified a homologous sequence (77.10%, E value $6e^{-66}$) to the *Cardinium* endosymbiont of *Aphytis* sp. 16S ribosomal RNA gene, partial sequence (S4 Tab. 5, Accession no. AY327473.1). Otherwise, the rP2 primer (16S rDNA) showed an affinity (77.57%, E value $1e^{-156}$) to the *Cardinium* endosymbiont of *Bemisia tabaci* partial 16S rDNA gene, clone 29_2_3 (S4 Tab. 5, Accession no. LN829689.2). Furthermore, rP2 sequencing sample had a 77.32% (E value $3e^{-148}$) homology to the *Cardinium* endosymbiont of *Pezothrips kellyanus* from Algeria, Spain, Turkey, Cyprus, New Zealand, and Australia (S4 Tab. 5). For the *Cardinium* endosymbiont of *P*. *kellyanus* from the Australia population was ascertained the strain *c*Pkel1 (Stouthamer et al. 2019). Despite, the relative low homology of *Cardinium* products to data base sequences, the analysis determined an alignment to *Bemisia tabaci*. The whitefly is known for their presence of single *Wolbachia* infection and/or multiple infections with other *Wolbachia* strains (Ahmed et al. 2010) or bacteria (Pan et al. 2012). In the whitefly*, Bemisia tabaci*, the enhancement of the *Cardinium* titre in *Wolbachia* and *Cardinium* co-infected lines was detected as a cause for a male-killing effect, which resulted in a higher female sex ratio in intraspecific amphigenetic progeny (Lv et al. 2020). In relation to the current study, crosses in which both partners are co-infected resulted in a female-bias sex ratio (1 female : 0.27 male) sex ratio. However, the results showed that female sex ratio in crosses between co-infected individuals (I_{WC} x I_{WC}) did not differ from matings with only *Wolbachia* infected partners (I_W x I_W). Instead, the presence of the *Cardinium*

symbiont in both crossing partner points to a rescue function of the *Cardinium* strain, so that the male sex ratio only discriminated in matings in which only one crossing partner owns a double infection (I_W x I_{WC}, I_{WC} x I_W).

These findings serve as a basis further study into reproductive incompatibility of *E*. *americanus*. In this work, we have focussed on *Cardinium* effect compared to double infected host lines. Our outcomes indicate a relevant impact of *Cardinium* on the number of male offspring. Furthermore, the homologous sequence alignments point to *Wolbachia* strains with a rescue factor, so that incompatible crosses have no detectable effect. The results of this study covered fundamental research into host and bacteria dynamics.

4.6 References

Ahmed, M.Z., S.-X. Ren, N.S. Mandour, J.M. Greeff, and B.-L. Qui. 2010. Prevalence of *Wolbachia* Supergroups A and B in *Bemisia tabaci* (Hemiptera: Aleyrodidae) and some of its natural enemies. J Econ Entomol 103: 1848–1859.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. J Mol Biol 215: 403–410.

Arakaki, N., T. Miyoshi, and H. Noda. 2001. *Wolbachia*-mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (Thysanoptera: Insecta). Proc R Soc Lond B 268: 1011–1016.

Baldo, L., N. Lo, and J.H. Werren. 2005. Mosaic nature of the *Wolbachia* surface protein. J Bacteriol 187: 5406–5418.

Baldo, L., J.C. Dunning Hotopp, K.A. Jolley, S.R. Bordenstein, S.A Biber, and et al. 2006. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Appl Environ Microbiol 72: 7098–7110.

Bordenstein, S.R., and J.H. Werren. 2000. Do *Wolbachia* influence fecundity in *Nasonia vitripennis*? Heredity 84: 54–62.

Bordenstein, S.R., and J.J. Wernegreen. 2004. Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and recombination rates. Mol Biol Evol 21: 1981–1991.

Bourtzis, K., H.R. Braig, and T.L. Karr. 2003. Cytoplasmic incompatibility, pp. 217–246. In K. Bourtzis, and T.A. Miller (eds.), Insect symbiosis. CRC Press, Boca Raton, London, New York, Washington D.C.

Breeuwer, J.A., and J.H. Werren. 1990. Microorganisms associated with chromosome destruction and reproductive associated with chromosome destruction and reproductive isolation between two insect species. Nature 346: 558–560.

Chiel, E., Y. Gottlieb, E. Zchori-Fein, N. Mozes-Daube, N. Katzir, and et al. 2007. Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. Bull Entomol Res 97: 407–413.

Cox, F.E.G. 2001. Concomitant infections, parasites and immune responses. Parasitology 122: S23–S38.

Dedeine, F., C. Bandi, M. Boulétreau, and L.H. Kramer. 2003. Insights into *Wolbachia* obligatory symbiosis, pp. 267–282. In K. Bourtzis, and T.A. Miller (eds.), Insect symbiosis, CRC Press, Boca Raton, London, New York, Washington D.C.

Dobson, S.L., E.J. Marsland, and W. Rattanadechakul. 2002. Mutualistic *Wolbachia* infection in *Aedes albopictus*: accelerating cytoplasmic drive. Genetics 160: 1087–1094.

Dong, J, XW Li, XC Zhang, and JN Feng. 2012. Sequencing and phylogenetic analysis of the *wsp* gene of *Wolbachia* in *Echinothrips americanus* Morgan. Journal of Northwest A & F University 3: 131–134.

Evans, J.D., D.C.A. Shearman, and B.P. Oldroyd. 2004. Molecular basis of sex determination in haplodiploids. Trends Ecol Evol 19: 1–3.

Giorgini, M., M.M. Monti, E. Caprio, R. Stouthamer, and M.S. Hunter. 2009. Feminization and the collapse of haplodiploidy in an asexual parasitoid wasp harbouring the bacterial symbiont *Cardinium*. Heredity 102: 365–371.

Gotoh, T., H. Noda, and S. Ito. 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. Heredity 98: 13–20.

Hoffmann, A.A. 1988. Partial cytoplasmic incompatibility between two Australian populations of *Drosophila melanogaster*. Entomol Exp Appl 48: 61–67.

Hoffmann, A.A., M. Turelli, and G.M. Simmons. 1986. Unidirectional incompatibility between populations of *Drosophila simulans*. Evolution 40: 692–701.

Holden, P.R, J.F.Y Brookfield, and P. Jones. 1993. Cloning and characterization of an *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. Mol Gen Genet 240: 213–220.

Hughes, G.L., and J.L. Rasgon. 2012. *Wolbachia* infections in arthropod hosts, pp. 351–366. In E.V. Fernando, and K.K. Harry (eds.), Insect pathology, 2nd edition, Academic press, Elsevier, St. Louis, MO, USA.

Hunter, M.S., S.J. Perlman, and S.E. Kelly. 2003. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. Proc Biol Sci 270: 2185–2190.

Jilge, M. 2016. Entwicklung und Einsatz künstlicher Diäten zur gezielten quantitativen Applikation spezifischer Wirksubstanzen bei phytosugen Terebrantia-Arten (*Frankliniella occidentalis*, *Echinothrips americanus*). M.S. thesis, Martin-Luther-University Halle-Wittenberg, Halle.

Jordan, K. 1888. Anatomie und Biologie der Physapoda. Zeitschr f wiss Zoologie 47: 542–620.

Kondo, N., M. Shimada, and T. Fukatsu. 2005. Infection density of *Wolbachia* endosymbiont affected by co-infection and host genotype. Biol Lett 1: 488–491.

Kumm, S., and G.B. Moritz. 2008. First detection of *Wolbachia* in arrhenotokous populations of thrips species (Thysanoptera: Thripidae and Phlaeothripidae) and its role in reproduction. Environ Entomol 37: 1422–1428.

Kumm, S., R. Kranz, and G. Moritz. 2006. Mikroorganismen-gesteuerte Parthenogenese bei Thysanopteren. Mitt Dtsch Ges allg angew Ent 15: 153–156.

Lewis, T. 1997. Pest thrips in perspective. In T. Lewis (ed.), Thrips as crop pests. CAB International, Oxon and New York, USA.

Lv, N., J. Peng, X.-Y. Chen, C.-F. Guo, W. Sang, and et al. 2020. Antagonistic interaction between male-killing and cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* in the whitefly, *Bemisia tabaci*. Insect Sci 00: 1–17.

McGraw, E.A., D.J. Merritt, J.N. Droller, and S.L. O'Neill. 2001. *Wolbachia*-mediated sperm modification is dependent on the host genotype in *Drosophila*. Proc R Soc Lond Ser B Biol Sci 268: 2565–2570.

Merçot, H., B. Llorente, M. Jacques, A. Atlan, and C. Montchamp-Moreau. 1995. Variability within the Seychelles cytoplasmic incompatibility system in *Drosophila simulans*. Genetics 141: 1015–1023.

Mound, L.A. 2005. Thysanoptera: diversity and interactions. Annu Rev Entomol 50: 247–269.

Mouton, L., F. Dedeine, H. Henri, M. Bouletreau, N. Profizi, and F. Vavre. 2004. Virulence, multiple infections and regulation of symbiotic populations in the *Wolbachia-Asobara tabida* symbiosis. Genetics 168: 181–189.

Nguyen, D.T., J.L. Morrow, R.N. Spooner-Hart, and M. Riegler. 2017. Independent cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* maintains endosymbiont coinfections in haplodiploid thrips populations. Evolution 71: 995–1008.

O'Neill, S.L., and T.L. Karr. 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature 348: 178–180.

O'Neill, S.L., R. Giordano, A.M.E. Colbert, T.L. Karr, and H.M. Robertson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc Natl Acad Sci USA 89: 2699–2702.

Pan, H., X. Li, D. Ge, S. Wang, Q. Wu, and et al. 2012. Factors affecting population dynamics of maternally transmitted endosymbionts in *Bemisia tabaci*. PLoS ONE 7: e30760.

Pannebakker, B.A., L.P. Pijnacker, B.J. Zwaan, and L.W. Beukeboom. 2004. Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). Genome 47: 299–303.

Perlman, S.J., S.E. Kelly, E. Zchori-Fein, and M.S. Hunter. 2006. Cytoplasmic incompatibility and multiple symbiont infection in the ash whitefly parasitoid, *Encarsia inaron*. Biol Control 39: 474–480.

Reynolds, K.T., and A.A. Hoffmann. 2002. Male age and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally-transmitted *Wolbachia*. Genet Res 80: 79–87.

Riegler, M., M. Sidhu, W.J. Miller, and S.L. O'Neill. 2005. Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. Curr Biol 15: 1428–1433.

Roberts, D.B. 1998. *Drosophila*, a practical approach, pp. 399. 2rd edn. Oxford University Press, Oxford, New York, Tokyo.

Ros, V.I.D., and J.A.J. Breeuwer. 2009. The effects of, and interactions between, *Cardinium* and *Wolbachia* in the double infected spider mite *Bryobia sarothamni*. Heredity 102: 413–422.

Sinkins, S.P., H.R. Braig, and S.L. O'Neill. 1995. *Wolbachia* superinfections and the expression of cytoplasmic incompatibility. Proc R Soc London 261: 325–330.

Stouthamer, C.M., S.E. Kelly, E. Mann, S. Schmitz-Esser, and M.S. Hunter. 2019. Development of a multi-locus sequence typing system helps reveal the evolution of *Cardinium hertigii*, a reproductive manipulator symbiont of insects. BMC Microbiol 19: 266.

Stouthamer, R., and D.J. Kazmer. 1994. Cytogenetics of microbe associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. Heredity 73: 317–327.

Tram, U., and W. Sullivan. 2002. Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. Science 296: 1124–1126.

Vavre, F., F. Fleury, J. Varaldi, P. Fouillet, and M. Bouletreau. 2000. Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: epidemiologic and evolutionary consequences. Evolution 54: 191–200.

Weeks, A.R., R. Velten, and R. Stouthamer. 2003. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. Proc Biol Sci 270: 1857–1865.

Weinert, L.A., M.C. Tinsley, M. Temperley, and F.M. Jiggins. 2007. Are we underestimating the diversity and incidence of insect bacterial symbionts? A case study in ladybird beetles. Biol Lett 3: 678–681.

Weinert, L.A., E.V. Araujo-Jnr, M.Z. Ahmed, and J.J. Welch. 2015. The incidence of bacterial endosymbionts in terrestrial arthropods. Proc R Soc Lond B 282: 20150249.

Werren, J.H. 1997. Biology of *Wolbachia*. Annu Rev Entomol 42: 587–609.

Werren, J.H., L. Baldo, and M.E. Clark. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741–751.

White, J.A., S.E. Kelly, S.J. Perlman, and M.S. Hunter. 2009. Cytoplasmic incompatibility in the parasitic wasp *Encarsia inaron:* disentangling the roles of *Cardinium* and *Wolbachia* symbionts. Heredity 102: 483–489.

White, J.A., S.E. Kelly, S.N. Cockburn, S.J. Perlman, and M.S. Hunter. 2011. Endosymbiont costs and benefits in a parasitoid infected with both *Wolbachia* and *Cardinium*. Heredity 106: 585–591.

Yen, J.H., and A.R. Barr. 1971. New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. Nature 232: 657–658.

Zabalou, S., A. Apostolaki, S. Pattas, Z. Veneti, C. Paraskevopoulos, and et al. 2008. Multiple rescue factors within a *Wolbachia* strain. Genetics 178: 2145–2160.

Zchori-Fein, E., and S.J. Perlman. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. Mol Ecol 13: 2009–2016.

Zchori-Fein, E., Y. Gottlieb, S.E. Kelly, J.K. Brown, J.M. Wilson, and et al. 2001. A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. Proc Natl Acad Sci USA 98: 12555–12560.

Zhou, W., F. Rousset, and S. O'Neill. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc Lond B 265: 509–515.

CHAPTER 5

Distribution patterns of *Wolbachia* and *Cardinium* during female progenesis and embryonic development of *Echinothrips americanus* and *Hercinothrips femoralis*

5.1 Summary

Many thrips species live in an endosymbiosis with specific bacteria. Both single and multiple infections with diverse symbionts are known in thrips, with differing effects on host reproduction. Maternal transmission paths and distribution patterns of endosymbionts during the early development are yet unknown in thrips. The present article investigates the occurrence of *Wolbachia* and *Cardinium* during female progenesis and embryonic development of *E*. *americanus* and *H*. *femoralis*. Electron-microscopic examinations localized bacterial structures in oocytes during vitellarium passage in both organisms. Bacteria were detected in the cytoplasm and follicular epithelial cells of developing oocytes and yolk mass of mature eggs for the oviposition. A characteristic localization of endosymbiotic bacteria was realised by *in situ* hybridization technique. The bacteria were detected during oogenesis of thrips in the nucleus, the cytoplasm, and the follicle cells of oocytes in the early vitellarium phase. Bacterial clusters in the later oocyte development were found in the transition area between the single oocytes and yolk mass of vitellogenic eggs. During the embryogenesis the endosymbionts were detected in all investigated developmental stages of each thrips species. In the early development, the symbionts were predominantly distributed in the head anlage. Later stages documented that the bacteria occurred in all body areas (head, thorax, abdomen), the appendages and the amnion-serosa complex of the developing prolarva.

Keywords: endosymbionts, thrips, oogenesis, embryogenesis

5.2 Introduction

Insects harbour a number of intracellular endosymbiotic bacteria. These microorganisms have a considerable impact on their host's biology (Moran 2006, Werren et al. 2008, Ma et al. 2014). It is estimated that more than 15% of all insect species live in an endosymbiosis with specific bacteria (Douglas 1998). In thrips, heritable endosymbionts that are responsible for various reproductive alterations such as cytoplasmic incompatibility (Nguyen et al. 2017) and parthenogenesis (Arakaki et al. 2001, Kumm and Moritz 2008) are known, while the vertical transmission routes of endosymbiotic bacteria are largely unknown in these insects.

Generally, the transmission of symbionts can take place horizontally, when the microorganism is transferred between organisms, or vertically, when the bacterium is immediately transferred from a parent individual to the offspring. Mainly, endosymbionts are vertically transmitted through the female germline in their insect hosts. The transmission routes of these bacteria can be subdivided into transovarial transfers, in which the bacteria are continuously associated with the reproductive organs by an infection of the oocytes, and transovum transfers, which are characterized by a bacterial egg surface contamination and a symbiont intake during larvae hatching process (Andreadis 1987).

Thrips females have panoistic ovarioles, which are divided into two paired ovaries. Each ovariole consists of three fundamental parts: the terminal filament, the germarium and the vitellarium (Fig. 5.1). Six developmental zones partitioned the ovariole, with diverse species-specific characteristic (Moritz 2006). Terminal filament and germarium form the zones I and II (Fig. 5.1). The germarium comprised the primordial germ cells, which later developed into oocytes and follicle cells. Zone III is located between the germarium and vitellarium (Fig. 5.1). In this zone, oocytes placed side by side begin to grow and were surrounded by follicle cells. The vitellarium (from zone IV to VI) is characterized by a series of successive developing oocytes (Fig. 5.1). Over the course of vitellogenesis, each oocyte grows intensive and built a layer of follicular epithelial cells, which generate a chorion (Tsutsumi 1996). Matured eggs for the oviposition are located towards the lateral oviducts (a structural conflation of the ovariole strands per ovary). The eggs, with strong flattened follicular cells, are transmitted via the lateral oviducts to the common oviduct, where the fertilization through spermatozoa takes place. Afterwards, the eggs pass on to the vagina and are deposited, in Terebrantia species within the host plant. In the most thrips species, the embryonic development starts after oviposition. In the beginning, a plasmodial preblastoderm is created which later built the blastoderm (Fig. 5.2A). The blastoderm undergoes cell differentiation and is subdivided into embryonic and extraembryonic (built the serosa) tissue parts (Moritz 1988). Following, the caudal germ band immigrates in the yolk mass (process anatrepsis) and causes the migration of the dorsal

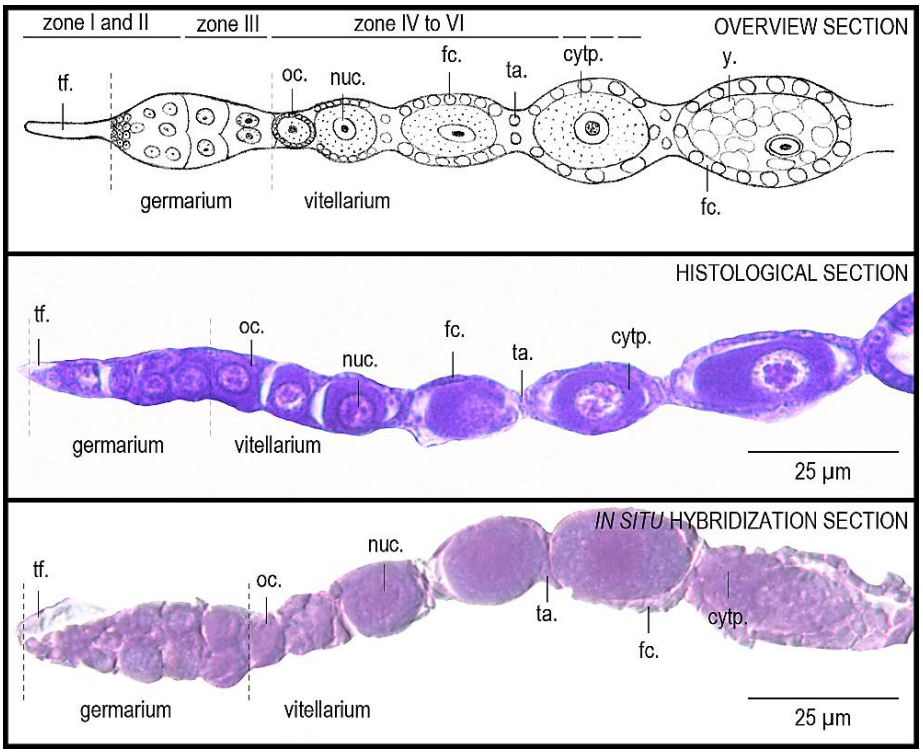


Fig. 5.1. Thrips ovariole. Overview section: schematic figure of a thrips ovariole with zoning in terminal filament, germarium and vitellarium. Histological section: *E*. *americanus* ovariole section, colored by Giemsa staining (S5 Suppl. method). *In situ* hybridization section: *F*. *occidentalis* ovariole after *in situ* hybridization procedure. (cytp.– cytoplasm, fc.– follicular epithelial cells, nuc.– nucleus, oc.– oocyte, ta.– transition area between oocytes, tf.– terminal filament, y.– yolk).

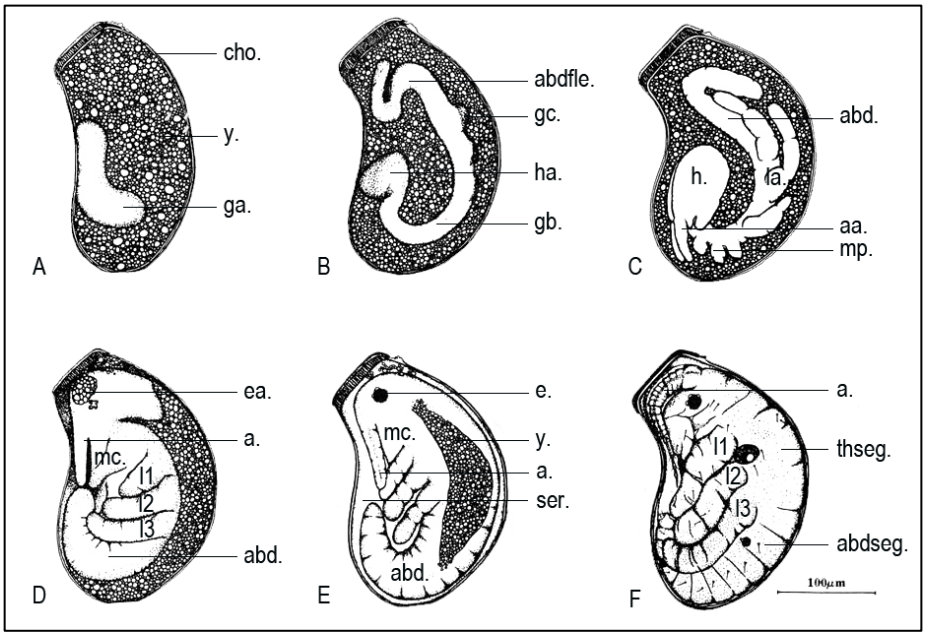


Fig. 5.2. Embryogenesis of a terebrantian thrips: (A) embryonic germ anlage, (B) germ band elongation, (C) embryo with appendages anlages before katatrepsis, (D) embryo after katatrepsis, (E) embryo during first dorsal closure, (F) embryo before moulting process to first instar larva. Modified figure after Moritz 1995. (a.– antenna, aa.– antenna anlage, abd.– abdomen, abdfle.– abdominal flexure, abdseg.– abdominal segment, cho.– chorion, e.– eye, ea.– eye anlage, ga.– germ anlage, gb.– germ band, gc.– germ cells, h.– head, ha.– head anlage, l1.–l3.– thoracic legs, la.– leg anlage, mc.– mouth cone, mp.– mouthparts, ser.– amnion-serosa, thseg.– thoracic segment, y.– yolk).

serosa tissue, the later embryo surrounding amnion. The s-shaped germ band elongates in the segment addition zone. First segmentation processes form extremities, mandible and maxilla anlages (Fig. 5.2B). Cell immigration mechanisms cause the development of ecto- and hyboblast (divided into meso- and entoderm). The formation of extremity, mouth and antennea anlages is completed just before the katatrepsis procedure (Fig. 5.2C). Katatrepis is characterized through a conspicuous dynamic processes that leads to a complete system transformation (also terms embryonic metamorphosis). At the beginning of this precedure, the dorsal organ is formed by amnional cells in the embryonic head area (Bournier 1960, Moritz 1988). Moreover, the event is characterized by nervous system differenziation and further organogenetic changes. After katatrepsis, the predominant organ cells are developed and functioning (Fig. 5.2D). The primary dorsal closure is caused by the amnion (Fig. 5.2E). Until hatching, the organogenesis marches and the embryo grows to an prolarvae (Fig. 5.2F).

The study aims to identify the endosymbiont distribution patterns during female progenesis and embryonic development in thrips. Two terebrantian species with different infection levels of facultative symbionts be used for the investigation: *E*. *americanus*, with a coinfection of *Cardinium* and *Wolbachia* (see chapter 3), and *H*. *femoralis*, with a single *Wolbachia* infection (Pintureau et al. 1999). Both thrips species possess endosymbiotic bacteria, whereby the reproductive effects are different. *E*. *americanus* presents a haplodiploid reproduction mode, despite the proven multiple infections and *H*. *femoralis* owns a *Wolbachia* induced thelytokous parthenogenesis (Kumm and Moritz 2008). Therefore, a further aspect for examination is the comparison of the bacterial distribution patterns of different symbionts within and between various thrips species. The vertical transfer through the female germline and the bacterial transmission during embryogenesis were ascertained using transmission electron microscopy and *in situ* hybridization technique.

5.3 Materials and methods

5.3.1 Breeding of thrips females

The determination of the *Wolbachia* and *Cardinium* distribution patterns during progenesis was realized on *Echinothrips americanus* and *Hercinothrips femoralis* females. Control thrips conduced *Frankliniella occidentalis* females, because this thrips species has no infection with *Wolbachia* or *Cardinium* (Kumm and Moritz 2008, Chapter 3). For breeding of the experimental animals, 20 females of each thrips species from the laboratory culture were transferred in rearing boxes (Take-away rect. food cont. 1000 ml, L-011201-4, 175.5 x 119 x 82 mm, Dai Dong Tien Corporation, Vietnam). The rearing boxes were equipped with two Petri plates, which were filled with 25 ml of 1.4% agar (*w*/*v*) (agar-agar, danish, Carl Roth GmbH + Co. KG, Germany), and in each plate a French bean leaf was placed. Additionally, a moist paper towel

was placed on the bottom of each rearing box. During the female egg deposition time (48 h), the boxes were held in the climatic chamber, in which the thrips were reared under following conditions: at 23 ± 1 °C, RH 50 ± 10%, and a 16 : 8 h L:D photoperiod, with lights on 6:00 a.m. CET. After the period the females were removed from the boxes. The boxes were closed again and placed back in the climate chamber. Finally, the adult stages hatched in the rearing boxes (after ~16 d *E*. *americanus*, ~28 d *H*. *femoralis*, ~14 d *F*. *occidentalis*). The adult females were utilized for the embedding procedure of the *in situ* hybridization and investigations on the electron microscope.

5.3.2 Rearing of the egg stages

The *Wolbachia* and *Cardinium* distribution patterns were identified during different embryonic developmental stages of *E*. *americanus* and *H*. *femoralis*. Similar embryonic stages of *F*. *occidentalis* were used as control. Thrips females of each species were placed in prepared 12-well Greiner plates (CELLSTAR® cell culture multiwell plates, 12 wells, Greiner Bio-One GmbH, Austria) for egg deposition. One female of the standard thrips culture was placed in each well of the Greiner plate, which was filled with 2 ml of 1.4% agar (*w*/*v*) and a leaf disc of *Phaseolus vulgaris* on the surface of the agar. Afterwards, the plate was closed with a glass lid and Parafilm® M (Pechiney Plastic Packaging, Chicago, IL, USA). The Greiner plate was transferred in the climatic chamber and kept under the same conditions as described for the adult females (see above). After 24 h the females were removed and the plate was closed again and relocated in the climatic chamber. To realize continuous embryonic stages, all 24 h a Greiner plate was scheduled over the complete embryonic developmental time (*E*. *americanus* ~8/9 d, *H*. *femoralis* ~11 d, *F*. *occidentalis* ~4/5 d), after the same procedure (as described before). The eggs were cut out from the leaf, with few leaf-materials in the direct surrounding of each egg. After which, the embryonic stages were used for the preparation of the *in situ* hybridization.

5.3.3 TEM/embedding for ultra-thin sections

The samples were fixed for 2 h in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde by room temperature (S5 Tab. 1). Then the specimens were washed four times for 20 min in 0.1 M Sorensen's phosphate buffer (pH 7.4) (S5 Tab. 1) and afterwards incubated overnight at 4 °C in the same buffer. The secondary fixation was carried out with 2% osmium tetroxide (S5 Tab. 1) by room temperature for 1 h. Subsequently, the samples were washed four times with ddH_2O. The dehydration of the specimens took place by an ascending alcohol series (S5 Tab. 2). Following this, the samples were incubated two times for 10 min in propylene oxide on a rotator and then two times overnight infiltrated in a 50 : 50 mixture of propylene

oxide and araldite (Epoxy resin, Araldite Cy212 Kit, Agar Scientific) on a rotator. For embedding the samples were transferred in silicone rubber molds filled with pure araldite for 2 d at 65 °C. After the polymerization of the epoxy resin, the samples were prepared for the ultra-thin sectioning. Before the sections were stained, the ultra-thin sections (70 nm) were transferred on formvar-coated specimen grids (Agar Scientific Ltd., Stansted, UK; copper grids). To contrast the sections, the grids were placed in a drop of uranyl acetate (2% aqueous) (S5 Tab. 1) for 30 min in the dark. Afterwards, the grids were carefully washed several times with ddH_2O and subsequently dried. In the next step, the grids were transferred to a Petri dish in a drop of lead citrate for 3 min. For the incubation time the Petri dish, equipped with NaOH small plates, was closed. After that, the grids were washed with ddH_2O and dried overnight. Contrasted specimen grids were used for the investigations on the transmission electron microscope (JEOL TEM 1010, 80 kV), which have an incorporated Megaview II camera.

5.3.4 *In situ* hybridization

For the *in situ* hybridization, the samples (15 adult females and 15 samples per egg stage) were fixed in a mixture of 4% paraformaldehyde and 0.25% glutaraldehyde (S5 Tab. 1) for 3 h at 4 °C on the shaker, washed in Sorensen's phosphate buffer (pH 7.2), dehydrated in an ascending alcohol series and embedded in paraffin. After sectioning of the samples (6 µm), the proteolysis with Proteinase K (Carl Roth GmbH + Co. KG, Germany) was realized (S5 Tab. 1). By means of the polymerase chain reaction, digoxigenin-labelled dUTPs were integrated with the newly synthesized DNA strand and afterwards detected by an antibody (anti-digoxigenin, Roche Diagnostics, Penzberg, Germany) and Fab fragments conjugated with an alkaline phosphatase (anti-digoxigenin-AP Fab fragments, Roche Diagnostics, Penzberg, Germany). The visualisation of the phosphatase and therewith of the bacteria DNA was enable through the addition of substrates. A detailed description of this method is presented in chapter 2. The observation of the bacteria was carried out under the light microscope (Leitz DMRBE, Leica, Germany) and documented with the Leica DFC450 C digital microscope camera. Afterwards, the images were processed with Photoshop CS6 (Adobe, Ireland). For the creation of the image plates, fig. 5.4, 5.5, and 5.6, also histological sections of Büttner 2019 and Hein 2019 were used.

5.4 Results

5.4.1 Bacterial microorganisms during oogenesis in *E. americanus* and *H. femoralis*

Bacterial microorganisms were observed by TEM investigations in both thrips species (Fig. 5.3). The bacteria were localized in the oocytes during vitellarium phase. In the cytoplasm, different bacterial structures were found in the areas of the cytoplasmic matrix (endoplasm and periplasm) in both thrips organisms (Fig 5.3A). Also, microorganisms were documented in the follicle cells of the strongly formed epithelium during the vitellarium stage (Fig 5.3B). The size of this bacterial microorganisms was 264 to 508 nm in length and 146 to 321 nm in width (S5 Tab. 3, large microorganism). In the later oocyte stages, bacterioid aggregations and single occurrences of bacteria were found in the cytoplasm of the thrips species (Fig. 5.3C). The vitellogenic eggs showed bacterial appearances in the protein yolk bodies and cytoplasm parts in both thrips individuals (Fig. 5.3D). These microorganisms possess a length from 84 to 412 nm and a width from 30 to 206 nm (S5 Tab. 3, small microorganism).

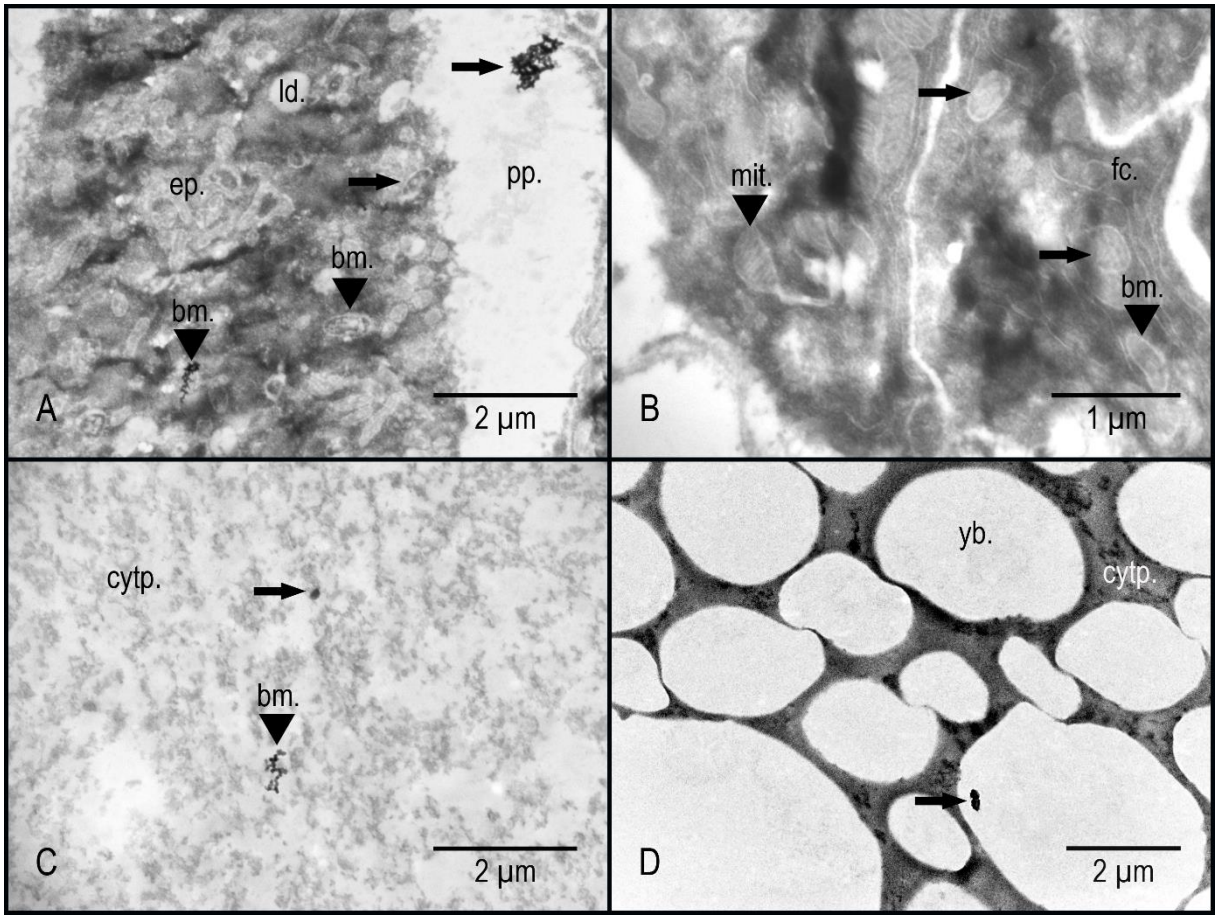


Fig. 5.3. Bacterial microorganisms in the developing oocytes during vitellarium phase in thrips (marked by arrows and triangles). Fig. 5.3 A–B *E. americanus*: bacterial structures (arrows) in the (A) cytoplasm and (B) follicle epithelium of oocytes during early vitellarium phase. Fig. 5.3 C–D *H. femoralis*: bacterial structures (arrows) in the (C) cytoplasm with accumulations of condensed material and (D) protein yolk bodies of an vitellogenic oocyte during later and end phase of oogenesis. (bm.– bacterial microorganisms, cytp.– cytoplasm, ep.– endoplasm, fc.– follicular epithelial cells, ld.– lipid droplet, mit.– mitochondrion, pp.– periplasm, yb.– protein yolk body).

5.4.2 Distribution patterns of *Cardinium* during female progenesis and embryonic development in *E. americanus*

The *Cardinium* bacteria were localized in the oocyte structures of the ovariole during the vitellarium phase in all tested samples (Fig. 5.4A–D). In figure 5.4A and B the distribution patterns of *Cardinium* are demonstrated within oocyte. The *Cardinium* bacteria were distributed in the nucleus, cytoplasm, and follicular epithelium of the oocyte during early vitellarium phase (Fig. 5.4B). Mainly, single findings and clusters of the endosymbiont were detected in the cytoplasm and strong flattened follicular cells (Fig. 5.4A/B). Also, a few bacteria were localized in the nucleus of the oocytes (Fig. 5.4B). In the figures 5.4C and D, oocytes of the ovariole are shown during the later passage of vitellarium. Clusters of the bacterium within the yolk mass were detected in oocytes with a strong formed follicular epithelium (Fig. 5.4C) and mature eggs for the oviposition, with flattened follicle cells (Fig. 5.4D). Furthermore, the endosymbiont was localized in the transition area between the single oocytes (Fig. 5.4C).

The *Cardinium* endosymbiont was detected in all stages of the embryonic development in *E. americanus* (Fig. 5.4E–H). At the beginning of the embryogenesis, the *Cardinium* bacteria were localized in single proofs and clusters in the periphery of the 1 d ± 12 h old egg in the most investigated specimens (Fig. 5.4E). After the katatrepsis, single occurrences of the symbiont were detected in the mouth cone and appendages of the 4 d ± 12 h old embryo (Fig. 5.4F) in mostly all samples. In the figures 5.4G and H the distribution patterns of *Cardinium* be shown in the 7 d ± 12 h old embryo. The endosymbiont was localized in the neuronal tissue of the head and abdominal segments of most 7 d ± 12 h old embryos (Fig. 5.4G). Moreover, the *Cardinium* clusters were occasionally detected in the serosa of this embryonic stage (Fig. 5.4H).

F. occidentalis, the control animal, shown no *Cardinium* appearances during oogenesis (S5 Fig. 1A/B) as well as in the 4 d ± 12 h old embryo (S5 Fig. 1C).

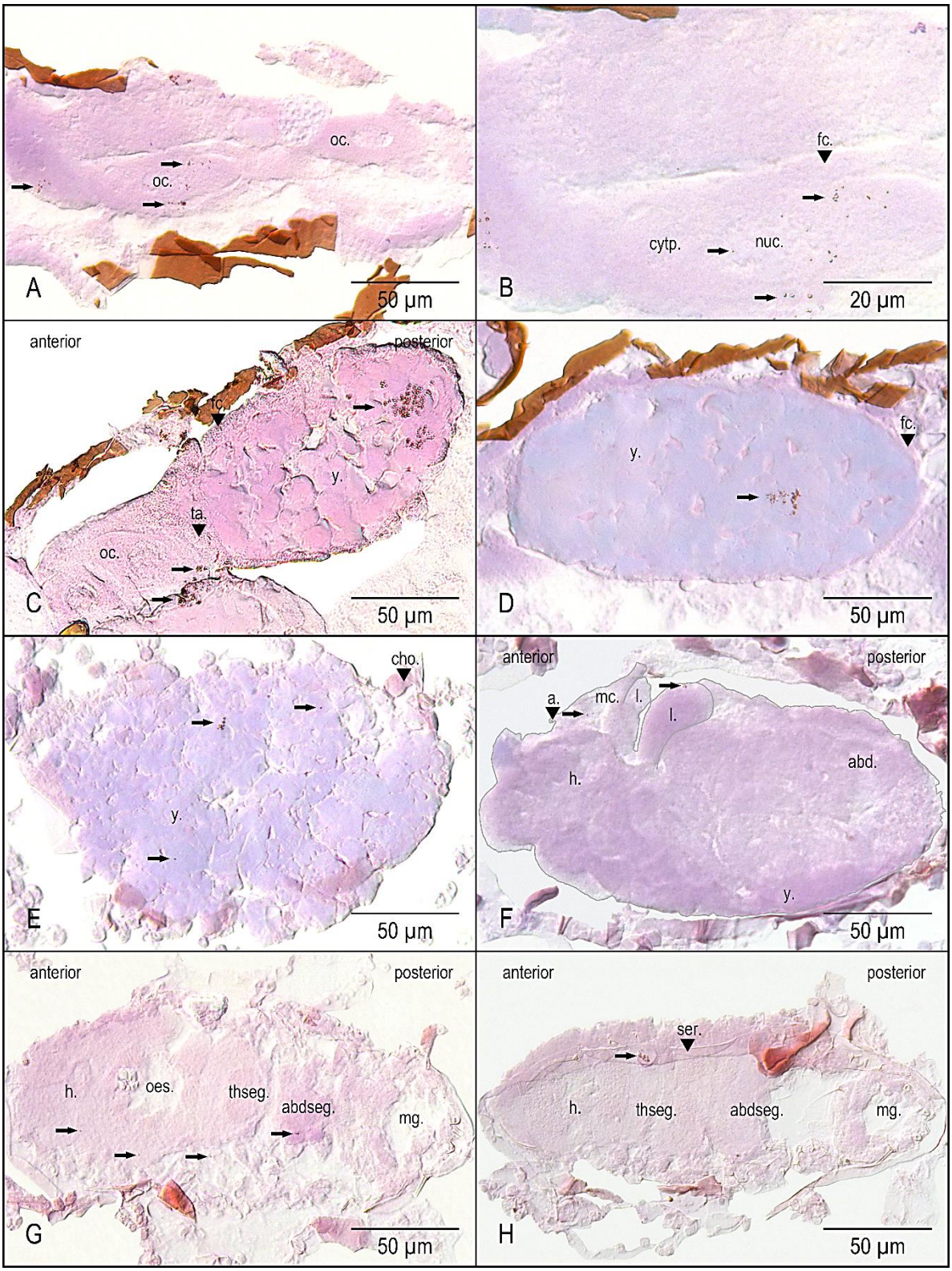


Fig. 5.4. Detection of *Cardinium* clusters during oogenesis and embryonic development of *E. americanus* by use of *in situ* hybridization (red-brown signals, arrows). Fig. 5.4A–D oogenesis: (A) part of ovariole (overview), (B) oocyte (detail view of fig. 5.4A), (C) oocyte with strong formed follicular epithelium, (D) vitellogenic oocyte with flattened follicle cells, Fig. 5.4E–H embryogenesis: (E) egg 1 d ± 12 h, (F) egg 4 d ± 12 h with embryo (sagittal sections), (G) and (H) egg 7 d ± 12 h with embryo (frontal sections). (a.– antenna, abd.– abdomen, abdseg.– abdominal segment, cho.– chorion, cytp.– cytoplasm, fc.– follicular epithelial cells, h.– head, l.– leg/extremity, mc.– mouth cone, mg.– midgut, nuc.– nucleus, oc.– oocyte, oes.– oesophagus, ser.– amnion-serosa, ta.– transition area between oocytes, thseg.– thoracic segment, y.– yolk).

5.4.3 Distribution patterns of *Wolbachia* during female progenesis and embryonic development in *E. americanus*

Figure 5.5A–E shows the *Wolbachia* detections in the oocyte structures of an ovariole during vitellarium passage. At the beginning of the vitellarium stage, *Wolbachia* clusters were found in the cytoplasm (Fig. 5.5A/B) and in the transition area between the oocytes (Fig. 5.5D) in the vast majority of samples. Moreover, single proofs were localized in the follicular epithelium (Fig. 5.5A) and nucleus in the early vitellaric eggs (Fig. 5.5C) in all examined samples. In the later egg development, accumulations of *Wolbachia* were found in the yolk mass of oocytes during chorion formation phase (Fig. 5.5D) and also in all observed vitellogenic eggs, just before egg deposition (Fig. 5.5E).

Within the embryonic development of *E. americanus*, *Wolbachia* bacteria were identified during all investigated egg stages (1 d ± 12 h, 4 d ± 12 h, 7 d ± 12 h) (Fig. 5.5F–H). In the early phase of the embryogenesis, the symbiont was detected in the yolk of all investigated 1 d ± 12 h old egg (Fig. 5.5F). The localized bacteria occurred as single findings and were unevenly distributed in the periphery of the egg (Fig. 5.5F). In most 4 d ± 12 h old embryos, *Wolbachia* clusters were detected in the developing neuronal tissue parts of the head (Fig. 5.5G). Likewise, clusters of the bacterium were often found in the thoracic legs of the 7 d ± 12 h old embryo (Fig. 5.5H).

In similar oogenic (S5 Fig. 1D–F) and embryonic (S5 Fig. 1G) stages of *F. occidentalis* no *Wolbachia* bacteria could be detected.

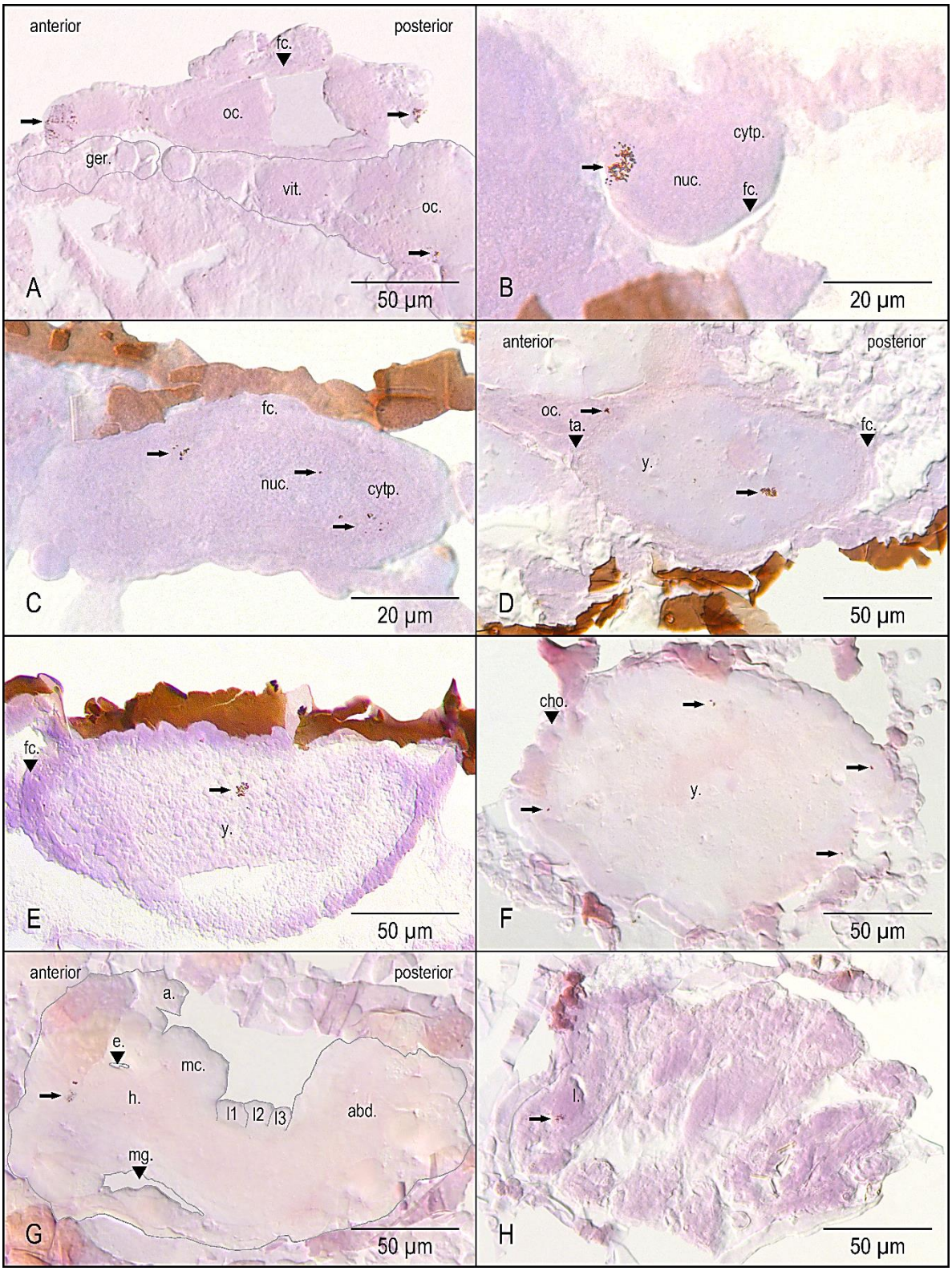


Fig. 5.5. Detection of *Wolbachia* clusters during oogenesis and embryonic development of *E. americanus* by use of *in situ* hybridization (red-brown signals, arrows). Fig. 5.5A–E oogenesis: (A) ovariole and oocytes, (B) oocyte, (C) oocyte, (D) oocyte during chorion formation, (E) vitellogenic oocyte. Fig. 5.5F–H embryogenesis: (F) egg 1 d ± 12 h, (G) egg 4 d ± 12 h with embryo (sagittal sections), (H) egg 7 d ± 12 h with embryo (transversal section). (a.– antenna, abd.– abdomen, cho.– chorion, cytp.– cytoplasm, e.– eye, fc.– follicular epithelial cells, ger.– germarium, h.– head, l.– leg/extremity, l1.–l3.– thoracic legs, mc.– mouth cone, mg.– midgut, nuc.– nucleus, oc.– oocyte, ta.– transition area between oocytes, vit.– vitellarium, y.– yolk).

5.4.4. Distribution patterns of *Wolbachia* during female progenesis and embryonic development of *H. femoralis*

Wolbachia bacteria were localized in the early oocyte structures of the ovarioles during vitellarium in all examined samples (Fig. 5.6A–B). Sparsely appearances of the endosymbiont were detected in the follicular epithelium (Fig. 5.6A) and inside the nucleus (Fig. 5.6B). Oocytes in the later vitellarium phase and particularly vitellogenic eggs, just before oviposition, harboured *Wolbachia* clusters in the yolk mass of all specimens (Fig. 5.6C).

During embryogenesis, *Wolbachia* bacteria were identified from the beginning to the end of this process (Fig. 5.6D–H) in the vast majority of the samples. The endosymbiont was localized as single findings in yolk mass of all 3 d ± 12 h old eggs (Fig. 5.6D). The bacteria DNA was detected inhomogeneous distributed in the periphery of the yolk mass (Fig. 5.6D). In *H. femoralis* the developmental point after the formation of the germ ligament was exemplarily demonstrated for all identified symbiotic distribution patterns in both organisms. The bacteria were primarily found in the later head (Fig. 5.6E, S5 Fig. 1H). Inside the head anlage the *Wolbachia* signals occurred as many single proofs near to the forming mouthparts in *H. femoralis* (S5 Fig. 1H). Additionally, some single occurrences could be localized in the further germ anlage. In *H. femoralis*, *Wolbachia* was also detected in the later thoracic region of the 4 d ± 12 h old embryo (Fig. 5.6E). Furthermore, in all investigated samples during the germ band formation, the symbionts occurred also in the surrounding yolk mass of the embryos (Fig. 5.6F, S5 Fig. 1H). In the 7 d ± 12 h old embryo, the bacterium was always localized as many single proofs in the thoracic and abdominal segments (Fig. 5.6F). Additionally, the endosymbiont appeared in the remaining yolk mass around the dorsal side of the embryo (Fig. 5.6F). Accumulations of *Wolbachia* were often found between the head and thoracic segments close to the midgut system of the 8 d ± 12 h old embryo (Fig. 5.6G). Furthermore, some samples showed single occurrences of the bacterium in the antennae (Fig. 5.6G). Finally, the 9 d ± 12 h old embryo had some single infections in the neuronal tissue parts of the head (Fig. 5.6H). A special accumulation of the *Wolbachia* endosymbiont was found in a part of the samples in the amnion-serosa tissue between the antennae and mouth cone of the later first instar larvae (Fig. 5.6H).

In *F. occidentals* neither the oocyte stages (S5 Fig. 1D–F) nor embryo (S5 Fig. 1G) showed a *Wolbachia* occurrence.

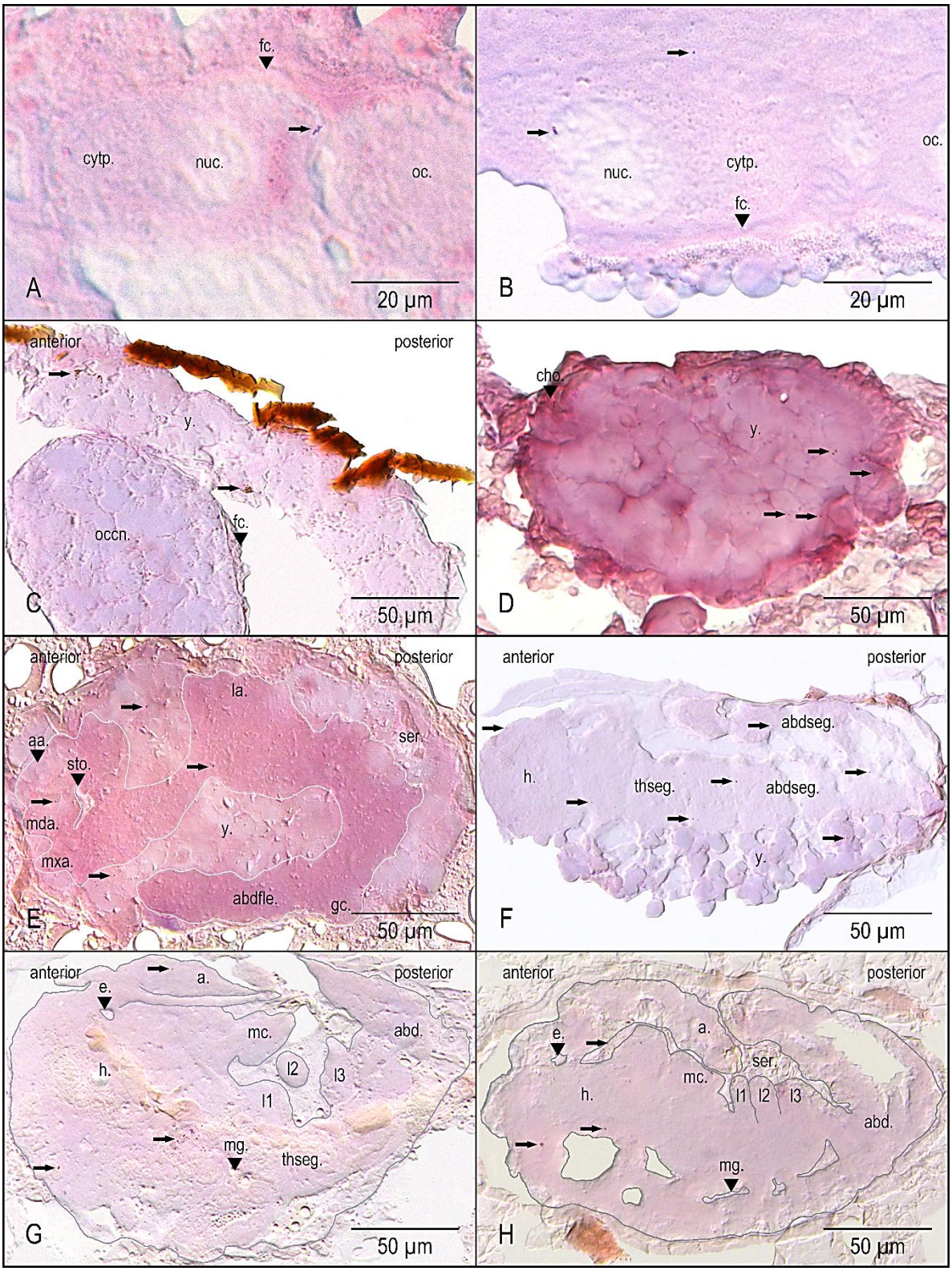


Fig. 5.6. Detection of *Wolbachia* clusters during oogenesis and embryonic development of *H. femoralis* by use of *in situ* hybridization (red-brown signals, arrows). Fig. 5.6A–C oogenesis: (A) oocyte, (B) oocyte, (C) vitellogenic oocyte, Fig. 5.6D–H embryogenesis: (D) egg 3 d ± 12 h, (E) egg 4 d ± 12 h with germ ligament (sagittal sections), (F) egg 7 d ± 12 h with embryo (sagittal sections), (G) egg 8 d ± 12 h with embryo (sagittal sections), (H) egg 9 d ± 12 h with embryo (sagittal sections). (a.– antenna, aa.– antenna anlage, abd.– abdomen, abdfle.–abdominal flexure, abdseg.– abdominal segment, cho.– chorion, cytp.– cytoplasm, e.– eye, fc.– follicular epithelial cells, gc.– germ cells, h.– head, l1.–l3.– thoracic legs, la.– leg anlage, mc.– mouth cone, mda.– mandible anlage, mg.– midgut, mxa.– maxilla anlage, nuc.– nucleus, oc.– oocyte, occn.– oocyte with cleavage nucleus, ser.– amnion-serosa, sto.– stomodaeum, thseg.– thoracic segment, y.– yolk).

5.5 Discussion

Plant sap-sucking insects, like thrips, are commonly infected with intracellular bacteria (Baumann 2005). Endosymbionts with a prevalent vertical transmission (transovarial route) are steadily present in the female germline stem cells of insects (Serbus et al. 2008). The distribution patterns of acquired symbionts often depend on the bacterial strain characteristics and/or insect species (reviewed in Russel et al. 2019). Several species of thrips are also infected with endosymbiotic bacteria (Tab. 1.1), whereby the vertical transmission routes are largely unknown. In this study, an examination of the maternal transmission routes and distribution patterns during embryonal development was conducted in two different thrips species with different reproduction modes.

5.5.1 Endosymbionts during oogenesis in *E. americanus* and *H. femoralis*

Thysanoptera possess typical panoistic ovarioles, which harbour no nurse cells (Moritz 1997). The germ cell differentiation is characterized through the formation of cell clusters via intercellular bridges in the thrips germaria (Pritsch and Büning 1989). Each cell arises from a stem oogonium through mitotic activities. In common with thrips, insects with meristic ovarioles have oocytes, which are interconnected by cytoplasmic bridges (Spradling 1993), and the prospective cells undergo mitosis events. Wherefore, the thrips ovariole type indicates to the secondary panoistic ovary. Germ cell divisions causes by mitosis events, and the germ cell cluster formation offers a potential symbiont transmission route between the cells. Investigations on *Drosophila* lines showed that *Wolbachia* bacteria distribute to oocytes and nurse cells by travelling on microtubules during early oogenesis (Ferree et al. 2005, Serbus et al. 2008), and move by microtubules-based motor proteins and elements, which are associated with the posterior cortical cytoskeleton, in the further development (Chang et al. 2011).

In the scope of this study, bacterial structures were found in the vitellarium passage of oocytes in both thrips species by TEM investigations. The microorganisms were detected in the areas of the cytoplasmic matrix (Fig 5.3A) and within the follicle cells of the strongly formed epithelium (Fig 5.3B). Further bacterial structures were observed in the later oocyte stages in the cytoplasm (Fig. 5.3C) and protein yolk bodies of vitellogenic eggs (Fig. 5.3D) in both thrips species. The intracellular bacteria *Wolbachia* and *Cardinium* often described as pleomorphic rods vary greatly in size (Hertig 1936, Zchori-Fein et al. 2004, Bordenstein et al. 2006). *Wolbachia* endosymbionts appear as small rods with a size of 0.2 to 1.8 µm (Hertig 1936, Wright et al. 1978, Louis and Nigro 1989, O'Neill et al. 1997, Bordenstein et al. 2006). By means of TEM examinations, Bordenstein et al. 2006, detected *Wolbachia* bacteria with a length of 0.3 to 1 µm and a diameter of 0.25 to 0.59 µm in the testes of *Nasonia vitripennis*. In contrast, *Cardinium* rods can have a size of 0.42–2.35 µm in length and 0.31–0.66 µm in width

(Zchori-Fein et al. 2004). The observed larger bacterial microorganisms in the cytoplasm and follicle epithelium during the earlier oocyte stages (Fig. 5.3A–B), possess a bacteria size similar to *Wolbachia* and *Cardinium* symbionts. Thus, the discovered bacteria could be the endosymbionts based on their size, despite the missing morphologic features such as parallel arrays of microfilament-like structures inside the cell body (*Cardinium*, Zchori-Fein et al. 2004) or up to three layers of membranes (*Wolbachia*, Stouthamer et al. 1999, Bordenstein et al. 2006).

In accordance with the TEM examinations, a *Wolbachia* or *Cardinium* finding couldn't be detected in the germaria of both thrips species by *in situ* hybridization technique. However, a strong *Wolbachia* presence in the germarium stage of *E*. *americanus* ovarioles is known (Chapter 2). *Wolbachia* and *Cardinium* are primarily inherited bacteria via cytoplasm of oocytes (Moran et al. 2008). The results show that both endosymbionts were localized in the cytoplasm of *E*. *americanus* (Fig. 5.4B, Fig. 5.5B). In comparison to the strong clusters of *Wolbachia* in the oocytes (Fig. 5.5B), the *Cardinium* bacteria was less distributed in the cytoplasm (Fig. 5.4B). It is particularly interesting that the *Wolbachia* bacteria couldn't be detected in the cytoplasm of *H*. *femoralis* by *in situ* hybridization but, in the nucleus. *Wolbachia* symbiont is known the causer of a thelytokous parthenogenesis in *H*. *femoralis*, through reduplication of chromosomes, so that a diploid organism develops from unfertilized eggs (Kumm and Moritz 2008). This fact could explain the occurrence of the bacterium in the oocyte nucleus (Fig. 5.6B). The presence of *Wolbachia* and *Cardinium* in the oocyte cell nuclei of *E*. *americanus* is potentially associated with reproductive incompatibility (Chapter 4). Symbionts in the female are able to reverse the sperm modification by producing of a “rescue factor”, so that successful fertilization takes place (Hughes and Rasgon 2012). Likewise, host molecules are recruited, which are necessary for normal sperm-egg interaction (Bourtzis et al. 2003). Examinations to determine the *Wolbachia* strain in *E*. *americanus* indicated a similar percentage homology to the *W*. *pipientis* strain *w*Mel and/or *Wolbachia* sp. *w*Ri (Chapter 4). Both *Wolbachia* strains are able to rescue their own effect (rescue function) (e.g. Zabalou et al. 2008). Therefore, an attendance of both symbionts in the oocyte nuclei could result from a rescue function of the induced effect. In this study, few appearances of both bacteria were detected in the follicle cells of the two organisms (Fig. 5.4B, Fig. 5.5A, Fig. 5.6A). A prevalent transfer of endosymbiotic bacteria via follicle cells can't be impossible for the investigated thrips species. Although, the occurrence of both bacteria in the follicle cells is also known in other insect hosts (e.g. Matalon et al. 2007, Serbus et al. 2008). An infection of the follicular epithelium can be generated through the somatic stem cell niche (SSCN). Frydman et al. (2006) demonstrated that *Wolbachia* bacteria accumulate in this region of the *Drosophila* germarium. The SSCN is an area of germaria, which pass all germ cells that subsequently produce follicle cells. *Wolbachia* contaminations in the SSCN enable a direct bacterial transfer to the germ cells, and

indirectly benefits the infection of follicle cell that develops from somatic stem cells in this niche. Furthermore, alternative transmission routes of symbionts via the follicle cells are also known in insects (reviewed in Bright and Bulgheresi 2010). During vitellarium stage of egg development, follicle cells support the vitellogenin transport from the hemolymph system into the oocyte (Nozaki and Matsuura 2016). Consequently, microorganisms can be potentially transmitted from the follicle cells (Terry et al. 1997) or transferred from the hemolymph into the oocyte (Michalik et al. 2009, Herren et al. 2013, Guo et al. 2018). Previous works show strong clusters of *Wolbachia* in the female fat body (area of vitellogenin synthesis) of *E*. *americanus* (Chapter 2) and *H*. *femoralis* (unpublished data). It might be that the vitellogenesis offers an option for bacterial transport. Another potential transmission path is the follicle cell function during postvitellogenesis. The cells secrete the vitelline membrane and chorion of oocytes (Heming 2003). Within this procedure, a transfer of bacteria is also conceivable to the later egg system of the embryo, especially to the extraembryonic structures.

The described transmission routes terminate with the end of egg maturation. After completion of vitellogenesis, the oocytes exhibited accumulations of *Wolbachia* and *Cardinium* in *E*. *americanus* (Fig. 5.4D, Fig. 5.5E) and *Wolbachia* clusters in *H*. *femoralis* (Fig. 5.6C). In this developmental phase, bacterial accumulations were observed inhomogeneous (more central distributed) in the yolk mass of the mature eggs. A number of researches show a posterior localization of bacteria in the oocytes during the last vitellarium stage, which is mainly associated with the later disposition of the primordial stem cell in the embryogenesis (reviewed in Russel et al. 2019). Already, Kose and Karr (1995) demonstrated a posterior concentration of *Wolbachia* in the mature eggs of *D*. *simulans*. Contrary to insect species like *Drosophila* sp., thrips possess other patterns of development. Thrips have a short germ development, so that the initially extraembryonic primordial germ cells are localized in the anterior of abdominal flexure (Moritz 2006). In addition to that, Veneti et al. (2004) detected that the posterior, anterior or cortical embryonic distribution of *Wolbachia* is depending on the strain and initiated even in the early oogenesis. Finally, the results of the current study documented an inhomogeneous distribution of both symbionts within the oocytes, which could be related to the later potential bacterial dispersion in the embryo.

5.5.2 Endosymbionts during embryogenesis in *E*. *americanus* and *H*. *femoralis*

Numerous observations provide clear evidence that *Wolbachia* bacteria have evolved mechanisms to integrate the embryonic fate maps (reviewed in Landmann 2019). Various somatic tissues and the germline of adult insects are infected with *Wolbachia*, whereby the bacterial dispersion is mostly defined during oogenesis and embryonic development. As mentioned previously, the distribution patterns of the investigated endosymbionts presented an inhomogeneous segmentation in the course of oogenesis. In the early embryogenesis of

thrips, a primordial short germ anlage develops at the posterior side of the egg system, which invaginated into the yolk mass (the process of anatrepsis) (Moritz 1997). At that moment, the bacteria were distributed peripherally in the yolk mass of the egg system (Fig. 5.4E, Fig. 5.5F, Fig. 5.6D). An incorporation in the developing germ anlage couldn't be observed during this developmental phase in both organisms. After the formation of the germ anlage, both bacteria could be localized in the germ ligament, primarily in the head anlage (exemplarily demonstrated for *H*. *femoralis*, Fig. 5.6E, S5 Fig. 1H). In addition, the symbionts were found in the ambient yolk mass of the germ ligament in both organisms (Fig. 5.6E). Various research articles describe a *Wolbachia* presence during symmetric and asymmetric stem cell divisions (O'Neill and Karr 1990, Callaini et al. 1994, Kose and Karr 1995). In this investigation, a localization of the *Wolbachia* bacterium in the primordial germ cells couldn't be documented in the *E*. *americanus* and *H*. *femoralis* (Fig. 5.6E) embryos. Likewise, the *Cardinium* symbiont in *E*. *americanus* also showed no distribution patterns into embryonic germ cells. Albertson et al. (2009) observed the *Wolbachia* bacterium in the early embryonic symmetric and asymmetric segregation of neuroblasts. In accordance with this fact, the examinations of Veneti et al. (2004) identified the *Wolbachia* strain *w*Ri with an even distribution throughout the cortex of embryos from different *Drosophila* lines. On the other side, the study determined a number of *Wolbachia* strains, including *Wolbachia w*Mel, which are concentrated mainly in the posterior region of the embryo, where pole cells are localized (Veneti et al. 2004). Previous works identified an affinity to homologous sequences of *Wolbachia* strains *w*Mel and *w*Ri for the investigated *E*. *americanus* laboratory population (Chapter 4). Furthermore, another observation determined also a presence of *Wolbachia* Mel subgroup in *E*. *americanus* (Dong et al. 2012). Consequently, it seems that the distribution patterns during early embryonic development are conserved in thrips. The findings of this study indicate a bacterial permeation through the embryonic head anlage, which is possibly associated with the short germ development. In the course of the early embryonic stage, elements of the head and thorax region segment offer an opportunity for symbionts to penetrate the embryo.

For the determination of the symbiotic distribution patterns in further development, embryonic stages were used directly after the katatrepsis (middle stage) and to the end of the embryogenesis (late stage). After the katatrepsis procedure, the *Cardinium* symbiont was detected in the formed mouth cone and legs of the *E*. *americanus* embryo (Fig. 5.4F). In contrast, the *Wolbachia* bacteria were only concentrated in the head area of the *E*. *americanus* embryo (Fig. 5.5G). Furthermore, the *Wolbachia* were localized in the head region, and in the thoracic and abdominal segments of the *H*. *femoralis* (Fig. 5.6F). The results of this study indicate a potential transmission path of the symbionts through the development of the nervous system. Differentiation of the nerve cord proceeds from the head to the abdominal segments, and starts early in the embryonic development with the formation of the germ layers. For

example, *Wolbachia* bacteria are known to accumulate in the brain tissue and nerve cord of insects (Albertson et al. 2009, Strunov et al. 2013). A bacterial transfer in the course of neurogenesis seems conceivable to distribute along the anterior-posterior axis of the embryo (after the katatrepsis) and ensures thereby a transmission to other body parts and appendages. Otherwise, a dispersion of symbionts within the nervous system opens the facility to an early influence of the host behaviour.

In addition to these findings, the bacteria were localized in extraembryonic tissues of both organisms during the middle and late embryonic stage. The occurrence of endosymbionts in the extraembryonic tissue could be associated with an additional transfer route through the dorsal closure. In thrips, the primary dorsal closure is realised by amnion and later replaced through peripheral epithelial cell growth (Moritz 1997). From the larval stages to the adult stages, for instance *Wolbachia* bacteria are distributed in the epithelial cell layer under the chitinous body surface and the adjacent fat body in *E*. *americanus* (Chapter 2) and *H*. *femoralis* (unpublished data). *Wolbachia* bacteria are known to use the vitellogenin mechanism (fat body secretes a large amount of vitellogenin components) to come inside the oocyte (Herren et al. 2013). A colonization of this body part during embryogenesis could ensure the later maternal transmission via hemolymph system into the germline (in females) and therewith to the next host generation.

5.5.3 Model of endosymbiotic distribution patterns during oogenesis and embryogenesis in thrips

The current study documented the endosymbiont distribution patterns during the female progenesis and embryonic development in thrips (investigations on *E*. *americanus* and *H*. *femoralis*). *Wolbachia* and *Cardinium* bacteria exhibit a similar distribution in both thrips species. A model of potential symbiotic transmission paths in thrips was created on the basis of detected bacterial findings by means of transmission electron microscope investigations and *in situ* hybridization.

At first, the bacteria are localized in the early vitellarium stage of the ovariole structures (Fig. 5.7B). The symbionts are mainly distributed in the oocyte nucleus, cytoplasm and follicle cells (Fig. 5.7B). Furthermore, the endosymbionts are accumulated in yolk mass of vitellogenic eggs during late egg maturation (Fig. 5.7B). Finally, the vitellarium passage of oocytes offers different opportunities to enter the germline (Fig. 5.7A–B). The primary vertical transmission is realized via the germ cell infection, although an infection of these cells couldn't be observed in this study (Fig. 5.7B). A further transmission path of endosymbionts via the vitellogenin transportation is conceivable and could explain the high infection status of vitellogenic eggs during the later egg maturation (Fig. 5.7A).

In the early embryonic stage, the bacteria are inhomogeneously distributed in the periphery of the egg system (Fig. 5.7C). After the formation of the germ ligament, symbiotic appearances are localized in the head anlage and appendages of limbs (Fig. 5.7D). The findings of this study indicate successive bacterial dispersion from the head to the abdomen (Fig. 5.7D). Possible infection of the body parts could be generated by the segmentation procedure. During late embryonic development, the symbionts have the opportunity to enter the further prolarva in the course of the dorsal closure (Fig. 5.7E). In the extraembryonic tissue (amnion) localized bacteria could be transferred in the developing embryo during this event.

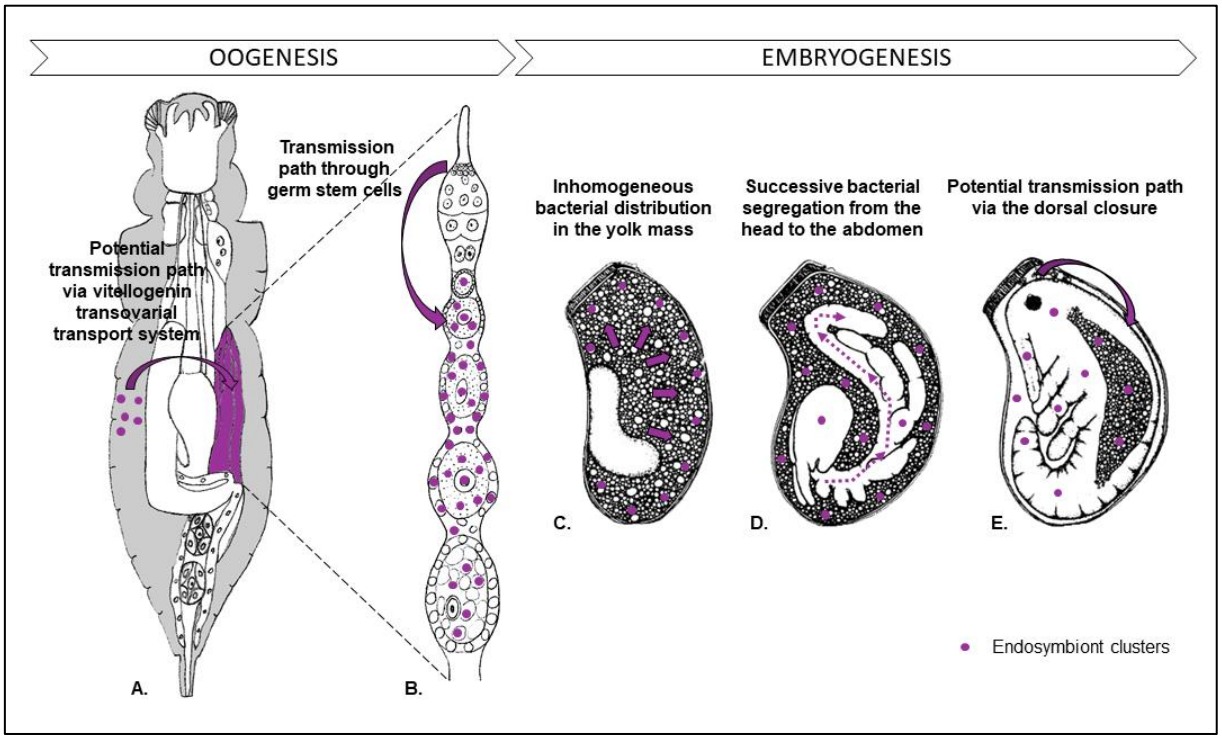


Fig. 5.7. Model of endosymbiont transmission routes during oogenesis and embryonal development in thrips. A. Potential transmission path into ovarioles via vitellogenin transport system in the adult female. B. Distribution patterns of symbionts in the ovarioles. C–E. Distribution patterns of symbionts in the early (C), middel (D) and late (E) embryoic development. Modified figures after Buffa 1898 (adult female thrips anatomy) and after Moritz 1995 (embryonic stages).

5.6 References

Albertson, R., C. Casper-Lindley, J. Cao, U. Tram, and W. Sullivan. 2009. Symmetric and asymmetric mitotic segregation patterns influence *Wolbachia* distribution in host somatic tissue. J Cell Sci 122: 4570–4583.

Andreadis, T.G. 1987. Transmission. In: Fuxa, J.R., and Y. Tanada (eds.), Epizootiology of insect diseases. New-York: John Wiley and Sons.

Arakaki, N., T. Miyoshi, and H. Noda. 2001. *Wolbachia*-mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (Thysanoptera: Insecta). Proc R Soc Lond B 268: 1011–1016.

Baumann, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol 59: 155–189.

Bordenstein, S.R., M.L. Marshall, A.J. Fry, U. Kim, and J.J. Wernegreen. 2006. The Tripartite Associations between Bacteriophage, *Wolbachia* and Arthropods. PLoS Pathog 2: 384–393.

Bournier, A. 1960. Sur l'existence et l'evolution du corps dorsal secondaire dans l'ontogenese de *Caudothrips buffai* Karny (Thysanoptera, Tubulifera). CR Acad Sc, Paris 250: 1347–1348.

Bourtzis, K., H.R. Braig, and T.L. Karr. 2003. Cytoplasmic incompatibility, pp. 217–246. In K. Bourtzis, and T.A. Miller (eds.), Insect symbiosis. CRC Press, Boca Raton, London, New York, Washington D.C..

Bright, M., and S. Bulgheresi. 2010. A complex journey: transmission of microbial symbionts. Nat Rev Microbiol 8: 218–230.

Buffa, P. 1898. Contributo allo studio anatomico della *Heliothrips haemorrhoidalis*. Riv Patolog Veget, Padova 7: 1–30, Tab. 5.

Büttner, C. 2019. Verteilungsmuster gramnegativer Bakterien (Wolbachien und *Candidatus cardinium*) während der Ei- und Embryonalentwicklung bei *Echinothrips americanus* (Thripidae, Thysanoptera). Bachelor thesis. Martin-Luther-University Halle-Wittenberg, Halle.

Callaini, G., M.G. Riparbelli, and R. Dallai. 1994. The distribution of cytoplasmic bacteria in the early Drosophila embryo is mediated by astral microtubules. J Cell Sci 107: 673–682.

Chang, C. W., D. Nashchekin, L. Wheatley, U. Irion, K. Dahlgaard, and et al. 2011. Anterior- posterior axis specification in *Drosophila* oocytes: Identification of novel bicoid and oskar mRNA localization factors. Genetics 188: 883–896.

Dong, J., X.W. Li, X.C. Zhang, and J.N. Feng. 2012. Sequencing and phylogenetic analysis of the *wsp* gene of *Wolbachia* in *Echinothrips americanus* Morgan. Journal of Northwest A & F University 3: 131–134.

Douglas, A.E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu Rev Entomol 43: 17–37.

Ferree, P.M., H.M. Frydman, J.M. Li, J. Cao, E. Wieschaus, and W. Sullivan. 2005. *Wolbachia* utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte. PLoS Pathog 1: e14.

Frydman, H.M., J.M. Li, D.N. Robson, and E. Wieschaus. 2006. Somatic stem cell niche tropism in *Wolbachia*. Nature 441: 509–512.

Guo, Y., A.A. Hoffmann, X.-Q. Xu, P.-W. Mo, H.-J. Huang, and et al. 2018. Vertical transmission of *Wolbachia* is associated with host vitellogenin in *Laodelphax striatellus*. Front Microbiol 9: 2016.

Hein, M. 2019. Verteilungsmuster von *Wolbachia* während der Progenese und Embryonalentwicklung bei *Hercinothrips femoralis* (Thripidae: Panchaeothripinae). Bachelor thesis. Martin-Luther-University Halle-Wittenberg, Halle.

Heming, B.S. 2003. The female reproductive system and oogenesis, pp. 29–64. In B.S. Heming (ed.), Insect development and evolution. Comstock Pub. Associates/Cornell University Press.

Herren, J.K., J.C. Paredes, F. Schupfer, and B. Lemaitre. 2013. Vertical transmission of a *Drosophila* endosymbiont via cooption of the yolk transport and internalization machinery. mBio 4: 1–8.

Hertig, M. 1936. The rickettsia, *Wolbachia pipientis* (gen. et sp. n.) and associated inclusions of the mosquito *Culex pipiens*. Parasitology 28: 453–486.

Hughes, G.L., and J.L. Rasgon. 2012. *Wolbachia* infections in arthropod hosts, pp. 351–366. In E.V. Fernando, and K.K. Harry (eds.), Insect pathology, 2nd edition, Academic press, Elsevier, St. Louis, MO, USA.

Kose, H., and T.L. Karr. 1995. Organization of *Wolbachia pipientis* in the *Drosophila* fertilized egg and embryo revealed by an anti-*Wolbachia* monoclonal antibody. Mech Dev 51: 275-288.

Kumm, S., and G. Moritz. 2008. First detection of *Wolbachia* in arrhenotokous populations of thrips species (Thysanoptera: Thripidae and Phlaeothripidae) and its role in reproduction. Environ Entomol 37: 1422-1428.

Landmann, F. 2019. The *Wolbachia* endosymbionts. Microbiol Spectrum 7: BAI-0018-2019.

Louis, C., and L. Nigro. 1989. Ultrastructural evidence of *Wolbachia* rickettsiales in *Drosophila simulans* and their relationships with unidirectional cross incompatibility. J Invertebr Pathol 54: 39–44.

Ma, W.J., F. Vavre, and L.W. Beukeboom. 2014. Manipulation of arthropod sex determination by endosymbionts: diversity and molecular mechanisms. Sex Dev 8: 59–73.

Matalon, Y., N. Katzir, Y. Gottlieb, V. Portnoy, and E. Zchori-Fein. 2007. *Cardinium* in *Plagiomerus diaspidis* (Hymenoptera: Encyrtidae). J Invertebr Pathol 96: 106–108.

Michalik, A., W. Jankowska, and T. Szklarzewicz. 2009. Ultrastructure and transovarial transmission of endosymbiotic microorganisms in Conomelus anceps and *Metcalfa pruinosa* (Insecta, Hemiptera, Fulgoromorpha). Folia Biol (Krakow) 57: 131–137.

Moran, N.A. 2006. Symbiosis. Curr Biol 16: R866–R871.

Moran, N.A., J.P. McCutcheon, and A. Nakabachi. 2008. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42: 165–190.

Moritz, G. 1988. Die Ontogenese der Thysanoptera (Insecta) unter besonderer Berücksichtigung des Fransenflüglers *Hercinothrips femoralis* (O.M. Reuter, 1891) (Thys., Thripidae, Panchaetothripinae) 2. Mitt. Erst- und Zweitlarve. Zoologische Jahrbücher, Anatomie 117: 299–351.

Moritz, G. 1995. Morphogenetic development of some species of the order Thysanoptera (Insecta), pp. 489–504. In B.L. Parker, M. Skinner, and T. Lewis (eds.), Thrips biology and management, Plenum Press, New York.

Moritz, G. 1997. Structure, growth and development. In T. Lewis (ed.), Thrips as crop pests. CAB International, Oxon and New York, USA.

Moritz, G. 2006. Thripse Fransenflügler, Thysanoptera. Die neue Brehm-Bücherei, Westarp Wissenschaft, Hohenwarsleben.

Nguyen, D.T., J.L. Morrow, R.N. Spooner-Hart, and M. Riegler. 2017. Independent cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* maintains endosymbiont coinfections in haplodiploid thrips populations. Evolution 71: 995–1008.

Nozaki, T., and K. Matsuura. 2016. Termite queens have disproportionately more DNA in their fat body cells: Reproductive division of labor and endoreduplication. Entomol Sci 19: 67–71.

O'Neill, S.L., and T.L. Karr. 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature 348: 178–180.

O'Neill, S.L., M.M. Pettigrew, S.P. Sinkins, H.R. Braig, T.G. Andreadis, and R.B. Tesh. 1997. In vitro cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. Insect Mol Biol 6: 33–39.

Pintureau, B., F. Lassabliére, C. Khatchadourian, and J. Daumal. 1999. Parasitoïdes oophages et symbiotes de deux thrips européens. Ann Soc Entomol Fr (N.S.) 35 (suppl.): 416–420.

Pritsch, M., and J. Büning. 1989. Germ cell cluster in the panoistic ovary of Thysanoptera (Insecta). Zoomorphology 108: 309–313.

Russell, S.L., L. Chappell, and W. Sullivan. 2019. A symbiont's guide to the germline. Curr Top Dev Biol 135: 315–351.

Serbus, L.R., C. Casper-Lindley, F. Landmann, and W. Sullivan. 2008. The genetics and cell biology of *Wolbachia*-host interactions. Annu Rev Genet 42: 683–707.

Spradling, A.C. 1993. Developmental genetics of oogenesis. The Development of *Drosophila melanogaster* 1: 1–70.

Stouthamer, R., J.A.J. Breeuwer, and G.D.D. Hurst. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu Rev Microbiol 53: 71–102.

Strunov, A., E. Kiseleva, and Y. Gottlieb. 2013. Spatial and temporal distribution of pathogenic *Wolbachia* strain *w*MelPop in *Drosophila melanogaster* central nervous system under different temperature conditions. J Invertebr Pathol 114: 22–30.

Terry, R.S., A.M. Dunn, and J.E. Smith. 1997. Cellular distribution of a feminizing microsporidian parasite: a strategy for transovarial transmission. Parasitology 115: 157–163.

Tsutsumi, T. 1996. Formation of the egg membranes of an idolothripine thrips, *Bactrothrips brevitubus* (Insecta: Thysanoptera). Proc Arthropod Embryol Soc Japan 31: 9–13.

Werren, J.H., L. Baldo, and M.E. Clark. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741–751.

Wright, J.D., F.S. Sjostrand, J.K. Portaro, and R.A. Barr. 1978. The ultrastructure of the rickettsia-like microorganism *Wolbachia pipientis* and associated virus-like bodies in the mosquito *Culex pipiens*. J Ultrastruct Res 63: 79–85.

Veneti, Z., M.E. Clark, T.L. Karr, C. Savakis, and K. Bourtzis. 2004. Heads or tails: host-parasite interactions in the Drosophila-*Wolbachia* system. Appl Environ Microbiol 70: 5366–5372.

Zabalou, S., A. Apostolaki, S. Pattas, Z Veneti, C. Paraskevopoulos, and et al. 2008. Multiple rescue factors within a *Wolbachia* strain. Genetics 178: 2145–2160.

Zchori-Fein, E., S.J. Perlman, S.E. Kelly, N. Katzir, and M.S. Hunter. 2004. Characterization of a 'Bacteroidetes' symbiont in Encarsia wasps (Hymenoptera: Aphelinidae): proposal of Candidatus *Cardinium hertigii*. Int J Syst Evol Microbiol 54: 961–968.

CHAPTER 6

Toxic impact of the entomopathogenic bacterium *Photorhabdus luminescens* on the survival rate of different Thripidae

6.1 Summary

In recent decades, various studies documented a broad spectrum of properties for entomopathogenic bacteria, which offer new opportunities for the usage in the biological pest management. For example, the gram-negative bacterium *P. luminescens* of nematodes (e.g. like *Heterorhabditis bacteriophora*) own specific toxin complexes, which can impair both cellular and humoral response and thus has a negative effect on the survival rate of the target insect. The current study explores the toxic impact of *P*. *luminescens* on the survival rate of different thrips species, which possess a diverse food spectrum, reproduction mode and presence of endosymbionts. For this purpose, a *P*. *luminescens* liquid culture was applied as a food source for 24 h on *Echinothrips americanus*, *Frankliniella occidentalis*, *Hercinothrips femoralis* and *Thrips tabaci*. Afterwards, the viability of the used thrips species was checked daily. The outcomes identified significant differences on the survival rate of *E*. *americanus* between the *P*. *luminescens* and control group. Interestingly, the presence of the immunoregulating *Wolbachia* bacterium had no protective effect on the viability of this thrips species. In contrast, the other tested thrips species show no significant difference in the probability of survival between *P*. *luminescens* and control group. By means of the *in situ* hybridization technique, bacterial DNA could not only be localized within the intestinal tract, but also within other internal tissues, after the application of the bacteria liquid culture.

Keywords: thrips, bacterial caused effects, viability, occurrences of bacteria in internal tissues

6.2 Introduction

Eco-friendly methods for biological pest control offer an effective alternative to chemical insecticides. Mainly two different concepts are known for pest controlling: the importing of natural enemies, which kill the pests (Howarth 1991) and the conservation or augmentation of natural enemies for the incessant reduce of the pest population (Messelink et al. 2014). Both methods provide a potential for plant and crop protection and the possibility to decrease the dependence on the utilization of pesticides.

A great deal agriculture damage is caused by species, which belong to the order Thysanoptera (Reitz 2009). Representatives of the family Thripidae, like *Frankliniella occidentalis*, number among to the most important insect pests on culture plants (Lewis 1997). The pest status of this thrips species is based on the reproduction potential, the broad host range, the function as virus vector of plants and other pathogens, the drifting in new habitats and the rapid development of tolerances or resistances against insecticides (Moritz 2006). Therefore, the biological pest control obtained on importance against pest thrips. For instance, in greenhouses predatory bugs (*Orius* spp.) are utilised for the control of thrips (Bosco et al. 2008, Cano et al. 2009, Huang et al. 2011). Unfortunately, this application is inefficient in agriculture. Another regulator against pest thrips could be the usage of nematodes. For example, the nematode *Thripinema nicklewoodi* is a natural enemy of *Frankliniella occidentalis*, but currently no in vitro procedure exists for the mass production of this nematode (Arthurs and Heinz 2006). In contrast, the nematodes of the genus *Heterorhabditis* are promising candidates against different insect species (Glazar et al. 1991). In nature, the nematodes life symbiotic with a bacteria species, which conduces as the main source of food for the nematode (Ehlers 2001). The pathogenic nematodes, particular the infective juvenile nematodes live in the soil and penetrate its target insects via integument, mouth opening, spiracle or anus (Ehlers 1989, ffrench-Constant and Bowen 2000). Normally the bacterium is housed in the intestine tract of the nematode (Endo and Nickle 1991) and during the infection process, the juveniles release its symbionts directly into the haemocoel of the insects (Ehlers 1989, ffrench-Constant and Bowen 2000, Goodrich-Blair and Clarke 2007). The microorganisms proliferate and inhibit a number of cellular and humoral responses of the host, whereby the target insect gradually dies (Gerritsen and Smits 1993). Following this, the bacteria augmented tissue of the host offers a possibility for nutrition and reproduction of the nematode (Lunau et al. 1993, Strauch and Ehlers 1998). Finally, the infective juveniles release the cadaver to find new suitable hosts in the soil (Ehlers 1989, ffrench-Constant and Bowen 2000). Various nematodes species of this genus could be produced in vitro for the utilisation in the plant protection (Ehlers 2001). However, the commercially used nematodes are too large for the infestation of thrips. A potential alternative or additional treatment for the biological pest control is provided by entomopathogenic microorganisms (e.g. bacteria, viruses, fungi) (Chandler et al. 2011). For

example, the symbiotic bacteria of nematodes (from the genus *Heterorhabditis*), which can be produced commercially in monoxenic liquid cultures (Ehlers et al. 1998), can provide a method for the application in greenhouses and in the field.

Previous articles of Gerritsen et al. (2004, 2005) demonstrated that entomopathogenic bacteria of nematodes have a toxic effect on thrips. The results of this studies showed that the bacterium *P*. *luminescens*, the symbiont of the nematode species *Heterorhabditis bacteriophora*, have a negative impact on the survival rate of different thripine species (Gerritsen et al. 2004, 2005). In the phytophagous sucking insects, *Frankliniella occidentalis* and *Thrips tabaci*, a mortality rate of 50% could be observed 7 days after the oral ingestion of the bacterium (Gerritsen et al. 2004, 2005). The gram-negative bacterium, *P*. *luminescens*, is able to produce antibiotic effective substances to inhibit other microorganisms (Ehlers 1989), and a number of virulence factors, including the ABC-type toxin complexes (ffrench-Constant and Bowen 2000, Gatsogiannis et al. 2013). Consequently, the bacterium can have significant impacts on the immunity of the host insect. Insects possess different physiological, molecular, morphological and behaviour relevant defence mechanisms to eliminate pathogens (Castillo et al. 2011). *P*. *luminescens* is capable to overcome different tissue barriers and the humoral and cellular immune defence (Vallet-Gely et al. 2008). Observations show that the toxins of *P*. *luminescens* have a negative impact on the survival rate of insects. It could document that less than 5 colony forming units (cfu) of the pathogen, by direct injection, suffice for a toxic effect within 48 h to 72 h (Goodrich-Blair and Clarke 2007).

In this study, we investigate the effects of the bacterium *P*. *luminescens* on different thrips species from the family Thripidae: *Echinothrips americanus*, *Frankliniella occidentalis*, *Hercinothrips femoralis* and *Thrips tabaci*. They possess a different food spectrum, reproduction mode and presence of endosymbionts. For our investigation, first we tested the successful ingestion of the bacterium in all thrips species. Following this, we examined the toxic impacts of *P*. *luminescens* on the survival rate of the thrips. Additionally, we identify the occurrence of the bacteria in the tissue parts of the tested species.

6.3 Materials and methods

6.3.1 Breeding of thrips species

The toxic impact of *P*. *luminescens* was tested on different thrips representatives of the family Thripidae (Tab. 6.1).

Tab. 6.1. Used thrips species.

Thrips species	Host plants	Reproduction mode	Endosymbionts
Echinothrips americanus, Morgan 1913	Laboratory culture on *Gossypium* spp. and *Hibiscus longiflora*	Arrhenotoky	*Wolbachia* (Kumm et al. 2006, Chapter 3) and *Cardinium* (Chapter 3)
Frankliniella occidentalis, (Pergande 1895)	Laboratory culture on *Phaseolus vulgaris* and *Chrysanthemum* spp.	Arrhenotoky	No (Kumm et al. 2006, Chapter 3)
Hercinothrips femoralis, (O.M. Reuter 1891)	Laboratory culture on *Ocimum basilicum* and *Citrus limon*	*Wolbachia* induced thelytoky	*Wolbachia* (Pintureau et al. 1999)
Thrips tabaci, Lindemann 1889	Laboratory culture on *Allium schoenoprasum* and *Allium ampeloprasum*	Thelytoky	No (Kumm et al. 2006)

For the experiments, we used adult females, 1 d ± 48 h after hatching. The specimens were bred in prepared rearing boxes (plastic boxes) with two Petri dishes filled with 25 ml of 1.4% (w/v) agar. A leaf of *Phaseolus vulgaris* was placed into the agar of each Petri dish. Following this, each box was stocked with 20 adult females from the laboratory cultures at the University of Halle-Wittenberg (at 23 ± 1 °C, RH 50 ± 10%, and a 16 : 8 h L:D photoperiod, with lights on 6:00 a.m. CET) and afterwards placed in the climate chamber for 48 h (under the same conditions as the laboratory cultures), before the females were removed. The boxes were closed again and placed back in the climate chamber. Depending on the developmental time of each thrips species, the adult females hatch out after 14 d (*F*. *occidentalis*), 16 d (*E*. *americanus* and *T*. *tabaci*) and 28 d (*H*. *femoralis*).

6.3.2 Cultivation of the entomopathogenic bacterium *P. luminescens*

For the experiments we used the *P*. *luminescens* bacteria strain BPl (e-nema, Schwentinental, Germany) of the nematode *Heterorhabditis bacteriophora*. The preparation of the bacteria was generated in a growth medium composed of: 15 g/l peptone, 3 g/l yeast extract, 6 g/l sodium chloride and 1 g/l glucose (pH 7.5). Afterwards, the liquid culture was incubated for 3 d at 23 °C on the shaker. Before preparing the *P*. *luminescens* solution, the number of bacteria per µl was determined by an optical density at 600 nm (apparatus: Jenway 6305 Spectrophotometer, UK; S6 Suppl. method 1). The application solution was prepared with a number of ~339500 bacteria per µl (bacteria number for each thrips species in S6 Tab. 1).

6.3.3 Survival analysis

Within the scope of the analysis, the survival rate of the different thrips species was determined after the ingestion of the *P*. *luminescens* or control solution. The toxic effect of *P*. *luminescens* was tested on ~48 adult females per thrips species (number of tested thrips per species in S6 Tab. 1). The control group consists of ~24 individuals per species (number of tested thrips per species in S6 Tab. 1).

6.3.3.1 Application and control solution components

The application solution consisted of 75 µl sucrose solution (10%), 50 µl patent blue V solution (4%) and 75 µl *P*. *luminescens* liquid culture. Instead of the bacteria liquid culture, the control solution contained only the growth medium.

6.3.3.2 Application conditions

The application of the bacteria and control solution was carried out with females of all thrips species starved for 24 h. In the first step, the females were transferred in a plastic container with gauze on one side (S6 Fig. 1A). Subsequently, the container was closed with Parafilm® M (Pechiney Plastic Packaging, Chicago, IL, USA) and the application or control solution was applied on parafilm (S6 Fig. 1B). After the application of the solution, the complete system was closed with a second layer of parafilm (S6 Fig. 1C). The application system was placed in a plastic container on a layer of agar (1.4%) at the bottom (S6 Fig. 1C). Finally, the system was transferred in a moist chamber and placed on a shaker at 110 rmp (at 23 ± 1 °C). After 24 h application time each thrips female was transferred in one well of a 12-well Greiner plate, which was filled with 2 ml of 1.4% (*w*/*v*) agar and a leaf disc of *P. vulgaris* on the surface of agar. The plates were sealed with a glass lid and parafilm and placed in the climate chamber (apparatus: MLR-352H Sanyo Electric Co., Japan; conditions: at 23 ± 1 °C, RH 50 ± 10%, and a 16 : 8 h L:D photoperiod, with lights on 6:00 a.m. CET). All three to five days the thrips species were relocated on new Greiner plates.

6.3.3.3 Documentation

The survival rate of the different thrips species was determined through a daily control of the viability of each thrips until death. For the determination of the survival rate, the generated data of Köcher (2018) were used jointly. The statistical evaluation of the result was generated by Kaplan-Meier-survival-analysis (program WinSTAT® for Excel, Fitch Software, Bad Krozingen, Germany). This statistical analysis ascertains the survival rate by revealing the number of survivors to a determined time point where no event has occurred yet. The subsequent log-rank test identifies the significant differences between two Kaplan-Meier curves.

6.3.4 *P. luminescens* detection

6.3.4.1 DNA extraction, gene detection, agarose gel electrophoresis

The evidence of the successful *P. luminescens* ingestion in all thrips species was the premise for the subsequent investigations of the bacterial toxic impacts. This was checked 24 h after application by gene detection in 6 females of each thrips species.

For the DNA isolation, a modified variant of the “100 fly method” after Roberts (1998) was used (described in Kumm et al. 2006). Each thrips was homogenized (under liquid nitrogen) in 100 µl extraction buffer (0.2 M Sucrose, 0.1 M TRIS, 0.1 NaCl, 0.05 M EDTA, 0.5% SDS, pH 9.2). Afterwards, the samples were incubated for 15 min at 70 °C, cooled off and added with 15 µl ice-cold 8 M potassium acetate, mixed, and incubated for 15 min on ice. After a 15 min centrifugation step (at 13200 rpm) the supernatant (90 µl) was transferred in a new tube with 2.5 vol. ethanol and 0.1 vol. 3 M sodium acetate (4 °C) and vigorously mixed. Following, the DNA was precipitated at -80 °C for 45 min. After this step, the DNA was sedimented by centrifugation at 13200 rpm (15 min) and the supernatant was discarded. The sediment was washed with 100 µl 70% ethanol for 5 min. Subsequently, the ethanol was discarded and the sediment dried at 45 °C for 5 min. In the end, the sediment was dissolved in 16 µl ddH_2O for 10 min at 45 °C.

The isolated bacteria DNA was identified by the evidence of the 16S rDNA. Therefore, we used the universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3', Weisburg et al. 1991) and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3' (Y=C : T), Lane 1991). The PCR reaction mixture comprised 5 µl 10x reaction buffer, 4 µl $MgCl_2$ (25 mM), 0.4 µl dNTPs (25 mM), 1.5 µl of each primer (5 pmol), 0.2 µl *Taq* polymerase and 2 µl of the template DNA in a total volume of 50 µl. Following program condition were utilized: 1 min at 94 °C initial denaturation, 35 cycles 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C and a last elongation step 7 min at 72 °C. The PCR products were verified through a 2% agarose gel and visualized with the gel documentation system DIAS-III from SERVA as well as documented with GelScan V6.0.

For comparison to the PCR results, the PCR products were used for a restriction fragment length polymorphism (RFLP). This technique enables a restriction digestion analysis between the *P. luminescens* strain (DNA extraction method described in S6 Suppl. method 2) and the bacterial DNA of the thrips samples. The complete method is described in S6 Suppl. method 3.

6.3.4.2 *In situ* hybridization

Additionally, we ascertained the distribution patterns of bacterial DNA after 24 h application in different thrips species by *in situ* hybridization. We localized bacteria by a modified method after Morel et al. 2001. They used immunohistochemistry *in situ* hybridization based on the detection of labelled DNA section. For this method, all specimens (20 female adults of each

thrips species) were fixed, embedded, and sliced. Afterwards, the pretreatment with Proteinase K of the sections took place. With the scope of the *in situ* hybridization (*in situ* PCR) digoxigenin-labelled dUTPs were integrated with the new synthesized DNA section. For amplification of bacteria DNA primers for the 16S rDNA (fD1: Weisburg et al. 1991, 1492r: Lane 1991) were used. Subsequently, the digoxigenin marked dUTPs were localized through an antibody and visualized through a colour reaction of the reporter enzyme. The complete method was realized after the instruction in chapter 2.

6.4 Results

6.4.1 Evidence of *P. luminescens* by gene detection

The primary purpose of this investigations part was the evidence of a successful *P. luminescens* ingestion of thrips. In most cases, the tested thrips samples exhibited a PCR product, with a fragment length of 1530 bp, after 24 h ingestion of *P. luminescens* (Fig. 6.1A and B). Particularly, bacterial DNA was detected in all tested females of *H. femoralis* (Fig. 6.1A) and *E. americanus* (Fig. 6.1B) after 24 h application time. Furthermore, 4 thrips of *F. occidentalis* (Fig. 6.1A) and 5 females of *T. tabaci* (Fig. 6.1B) showed a PCR result, after 24 h ingestion of the bacteria liquid culture. In the control group, no PCR product was observed for thrips females (data not shown). The performed RFLP showed a compliant restriction fragment pattern for the *P. luminescens* DNA and the thrips DNA samples (S6 Fig. 2).

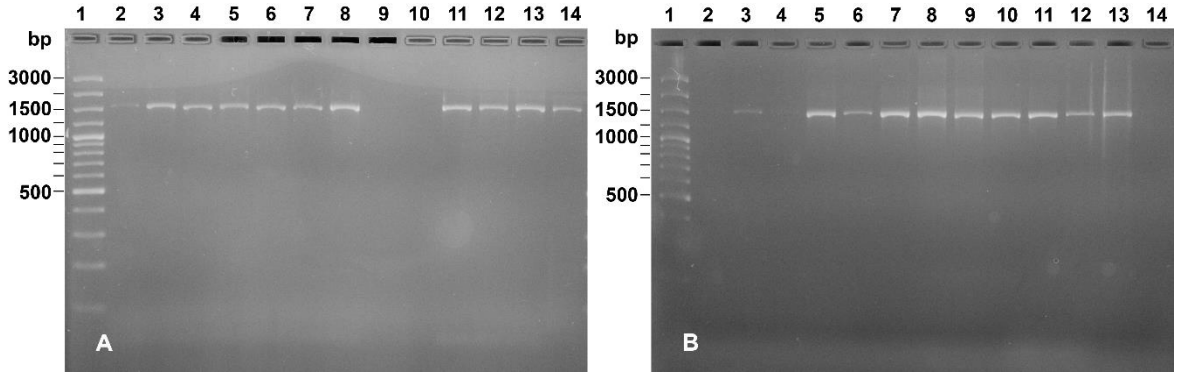


Fig. 6.1. Detection of bacterial DNA (by use of primers (fD1/1492r) for the 16S rDNA) in different Thripidae after 24 h *P. luminescens* ingestion. DNA fragment length of 1530 bp. Fig. 1A: lanes 2–7 *H. femoralis*, lanes 8–13 *F. occidentalis*, and lane 14 positive control *P. luminescens* strain DNA. Fig. 1B: lanes 2–7 *T. tabaci*, lanes 8–13 *E. americanus*, and lane 14 negative control. In each agarose gel lane 1 marker (Gene Ruler^{TM} 100 bp (Thermo Scientific, Waltham)).

6.4.2 Detection of the bacterial distribution patterns by *in situ* hybridization

Generally, all investigated Thripidae showed similar bacterial distribution patterns. The distribution patterns of bacteria DNA were displayed for *F. occidentalis* were exemplifying for all investigated thrips species in this work (Fig. 6.2). Females showed mainly four different

localization points of the bacterium. Few bacteria were localized in the neuronal tissue, especially around the oesophagus in the areas of central brain in some samples (Fig. 6.2A). Furthermore, bacterial DNA was detected in the oscillatoric flight muscles in the pterothorax of the adult females in the most specimens (Fig. 6.2B). Considerable changes in the muscle structures of the direct surrounding of the localized bacteria (Fig. 6.2B) took place. Prevalent distributed was the bacteria in the intestinal system in all parts of the gut (foregut, midgut, hindgut). In the midgut the bacteria were localized as accumulations in the intestinal epithelium of all tested females (Fig. 6.2C). A few bacteria were detected in the female gonads (Fig. 6.2D). The bacteria occurred as clusters in the different parts of the ovarioles, for example in the germarium (Fig. 6.2D) or oocytes during vitellogenesis (data not show).

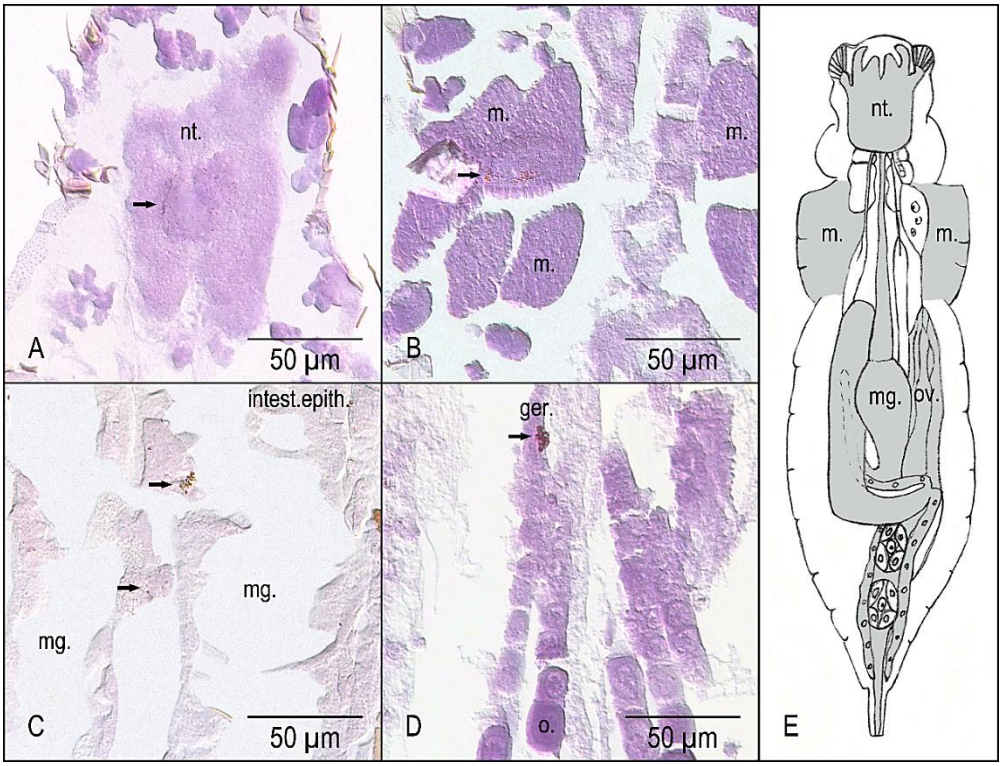


Fig. 6.2. Detection of bacterial clusters in adult *F. occidentalis* females (frontal sections) by use of *in situ* hybridization. The bacteria DNA is clearly recognizable as red-brown signals (arrows). (A) neuronal tissue, (B) oscillatoric flight muscles, pterothorax, (C) loops of midgut, intestinal epithelium, (D) germarium and vitellarium, (E) overview thrips anatomy (female) with marked tissues of bacterial distribution patterns (grey coloured). Overview of female thrips anatomy, modified figure after Buffa 1898. (ger.– germarium, m.– muscle, mg.– midgut, intest.epith.– intestinal epithelium, nt.– neuronal tissue, o.– oocyte, ov.– ovariole).

6.4.3 Survival analysis by Kaplan-Meier

A significant difference in the survival rate between *P. luminescens* and control group was determined by means of the Kaplan-Meier analysis for *E. americanus* (Fig. 6.3A, $p < 0.05$). Especially, 48 h after the application of the bacteria liquid culture, the mortality rate reached 68%, whereas the survival rate in the control group remained by 100% (Fig. 6.3A).

No significant difference in probability of survival between *P. luminescens* and control group of *F. occidentalis* could be detected (Fig. 6.3B, $p > 0.05$). The tested thrips females of the application group have an average lifespan of 12 d, in contrast to the control group with 10 d. Furthermore, both groups showed a mortality rate of more than 50% (application group 52%, control group 62%) 10 d after the observation started (Fig. 6.3B).

Likewise, *H. femoralis* females showed no significant difference in the survival probability of the *P. luminescens* and control group (Fig. 6.3C, $p > 0.05$). The average lifespan was 44 d for the *P. luminescens* ingested females and 43 d for the control females. Moreover, present in both groups, a few thrips females lived over 100 d (maximal lifespan of each thrips 126 d (*P. luminescens* group) and 128 d (control group)).

The statistical evaluation found no significant differences between the *P. luminescens* and the control group for *T. tabaci* (Fig. 6.3D, $p > 0.05$). It is particularly interesting that the average lifespan of both groups with 7 d is similar. What is also notable is the similar graph shape within 10 d after the application time (Fig. 6.3D).

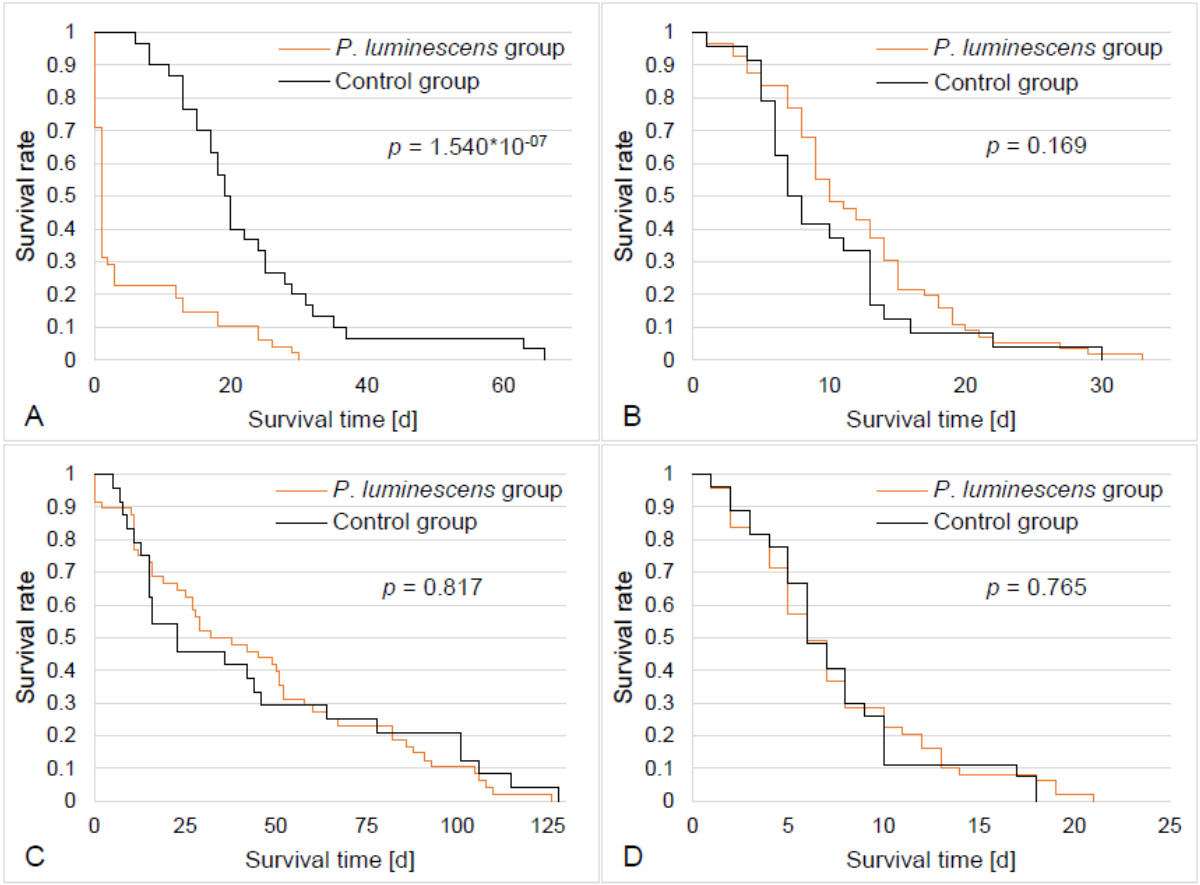


Fig. 6.3. Kaplan-Meier curves of (A) *E. americanus*, (B) *F. occidentalis*, (C) *H. femoralis* and (D) *T. tabaci* after 24 h ingestion of the liquid culture with *P. luminescens* (*P. luminescens* group) and without the bacterium (control group). $P < 0.05$.

6.5 Discussion

Every year insects cause a lot of economic and ecological damage worldwide. The control of pest insects, like thrips, is increasingly more difficult through the usage of pesticides. Thrips are able to develop rapid tolerances or resistances to these chemical compounds, making the use of alternative methods important (Immaraju et al. 1992, Brødsgaard 1994). The last decades, documented that the usage of natural enemies in greenhouses resulted in a successful control of pests (Pilkington et al. 2010). However, the utilization of effective natural enemies is dependent on different factors, for example the availability of pest antagonists, the application costs, the application area, or the effectivity on all plants (Messelink et al. 2014). Hence, entomopathogenic microorganisms (bacteria, fungi, viruses etc.) offers an alternative or back-up treatment when the natural enemy approach is ineffective for pest control (Chandler et al. 2011, Lacey et al. 2015).

Previous investigations demonstrate the successful oral application of the entomopathogenic bacterium *P. luminescens* with a toxic impact on the survival rate in thrips (Gerritsen et al. 2004/2005). Gerritsen et al. (2004) ascertained an effective oral toxicity to *Frankliniella occidentalis* for the *P. luminescens* P2 strain, by which means less than 50% of thrips females died after 7 d. Additionally, the study showed that other tested *P. luminescens* bacteria strains (Pmol, P4, PP88, Psie, P23, PNJ, PDa I) have no impact on the survival rate of *F. occidentalis* (Gerritsen et al. 2004). In this study, we tested the toxic impact of the *P. luminescens* strain BPl (of *Heterorhabditis bacteriophora*) on different representatives of the family Thripidae. What is particularly interesting is the different food spectrum, reproduction mode and presence of endosymbionts in the used thrips species. By means of the Kaplan-Meier analysis, a significant difference of the survival rate could be determined between *P. luminescens* and control group for *E. americanus* (Fig. 6.3A, $p < 0.05$). The observations show a 68% mortality rate of the thrips females 48 h after the application of the bacteria liquid culture, whereas the survival rate in the control group remained by 100% (Fig. 6.3A). In contrast, the other tested thrips females (*F. occidentalis*, *H. femoralis* and *T. tabaci*) showed no significant difference of the survival probability of the *P. luminescens* and control group (Fig. 6.3B–D, $p > 0.05$). Connected with prior researches the toxic impact on the survival rate of the bacterium seems to depend on the applied bacteria strain and target thrips species. *P. luminescens* produce four major toxin groups (reviewed in Rodou et al. 2010), by which the bacterium is able to overcome the cellular and humoral immune response of the target insect (Vallet-Gely et al. 2008, Nielsen-LeRoux et al. 2012). Particularly the toxin complexes (Tcs) have an oral and injectable toxic activity and function as class of insecticidal toxins (Blackburn et al. 2005).

Insects possess different physiological, molecular, morphological and behaviour relevant defence mechanisms to eliminate pathogens (Castillo et al. 2011). By means of the *in situ* hybridization, we tested the ability of *P. luminescens* to overcome the tissue barriers within the

intestinal system. For these examinations, we used primers for the 16S rDNA, which detected a great number of eubacteria (Lane 1991, Weisburg et al. 1991). Moghaieb et al. 2017 conducted a 16S rDNA analysis by means of the used primers and ascertained *P*. *luminescens* strains. In the different thrips species, we observed prevalent four various localization points for bacterial DNA, with similar distribution patterns in all Thripidae (Fig. 6.2). Primarily bacteria were localized within the intestinal epithelium of the midgut after 24 h of *P*. *luminescens* application (Fig. 6.2C). Furthermore, the bacteria were detected in the neuronal tissue (Fig. 6.2A), in the oscillatoric flight muscles in the pterothorax (Fig. 6.2B) and in the gonads (Fig. 6.2D) of the females. In contrast to that, females without *P*. *luminescens* application showed only a few appearances of bacteria in the intestinal tract and reproductive system (unpublished data). Thrips, like *F*. *occidentalis*, naturally harboured a broad spectrum of symbiotic bacteria, for example BFo (Chanbusarakum and Ullman 2009) or *Erwinia* sp. (de Vries et al. 2004). In the most insects, the midgut plays an important part for the digestion and absorption (Engel and Moran 2013). Especially, the midgut epithelial cells possess various functions, for instance the cells secrete digestive enzymes, absorb nutrients and in the most case produce a peritrophic matrix (or peritrophic membrane) and also a basal lamina (or basal membrane). The peritrophic matrix (based on structural variations in some insects also termed as “plexiform surface coat” or “extracelluar membrane layers”) is a natural protective film and function among others as barrier against pathogens (Shao et al. 2001). Silva et al. (2002) demonstrated in *Manduca sexta* that *P*. *luminescens* utilize a specific niche during the midgut colonization. The bacterium occupies the area of midgut epithelium between the basal membrane and extracellular matrix by a direct injection. Inside the niche, the bacterium produces the gut active Toxin complex A (Tca) and the RTX-like metalloprotease PrtA. Both toxin and protease lead to programmed death of the midgut epithelium cells (Silva et al. 2002). Furthermore, Daborn et al. (2001) showed that an intestinal infection of *P*. *luminescens* leads to an up-regulation of the nitric oxide synthase (NOS) in *Manduca sexta* only in the gut tissue. The midgut cells produced NOS function as direct elimination mechanism of pathogens in insects. Also, in *Drosophila*, an oral infection of the entomopathogenic bacterium *Serratia marcescens* activated the expression of the antimicrobial peptides in the gut only (Nehme et al. 2007). Hence, the bacteria find its way into the hemolymph system and overcomes the humoral immune response of the fly (Nehme et al. 2007). This process could explain the distribution patterns of bacterial DNA in the nervous system and in the oscillatoric flight muscles of the pterothorax in the tested thrips females (Fig. 6.2A–B). That fact could indicate to a potential penetration opportunity of *P*. *luminescens* via the gut system into other parts of the insect body. Despite of the widely distribution of the bacteria within the body of all thrips species, a toxic impact on the survival rate could only ascertained in *E*. *americanus* (Fig. 6.3A).

A possible explanation for the different immune reaction between the tested species could be the presence of the endosymbionts. Microbial symbionts are able to affect the ecology, life history and evolution of their host insects (Zchori-Fein and Bourtzis 2012). Phloem-feeding pests, like thrips, harbour vertically transmitted, intracellular bacterial microorganisms (also called endosymbionts). In phloem-feeding insects these bacteria could protect the insect against natural enemies (Oliver et al. 2003, Teixeira et al. 2008), which support the presence on crops (Hosokawa et al. 2007) and promotes the competence to plant virus transmission (van den Heuvel et al. 1994, Gottlieb et al. 2010). The tested species *E*. *americanus* is infected with *Wolbachia* and *Cardinium* (Chapter 3). Also, a second thrips species, *H*. *femoralis*, owns a endosymbiosis with *Wolbachia*. Kumm and Moritz (2008) demonstrated that present thelytoky in this thrips species is based on a *Wolbachia* infection. The endosymbiont induced a thelytokous parthenogenesis, by which virgins produce only female offspring (Stouthamer et al. 1990). *Wolbachia* are able to interact with pathogens, because of the occurrence in a variety of tissues and cell types within insects (Dobson et al. 1999). For example, *Wolbachia* infected flies and mosquitoes are able to generate resistance against a wide range of viruses, bacteria, pathogens, and fungi (Eleftherianos et al. 2013). In contrast to the disadvantageous immune reaction in *E*. *americanus*, the observed *H*. *femoralis* females show no significant differences on the survival rate between the *P*. *luminescens* and control group. This result might indicate to a *Wolbachia*-induced host protection in *H*. *femoralis*. However, the study of Shokal et al. (2016) shows that the *P*. *luminescens* infection in *D*. *melanogaster* leads to a death of all infected lines within 24 h, independent of an endosymbiont presence. Likewise, the multiple infection of *Wolbachia* and *Spiroplasma* shown neither a protective effect on the survival response nor affected the replication activity of *P*. *luminescens* (Shokal et al. 2016).

The findings of the present study document that the entomopathogenic bacterium potentially has the ability to overcome the cellular barriers of the intestinal system of the different thrips species. But a toxic impact on the survival rate was only ascertained for *E*. *americanus*. It seems that the toxic impact of the *P*. *luminescens* is dependent on the bacteria strain specific toxin complexes and the target insect. Consequently, the application of *P*. *luminescens* offers an opportunity for the usage in the biological control of *E*. *americanus*.

6.6 References

Arthurs, S., and K.M. Heinz. 2006. Evaluation of the nematodes *Steinernema feltiae* and *Thripinema nicklewoodi* as biological control agents of western flower thrips *Frankliniella occidentalis* infesting chrysanthemum. Biocontrol Sci Technol 16: 141–155.

Blackburn, M.B., J.M. Domek, D.B. Gelman, and J.S. Hu. 2005. The broadly insecticidal *Photorhabdus luminescens* toxin complex a (Tca): Activity against the Colorado potato beetle, *Leptinotarsa decemlineata*, and sweet potato whitefly, *Bemisia tabaci*. J Insect Sci 5: 32.

Bosco, L., E. Giacometto, and L. Tavella L. 2008. Colonization and predation of thrips (Thysanoptera: Thripidae) by *Orius* spp. (Heteroptera: Anthocoridae) in sweet pepper greenhouses in Northwest Italy. Biol Control 44: 331–340.

Brødsgard, H.F. 1994. Insecticide resistance in European and African populations of western flower thrips tested in a new residue on glass test: Econ Entomol 87: 1141–1146.

Buffa, P. 1898. Contributo allo studio anatomico della *Heliothrips haemorrhoidalis*. Riv Patolog Veget Padova 7: 1–30, Tab. 5.

Cano, M., E. Vila, D. Janssen, D. Bretones, E. Salvador, L. Lara, and M.M. Tellez. 2009. Selection of refuges for *Nesidiocoris tenuis* (Het.: Miridae) and *Orius laevigatus* (Het.: Anthocoridae): Virus reservoir risk assessment. IOBC/WPRS Bull 49: 281–286.

Castillo, J.C., S.E. Reynolds, and I. Eleftherianos. 2011. Insect immune responses to nematode parasites. Trends Parasitol 27: 537–547.

Chanbusarakum, L.J., and D.E. Ullman. 2009. Distribution and ecology of *Frankliniella occidentalis* (Thysanoptera: Thripidae) bacterial symbionts. Environ Entomol 38: 1069–1077.

Chandler, D., A.S. Bailey, G.M. Tatchell, G. Davidson, J. Greaves, and W.P. Grant. 2011. The development, regulation and use of biopesticides for integrated pest management. Philos Trans R Soc B-Biol Sci 366: 1987–1998.

Daborn, P.J., N. Waterfield, M.A. Blight, and R.H. ffrench-Constant. 2001. Measuring virulence factor expression by the pathogenic bacterium *Photorhabdus luminescens* in culture and during insect infection. J Bacteriol 183: 5834–5839.

Dobson, S.L., K. Bourtzis, H.R. Braig, B.F. Jones, W. Zhou and et al. 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem Mol Bio 29: 153–160.

de Vries, E.J., G. Jacobs, M.W. Sabelis, S.B.J. Menken, and J.A.J. Breeuwer. 2004. Diet-dependent effects of gut bacteria on their insect host: the symbiosis of *Erwinia* sp. and western flower thrips. Proc R Soc Lond B 271: 2171–2178.

Ehlers, R.-U. 1989. Untersuchungen zur Taxonomie und Vermehrung entomophager Nematoden der Gattungen *Steinernema* und *Heterorhabditis* und ihrer symbiontischen Begleitbakterien *Xenorhabdus* spp. Ph.D. dissertation. Christian-Albrechts-University, Kiel.

Ehlers, R. U. 2001. Mass production of entomopathogenic nematodes for plant protection: Appl. Microbiol. Biotechnol. 56: 623–633.

Ehlers, R.-U., S. Lunau, K. Krasomil-Osterfeld, and K.H. Osterfeld. 1998. Liquid culture of the entomopathogenic nematode-bacterium-complex *Heterorhabditis megidis/Photorhabdus luminescens.* BioControl 43: 77–86.

Eleftherianos, I., J. Atri, J. Accetta, and J.C. Castillo. 2013. Endosymbiotic bacteria in insects: guardians of the immune system? Front Physiol 4: 46.

Endo, B.Y., and W.R. Nickle. 1991. Ultrastructure of the intestinal epithilium, lumen and associated bacteria in *Heterorhabditis bacteriophora.* J Helminthol Soc Wash 58: 202–212.

Engel, P., and N. Moran. 2013. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev 37: 699–735.

ffrench-Constant, R. H., and D.J. Bowen. 2000. Novel insecticidal toxins from nematode-symbiotic bacteria. Cell Mol Life Sci 57: 828–833.

Gatsogiannis, C., A.E. Lang, D. Meusch, V. Pfaumann, O. Hofnagel, and et al. 2013. A syringe-like injection mechanism in *Photorhabdus luminescens* toxins. Nature 495: 520–523.

Gerritsen, L.J.M., and P.H. Smits. 1993. Variation in pathogenicity of recombinations of *Heterorhabditis* and *Xenorhabdus luminescens* strains. Fundam appl Nematol 16: 367–373.

Gerritsen, L.J.M., J. Georgieva, and G.L. Wiegers. 2004. Oral toxicity of bacterial toxins against thrips species. Pest Control 15: 75–80.

Gerritsen, L.J.M., J. Georgieva, and G.L. Wiegers. 2005. Oral toxicity of *Photorhabdus* toxins against thrips species. J Invertebr Pathol 88: 207–211.

Glazar, I., S. Galper, and E. Sharon. 1991. Virulence of the nematode (*Steinernematids* and *Heterorhabditis*)-bacteria (*Xenorhabdus spp.*) complex to the Egyptian cotton leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae). J Invertebr Pathol 57: 94–100.

Goodrich-Blair, H., and D.J. Clarke. 2007. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: Two roads to the same destination. Mol Microbiol 64: 260–268.

Gottlieb, Y., E. Zchori-Fein, N. Mozes-Daube, S. Kontsedalov, M. Skaljac, and et al. 2010. The transmission efficiency of tomato yellow leaf curl virus by the whitefly *Bemisia tabaci* is correlated with the presence of a specific symbiotic bacterium species. J Virol 84: 9310–9317.

Hosokawa, T., Y. Kikuchi, M. Shimada, and T. Fukatsu. 2007. Obligate symbiont involved in pest status of host insect. Proc R Soc B-Biol Sci 274: 1979–1984.

Howarth, F.G. 1991. Environmental impacts of classical biological control. Annu. Rev. Entomol. 36: 485–509.

Huang, N.X., A. Enkegaard, L.S. Osborne, P.M.J. Ramakers, G.J. Messelink, and et al. 2011. The banker plant method in biological control. Crit Rev Plant Sci. 30: 259–278.

Immaraju, J. A., T.D. Paine, J.A. Bethke, K.L. Robb, and J.P. Newman. 1992. Western Flower Thrips (Thysanoptera. Thripidae) Resistance to Insecticides in Coastal California Greenhouses. J Econ Entomol 85: 9–14.

Köcher, C. 2018. Toxikologische Wirkung des entomopathogenen Bakteriums *Photorhabdus luminescens* auf verschiedene Thripidae (*E. americanus*, *F. occidentalis*, *H. femoralis*, *T. tabaci*). Bachelor thesis. Martin-Luther-University Halle-Wittenberg, Halle.

Kumm. S., and G. Moritz. 2008. First detection of *Wolbachia* in arrhenotokous populations of thrips species (Thysanoptera: Thripidae and Phlaeothripidae) and its role in reproduction. Environ Entomol 37: 1422–1428.

Kumm, S., R. Kranz, and G. Moritz. 2006. Mikroorganismen-gesteuerte Parthenogenese bei Thysanopteren. Mitt Dtsch Ges allg angew Ent 15: 153–156.

Lacey, L.A., D. Grzywacz, D.I. Shapiro-Ilan, R. Frutos, M. Brownbridge, and M.S. Goettel. 2015. Insect pathogens as biological control agents: Back to the future. J Invertebr Pathol 132: 1–41.

Lane, D.J. 1991. 16s/23s RNA sequencing, pp. 115–176. In E. Stackebrandt, and M. Goodfellow (eds.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd, Baffins Lane, Chichester, West Sussex PO19 1UD, England.

Lewis, T. 1997. Pest thrips in perspective. In T. Lewis (ed.), Thrips as crop pests. CAB International, Oxon and New York, USA.

Lunau, S., S. Stoessel, A.J. Schmidt-Peisker, and R.-U. Ehlers. 1993. Establishment of monoxenic inocula for scaling up *in vitro* cultures of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis* spp. Nematologica 39: 385–399.

Messelink, G.J., J. Bennison, O. Alomar, B.L. Ingegno, L. Tavella, and et al. 2014. Approaches to conserving natural enemy populations in greenhouse crops: current methods and future prospects. BioControl 59: 377–393.

Moghaieb, R.E.A., A.A. Abdelhadi, H.A. El-Sadawy, N.A.T. Allam, B.A. Baiome, and M.H. Soliman. 2017. Molecular identification and genetic diversity among *Photorhabdus* and *Xenorhabdus* isolates. 3 Biotech 7: 6.

Morel, G., A. Cavalier, and L. Williams. 2001. *In situ* hybridization in electron microscopy. CRC Press, USA.

Moritz, G. 2006. Thripse Fransenflügler, Thysanoptera. Die neue Brehm-Bücherei, Westarp Wissenschaft, Hohenwarsleben.

Nehme, N.T., S. Liégeois, B. Kele, P. Giammarinaro, E. Pradel, and et al. 2007. A model of bacterial intestinal infections in *Drosophila melanogaster*. PLOS Pathog 3: e173.

Nielsen-LeRoux, C., S. Gaudriault, N. Ramarao, D. Lereclus, and A. Givaudan. 2012. How the insect pathogen bacteria *Bacillus thuringiensis* and *Xenorhabdus/Photorhabdus* occupy their hosts. Curr Opin Microbiol 15: 220–231.

Oliver, K.M., J.A. Russell, N.A. Moran, and M.S. Hunter. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci USA 100: 1803–1807.

Pilkington, L.J., G. Messelink, J.C. van Lenteren, and K. Le Mottee. 2010. “Protected Biological Control”–Biological pest management in greenhouse industry. Biol. Control 52: 216–220.

Pintureau, B., F. Lassablière, C. Khatchadourian, and J. Daumal. 1999. Parasitoïdes oophages et symbiotes de deux thrips europeens. Ann Soc Entomol Fr 35: 416–420.

Reitz, S.R. 2009. Biology and Ecology of the Western Flower Thrips (Thysanoptera: Thripidae): The Making of a Pest. Florida Entomologist 92: 7–13.

Roberts, D.B. 1998. *Drosophila*, a practical approach, pp. 399. In 2rd edn., Oxford University Press, Oxford, New York, Tokyo.

Rodou, A., D.O. Ankrah, and C. Stathopoulos. 2010. Toxins and secretion systems of *Photorhabdus luminescens*. Toxins. 2: 1250–1264.

Shao, L., M. Devenport, and M. Jacobs-Lorena. 2001. The peritrophic matrix of hematophagous insects. Arch Insect Biochem Physiol 47: 119–125.

Shokal, U., S. Yadav, J. Atri, J. Accetta, E. Kenney, and et al. 2016. Effects of co-occurring *Wolbachia* and *Spiroplasma* endosymbionts on the *Drosophila* immune response against insect pathogenic and non-pathogenic bacteria. BMC Microbiol. 16: 16.

Silva, C.P., N.R. Waterfield, P.J. Daborn, P. Dean, T. Chilver, and et al. 2002. Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. Cell Microbiol. 4: 329–339.

Stouthamer, R., R. Luck, and W.D. Hamilton. 1990. Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/ Trichogrammatidae) to revert sex. PNAS 87: 2424–2427.

Strauch, O., and R.-U. Ehlers. 1998. Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. Appl. Microbiol. & Biotechnol. 50: 369–374.

Teixeira, L., A. Ferreira, and M. Ashburner. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. Plos Biol 6: 2753–2763.

Vallet-Gely, I., B. Lemaitre, and F. Boccard. 2008. Bacterial strategies to overcome insect defences. Nature Reviews Microbiology 6: 302–313.

van den Heuvel, J.F.J.M., M. Verbeek, and F. Vanderwilk. 1994. Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by *Myzus persicae*. J Gen Virol 75: 2559–2565.

Weisburg, W.G., S.M. Barns, D.A. Pelletier, and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697–703.

Zchori-Fein, and E., K. Bourtzis. 2012. Manipulative tenants: bacteria associated with arthropods. Frontiers in microbiology. CRC Press, Boca Raton.

CHAPTER 7

General discussion on the importance of endosymbionts and other bacteria for *Echinothrips americanus*

Associations with heritable endosymbionts can benefit the insect biology, with a range of consequences for their host. These symbionts are mainly maternally transmitted with a high efficiency but also undergo extensive horizontal transfers in nature. Coinfection of individuals result from horizontal transfers between host species and offers the possibility for recombination and gene transfer. Numerous observations document the presence of *Wolbachia* and *Cardinium* within an insect species, which can be responsible for a variety of induced phenomena, including alterations of the reproduction mode. The current thesis described the occurrence of endosymbionts and its importance, distribution, and association with *E*. *americanus*. Molecular examinations ascertained that the thrips species is naturally co-infected with maternally transmitted facultative symbionts. By means of *in situ* hybridization technology the bacterial distribution patterns of these endosymbionts could be documented during the single development stages in this thrips species. Investigations on thrips-symbiont associations identified a beneficial impact on the male production for a naturally occurring endosymbiotic bacterium in *E*. *americanus*. Survival analyses showed that the present endosymbionts have no impact on protection against other bacteria in this thrips species.

7.1 Distribution patterns of *Wolbachia* within the developmental stages

A *Wolbachia* infection is already known in *E*. *americanus* (Kumm et al. 2006, Kumm und Moritz 2008, Chapter 3), whereas the bacteria-dependent distribution patterns and effects were so far undiscovered. Previous studies on thrips show that microorganisms like *Wolbachia*, can be maternally transmitted (Saurav et al. 2016, Nguyen et al. 2017, Ambika and Rajagopal 2018) and occur in the total DNA of different developmental stages (Kaczmarczyk et al. 2017, Kaczmarczyk et al. 2018, Chapter 3). Until now, the bacterium was localized in the abdominal region of larvae and adults of different thrips species (Saurav et al. 2016, Ambika and Rajagopal 2018). Generally, bacterial infections can be generated through a continuous persistence in the female germline and its precursor cells in the embryonic, larval, and adult stages. Another option of symbionts to enter the germline is the transmission via soma cells. Many observations identified *Wolbachia* bacteria in a number of somatic tissues, which were infected though the embryonic development or transfers from cell to cell (reviewed in Pietri et

al. 2016). The bacterium can be present in the germlines of both sexes, whereby the maternal transmission through the female germline seems essential for a transfer to the next host generation (Serbus et al 2008).

Within the scope of the present work, the *Wolbachia* distribution patterns were detected during the development of both sexes in *E*. *americanus*. (Chapter 2). In the larval stages of *E*. *americanus*, the endosymbiont was presented in the neuronal tissue, in the midgut epithelium, the salivary gland, in muscle structures of the legs and in the fat body of the abdomen (Chapter 2). The bacteria were already located in the body segments of later tissues during the embryonic development of this thrips species (Chapter 5). These aspects seem to be associated with the developmental type of thrips and the mode of the *Wolbachia*-dependent distribution. The strong presence of *Wolbachia* in the nervous system in the larval stages (Chapter 2) and the invasion of *Wolbachia* through the embryonic head anlage (Chapter 5) particularly supports this assumption. Furthermore, the *Cardinium* symbiont shows similar distribution patterns in the larval neuronal tissue parts (Chapter 3) and also during the embryonic development of *E*. *americanus* (Chapter 5). Thrips possess a short germ development, which is characterized by a successive development of the body parts. In the process of embryogenesis, the head-thorax complex is formed at first and the abdominal structures develop through the elongation of the germ band in the posterior grow zone (Moritz 1997). Therefore, the bacterial distribution in the head anlage offers an opportunity to enter the embryo and the integration in the nervous system during the early thrips development. Numerous studies document the presence of *Wolbachia* within the embryonic and larval nervous cells (e.g. Dobson et al. 1999, Clark et al. 2005, Albertson et al. 2009, Strunov et al. 2013/2017). Albertson et al. (2009) detected the bacteria between the cells in the early embryonic symmetric and asymmetric segregation of neuroblasts. The occurrence of *Wolbachia* bacteria in the nervous system can have consequences on the hormone availability, physiology, metabolism, and behaviour of insects (Nässel 1993, Arrese and Soulages 2010, Albertson et al. 2013). Furthermore, the *Wolbachia* symbiont was detected in the in the salivary gland and midgut epithelium in this thesis (Chapter 2). During the larval development, the salivary glands move towards the anterior midgut sections (Moritz 2006). This anatomic dislocation is important to obtain the ability as virus vector (e.g. Amin et al. 1981, Wijkamp and Peters 1993). Until now, a function as virus vector couldn't be observed in *E*. *americanus*. *Wolbachia* are known to develop resistances against viral, bacterial, parasitic, and fungal pathogens (Eleftherianos et al. 2013). Consequently, the *Wolbachia* findings during larval salivary gland and midgut development indicate the presence of a protective effect against pathogens. Otherwise, the existence of the symbiont in gland and midgut parts could be also connected with a plant-mediated horizontal transfer. During the feeding process of thrips, the saliva, and its components, finds its way into the plant cells (Miles 1968, Gray and Banerjee

1999). In phytophagous insect species, like *E*. *americanus*, *Wolbachia* bacteria can be transmitted through infected plant material (Li at al. 2017). Based on the anatomic dislocation during larval stages, an infection pathway via the salivary gland to the midgut tissue is possible for the bacterium. However, molecular analyses of the plant material (on which *E*. *americanus* lives) so far show no indications of the *Wolbachia* symbiont and thereby for a potential horizontal transfer (data not shown). Finally, the *Wolbachia* symbiont was detected in the muscles of the legs in the larval development (Chapter 2). Peng et al. (2008) documented an impact on the locomotor activity depending on the host species and *Wolbachia* strain in different *Drosophila* lines. Therefore, it is possible that the distribution of the symbiont in the leg muscles has an effect on the local cell and tissue function of the nervous system, which alter the energy demands of the thrips.

In the subsequent resting stages, the distribution patterns of *Wolbachia* in the muscular body parts alter with the beginning of the metamorphose process in this thrips species (Chapter 2). During pupal development, the bacteria were located only in the muscular parts of the wings. In the later adult stages, the symbiont was additional present in the thoracic muscles of both sexes. This developmental phase causes a vast alteration of the muscle system (Moritz 1997). Thus, the metamorphosis offers an opportunity for the bacteria to transfer to new tissues, and in the case of this work to the thoracic muscular system of the later adult stages. The *Wolbachia* distribution through the musculature within metamorphosis was also detected in the leaf-cutting ant *Acromyrmex octospinosus* (Andersen et al. 2012). After the metamorphose process, *Wolbachia* bacteria were localized in the muscle fibres only in adult workers (Andersen et al. 2012). In addition to this, the symbiont was also detected in different parts of the central brain (dependent on the sex), in the fat body cells of all body parts and in the reproductive tissues of both sexes (see above) in the resting and adult stages (Chapter 2). Especially, the sex-dependent bacterial distribution in the nervous system can have a direct effect on the behaviour of this thrips species. Females exhibit *Wolbachia* accumulations superior to the oesophagus, in the central body of the protocerebrum. This part of the brain is associated with the control of flight, visual memory, and courtship (Hanesch et al. 1989, Zars et al. 2000). Moreover, the females present the symbiont in the basal segments of the antennae in the scape pedicel area. In contrast to that, *Wolbachia* were detected in antennal lobes of the deutocerebrum in males. This area is associated with the scape muscles (movements of the antennae) and signal receiving from a range of antennal sensilla (including perception of pheromones). The occurrence of the endosymbiont in specific areas of the central brain can have various consequences on the host biology. For example, *Wolbachia* are able to alter the sexual behaviour (Miller et al. 2010, Strunov et al. 2017) or the specific pheromone profiles (Kim et al. 2004, Chao et al. 2010). The appearance of the bacterium in the specific areas of the *E*. *americanus* female brain might have a potential effect on the

movements of the female antennae, and also potentially for the courtship and behaviour of the thrips species. Whereas, the specific *Wolbachia* distribution in the male central brain could be associated with the perception of sex-specific pheromones. Thus, the bacterium might have a contribution on the *E*. *americanus* sex behaviour. Furthermore, the *Wolbachia* symbiont was detected in the reproductive tissues of both sexes (Chapter 2). During resting stages, the bacterium was first detected in the developing testes and accessory glands of the males. Afterwards, the symbiont was present in the reproductive tissues of the adult males, which is also known in different *Drosophila* lines (reviewed in Serbus et al. 2008). *Wolbachia* bacteria can not be transmit throughout the male germline (e.g. Riparbelli et al. 2007). The endosymbiont was located in the cyst of postmeiotic spermatids (Clark et al. 2003, Riparbelli et al. 2007) and is later eliminated during sperm development (Bressac et al. 1993, Fuller 1993, Clark et al. 2002). This uneven presence seems effective to induce a cytoplasmic incompatibility (Serbus et al. 2008). In the *E*. *americanus* population of our laboratory culture, the occurrence of a reproductive incompatibility has long been presumed (Kumm and Moritz 2012), because of the female-biased sex ratio (Kumm 2002) and the existing *Wolbachia* infection (Kumm et al. 2006, Kumm und Moritz 2008, Chapter 3). Numerous observations identify that the presence of *Wolbachia* in the reproductive system have an impact on the fitness of their hosts (e.g. O'Neill et al. 1997, Hatcher 2000). *Wolbachia* localized in the reproductive tissues can decrease the fertility of males by influencing sperm production and accessory gland proteins (for example Turelli and Hoffmann 1991, Snook et al. 2000). Therefore, *Wolbachia* occurrences in the male ejaculate can decrease the cytoplasmic incompatibility induction, in consequence of the sperm competition between infected and uninfected males and benefit the infection of females. Hence, the continuous infection of the females possesses an advantage in the transmission of the endosymbiont to the offspring (Champion de Crespigny et al. 2005). In *E*. *americanus* females, the bacterium was located in the germarium, cytoplasm, follicle epithelium and the yolk mass of mature eggs. A detailed documentation and discussion of the *Wolbachia* findings within female germline and the embryonic development is described under 7.3 (Distribution patterns of endosymbionts during oogenesis and embryonic development). *Wolbachia* have developed mechanisms to incorporate into embryonic fate map to infect further somatic target tissues and the germline of the adults. The bacterial distribution patterns during female progenesis and embryogenesis are mostly responsible for the *Wolbachia*-dependent dispersion during the developmental stages (reviewed in Landmann 2019).

7.2 Existence of a coinfection, the impact of the *Cardinium* endosymbiont and the occurrence of a possible cytoplasmic incompatibility

Within the scope of this thesis, a maternally transmitted coinfection with the endosymbionts *Wolbachia* and *Cardinium* was detected in all developmental stages (from egg to adult) of both sexes in *E*. *americanus* (Chapter 3). Our *E*. *americanus* laboratory population has a female-biased sex ratio under normal breeding conditions (1 : 0.3, Kumm 2002), whereas a cytoplasmic incompatibility (CI) has long been suspected in the thrips species (Kumm and Moritz 2012). Normally, in haplodiploid organisms, like thrips, the CI results in male-biased sex ratio through the elimination of the paternally chromosomes (Breeuwer and Werren 1990) or embryonic mortality of fertilized eggs (Vavre et al. 2000). Nguyen et al. (2017) discovered in *Pezothrips kellyanus* a coinfection with *Wolbachia* and *Cardinium*, which induce a complete reproductive incompatibility independent of each other. Both endosymbionts cause the death of the female offspring and result in male development (Nguyen et al. 2017). A further finding of this study was the evidence of a female-biased sex ratio in mating conditions between double infected individuals (Nguyen et al. 2017). Also, other observations on haplodiploid insects identify that the increased amount on females in a population can be the result of a coinfection (e.g. ash whitefly *Encarsia inaron*, Perlman et al. 2006).

Therefore, a further aim of this work was to determine the impact of the endosymbionts, especially of the *Cardinium* bacterium, on the sex ratio of *E*. *americanus*. Crossing experiments detected a beneficial effect on the male production for the *Cardinium* symbiont in *E*. *americanus* (Chapter 4). In the naturally multiple infected population of our laboratory, the symbiont induced an increased male production, if one crossing partner was infected with both symbionts and one with *Wolbachia* (crossing combination: I_{WC} x I_W, I_W x I_{WC}). Moreover, the number of females and unhatched eggs were unaffected of symbiont-induced impact. Similar findings could be discovered for the *Cardinium* endosymbiont in the parasitoid wasp *Encarsia inaron* (White et al. 2009). *Cardinium* increased the number of males through the enhancement of male survivorship. At this, the increased male rate has no effect on the female production in this wasp. Furthermore, White et al. (2009) observed that the additional presence of the *Wolbachia* symbiont in *Cardinium* infected organisms resulted in an absence of this beneficial effect. In contrast to the study of White et al. (2009), a beneficial effect on the male production could only be detected, if one crossing partner had the coinfection and one a single infection with *Wolbachia*. This aspect indicates to a *Cardinium* strain with a rescue function, if both crossing partners are infected with this symbiont.

In this work, a characterization of possible *Cardinium* and *Wolbachia* strain took place (Chapter 4). For the *Cardinium* symbiont of *E*. *americanus*, a high affinity could not be found with the *Cardinium* strain sequences of other insects. By means of the *Wolbachia* strain analyses, a high sequence homology was found in the *Wolbachia pipientis* strain *w*Mel and

the *Wolbachia* sp. *w*Ri complete genome. Another observation on *E*. *americanus* also reported on the presence of *Wolbachia* Mel subgroup (Dong et al. 2012). The study of Zabalou et al. (2018) on *Drosophila simulans* showed no CI induced effect, when both crossing partners had an infection with *w*Mel strain. Likewise, the infection with the *w*Ri strain of both crossing partner resulted in the absence or low level of CI in *D*. *simulans* (Zabalou et al. 2008). Whereby, numerous studies observed diverse levels of CI in natural *Wolbachia* infected insects depending on the bacteria and insect genetic background and male age (Hoffmann 1988, McGraw et al. 2001, Reynolds and Hoffmann 2002, Riegler et al. 2005).

7.3 Distribution patterns of endosymbionts during oogenesis and embryogenesis

Endosymbionts are mainly maternally transmitted via the cytoplasm of the oocytes (Werren and O'Neill 1997, Moran 2008). In the recent years, the vertical transmission of the *Wolbachia* and *Cardinium* symbiont was detected in various thrips species (Saurav et al. 2016, Nguyen et al. 2017, Ambika and Rajagopal 2018), whereas the bacterial dispersion is mostly unknown. For the first time, the current work presents the distribution patterns of *Wolbachia* and *Cardinium* during oogenesis and embryogenesis in *E*. *americanus* (Chapter 5).

In the course of oogenesis, *Wolbachia* and *Cardinium* were primary ascertained in the vitellarium passage (Chapter 5). Both bacteria were located in the nucleus, cytoplasm, follicle epithelium and yolk mass of oocytes in this thrips secies. Additionally, *Wolbachia* bacteria were found few times in the germarium (Chapter 2). Endosymbionts use different paths to enter the germline of insects (reviewed in Russel et al. 2019). The continuous presence of symbionts in the female germline is variable and dependent on the bacteria strain, whereby different strains seem to share various interaction mechanisms (Russel et al. 2019). Symbionts can be transmitted directly with the stem cell division or indirectly from soma cell into the germline (reviewed in Landmann 2019). In the germarium passage the endosymbionts can be present in the stem cells (Serbus et al. 2008) or in the somatic stem cell niche (SSCN) (Frydman et al. 2006). A continuous presence of the symbiont is realized by equally distributed between the self-renewing stem cells and the differentiating daughter cells. Genetic and cellular studies in fruit flies showed that the *Wolbachia* symbiont attained their dynamic dispersion through microtubules associations. The endosymbiont uses the dynein and kinesin motors to move from the anterior end to the posterior pole (Ferree et al. 2005, Serbus and Sullivan 2007). *Wolbachia* bacteria are also known for the usage of the SSCN to contaminate the female germline. All germ cells have contact with this area of the insect germarium. The stem cells in this niche produce the follicle cells, which are associated with the germ cells during the entire oocyte maturation. *Wolbachia* symbionts can accumulate in this niche and are able to infect

the germline directly through the germ cell infection or indirectly through of the follicle cells (Frydman et al. 2006, Pfarr and Hoerauf 2007). Other investigations document that the actin cytoskeleton during later oogenesis is utilized for the distribution within the oocytes and for the transfer to the next host generation (Newton et al. 2015). Furthermore, biochemical experiments on filariae (phylum Nematoda) point to an interaction pattern between the actin cytoskeleton and the *Wolbachia* surface proteins (Melnikow et al. 2013). Alternative transmission routes of endosymbionts (indirectly transfers) via the follicle cells are also known in insects, which are associated with high infection of the follicle cells. During transovarial, endosymbiotic bacteria, housing in the fat body cells or hemolymph of adult females, can utilize the vitellogenin transovarial transportation system to enter the ovaries (e.g. Guo et al. 2018, Herren et al. 2013). For example, microscopic observations on the ovary of *Laodelphax striatellus* suggest that the *Wolbachia* bacteria invade the ovarioles from the tropharium (anterior side) to pedicel side through the nutritive cords (Guo et al. 2018). Otherwise, *Spiroplasma* use the yolk uptake process to incorporate in the germline of *D*. *melanogaster* (Herren et al. 2013). The bacterium passes the oocytes through the intercellular spaces, which surround the ovarian follicle cells. Afterwards the bacterium is endocytosed within the yolk granules during vitellogenic stages of egg maturation (Herren et al. 2013). The findings of the current work support the presumption that an alternative transmission route via the transovarial transport mechanism of vitellogenin is possible in *E*. *americanus* (indirectly transmission through soma cells into the germline). Bacterial occurrences in the ovary of this thrips show a dominant distribution of the *Wolbachia* and *Cardinium* symbiont in the vitellogenic stages of the oogenesis. In this developmental phase, the endosymbionts were conspicuously strongly distributed in the follicle cells and yolk mass of mature eggs. The accumulated bacteria within yolk mass were non-homogeneously distributed or more towards the centre of the egg. Further investigations on the thrips species *H*. *femoralis* showed similar distribution patterns for the *Wolbachia* symbiont during this oogenesis stage (Chapter 5). Moreover, *E*. *americanus* (Chapter 2) and *H*. *femoralis* (unpublished data) show strong clusters of *Wolbachia* in the female fat body (area of vitellogenin synthesis). Thus, a transfer of both symbionts via the vitellogenin transport system seems to be possible and may explain the intensified bacterial occurrence with increasing egg maturation in the thrips. However, in this work a potential transmission path via the stem cells was detected few times for the *Wolbachia* endosymbiont in *E*. *americanus* (Chapter 2), therefore it cannot be ruled out that both symbionts use this infection also.

Endosymbiont transmissions to the next generation can vary through different parameters: the type of transfer partner, the developmental stage of transmission and type of offspring, the mode of translocation and the localization in the egg (reviewed in Bright and Bulgheresi 2010). Many studies observed that endosymbionts, like *Wolbachia*, are integrated into the female

germline and primarily located in the germ plasm of the embryo (Breeuwer and Werren 1990, Stouthamer et al. 1993, Zchori-Fein et al. 1998, Hadfield and Axton 1999, Pintureau et al. 2000). In this work, a definitive localization of both symbionts couldn't be documented during early embryonic development (Chapter 5). The symbionts appeared as single proofs in the yolk filled egg system, mainly in the periphery. After the elongation of the germ ligament, both bacteria were primary detected in the head anlage of the embryos and additionally in the individuals surrounding yolk mass. With the beginning of the segmentation process, the bacteria were distributed in the thorax and abdomen. It is already known that symbionts enter into egg structures during egg development and can later be distributed within cells of the embryo before gastrulation, whereby the bacterial dispersion depends on a combination of insect and symbiont factors (Veneti et al. 2004, Pietri et al. 2016). The study of Veneti et al. (2004) identified a homogenous distribution throughout the cortex of the embryo for the *Wolbachia* *w*Ri strain in different *Drosophila* lines (*D. simulans*, *D*. *yakuba*, *D*. *teissieri*, *D*. *santomea*). Moreover, the evenly located bacteria were mainly distributed in the follicle cells during egg maturation (Veneti et al. 2004). Other observations found the cytoplasmic bacteria in the cortical layer of early *D*. *melanogaster* embryos and scattered in the yolk regions of deposited eggs (Callaini et al. 1994). The results of the present thesis also indicate a cortical distribution of both endosymbionts, which initially begins in the head anlage of the embryo. The bacteria could be transmitted through the gradual elongation of the germ band to the later abdominal segments. Moreover, the development of thrips seems related with the same bacterial dispersion patterns. Likewise, further investigations to the *Wolbachia* occurrence during embryogenesis in *H*. *femoralis* show similar distribution patterns (Chapter 5). This phenomenon may be explained with the short germ development of thrips. The embryonic development of the most thrips species starts after egg deposition. A uniform plasmodial preblastoderm is formed through the penetration of the cleavage energids into periplasma (Moritz 1997). With the end of the cell differentiations, the blastoderm is divided into embryonic and extra-embryonic parts through asynchronous mitotic activities. The further development of thrips is characterized through different events: the first invagination of embryonic cells into the yolk and the process of anatrepsis with the result of an S-shaped germ band elongation (Moritz 1997). Consequently, the distribution of both symbionts seems associated with the early bacterial invagination in the cortical layers of the shaped head-thorax anlage during the embryonic development of *E*. *americanus*. The outcomes indicate along with further examinations on *H*. *femoralis* to a consistent bacterial dispersion during the embryogenesis in thrips. However, it must be considered that in this context other observations point to control factors of both host and symbiont (Veneti et al. 2004).

7.4 The entomopathogenic bacterium *P. luminescens* impacts the survival rate

The outcomes of this work identified an impact of *P. luminescens* on the survival rate of *E. americanus* (Chapter 6). Significant difference could be detected in the survival rate between the *P. luminescens* and the control group. *E. americanus* females which ingested the bacteria liquid culture (for 24 h) possessed a 68% mortality rate after 48 h, whereas the survival rate in the control group remained 100%. The consequences of the entomopathogenic bacterium was also test in other thrips females (*F. occidentalis*, *H. femoralis* and *T. tabaci*) with different food spectrum, reproduction mode and presence of endosymbionts. Compared to *E. americanus*, the other tested thrips females showed no significant difference in survival probability of the *P. luminescens* and control group (Chapter 6). The impact of *P. luminescens* could be already detected in other studies of thrips (Gerritsen et al. 2004, Gerritsen et al. 2005, Uma et al. 2010). For example, Gerritsen et al. (2004) documented an effective oral toxicity to *Frankliniella occidentalis* for the *P. luminescens* P2 strain, which resulted in a mortality rate of less than 50% after 7d. In *Thrips palmi*, the *P. luminescens* symbiont of *Heterorhabditis indica* caused a high mortality within 24 h to 48 h after treatment with this bacterium (Uma et al. 2010). Generally, *P. luminescens* possesses the ability to overcome the cellular and humoral immune response of the target insect (Vallet-Gely et al. 2008, Nielsen-LeRoux et al. 2012). Especially, the toxin complexes of the bacterium play an important role in the oral and injectable toxic activity on insects (Blackburn et al. 2005). Furthermore, the entomopathogenic bacterium is able to use a specific niche during the midgut colonization (Silva et al. 2002). Within the niche, the bacterium produces a toxin and protease, which lead to programmed death of the midgut epithelium cells (Silva et al. 2002).

To verify the bacterial distribution patterns within the *E. americanus* thrips body, after the 24 h application of the bacteria liquid culture, an *in situ* hybridisation was used (primers for the 16S rDNA was used, which detected a great number of eubacteria, Chapter 6). The results ascertained a dispersion of bacteria mainly within the midgut epithelium, but also in the neuronal tissue, in the thoracic muscles and gonads of the *E. americanus* females. Also, the investigations in all other tested species of this work showed similar bacterial distribution patterns in the female thrips bodies (Chapter 6). Based on these findings, it seems that *P. luminescens* overcomes the natural barrier within the midgut to enter the hemolymph system and distribute via hemolymph system to other tissue parts of the thrips body. This infection way is known for other entomopathogenic bacteria (Nehme et al. 2007). However, it should be noted that the used primers for the 16S rDNA detected a great number of eubacteria (Lane 1991, Weisburg et al. 1991). Furthermore, females without *P. luminescens* application (control group) showed also a few appearances of bacteria in the intestinal tract and reproductive system in *E. americanus* and in all other tested thrips species (unpublished data). Thus, it cannot be ruled out that the detected microorganisms belongs to other bacteria

species, because thrips like *F*. *occidentalis*, naturally harboured a broad spectrum of symbiotic bacteria (de Vries et al. 2004, Chanbusarakum and Ullman 2009).

Endosymbionts like *Wolbachia* and *Cardinium*, are able to protect their host against natural enemies. The symbionts can have a positive influence on specific characteristics or increase the fitness (Lively et al. 2005, Jones et al. 2007). Moreover, the bacteria have been shown defends their host against bacteria and viruses (Davidson et al. 2001, Lopanik et al. 2004, Brownlie and Johnson 2009, Eleftherianos et al. 2013). In this work, a toxic impact of the entomopathogenic bacterium *P*. *luminescens* on the survival rate could be only documented in the co-infected *E*. *americanus* (*Cardinium* and *Wolbachia* coinfection, Chapter 3), in contrast to other endosymbiont-infected (*H*. *femoralis*, *Wolbachia* infection, Pintureau et al. 1999) and uninfected thrips species (*Frankliniella occidentalis* and *Thrips tabaci*, Kumm and Moritz 2008). The occurrence of *Wolbachia* and *Cardinium* seems not to have an impact on the survival rate in *E*. *americanus*. Shokal et al. (2016) observed that the *P*. *luminescens* infection in *Drosophila melanogaster* leads to death of all infected lines within 24 h, independent of an endosymbiont presence (single and co-infected individuals). The results of this work and the findings of other thrips studies, indicate that the toxic impact of *P*. *luminescens* against thrips dependents on bacterial strain characteristics and the target thrips species. Furthermore, the presence of endosymbionts seems to be independent of the *P*. *luminescens* success, although a negative effect based on the coinfection cannot be ruled out.

7.5 References

Albertson, R., C. Casper-Lindley, J. Cao, U. Tram, and W. Sullivan. 2009. Symmetric and asymmetric mitotic segregation patterns influence *Wolbachia* distribution in host somatic tissue. J Cell Sci 122: 4570–4583.

Albertson, R., V. Tan, R.R. Leads, M. Reyes, W. Sullivan, and C. Casper-Lindley. 2013. Mapping *Wolbachia* distributions in the adult *Drosophila* brain. Cell Microbiol 15: 1527–1544.

Ambika, S., and R. Rajagopal. 2018. Lumen anatomy and localization of *Wolbachia* sp. in the thrips, *Plicothrips apicalis* (Bagnall). Curr Sci 115: 1297–1304.

Amin, P.W., D.V.R. Reddy, and A.M. Ghanekar. 1981. Transmission of tomato spotted wilt virus, causal agent of bud necrosis of peanut, by *Scirtothrips dorsalis* and *Frankliniella schultzei*. Plant Disease 65: 663–665.

Andersen, S.B., M. Boye, D.R. Nash, and J.J. Boomsma. 2012. Dynamic *Wolbachia* prevalence in *Acromyrmex* leaf-cutting ants: potential for nutritional symbiosis. J Evol Biol 25: 1340–1350.

Arrese, E.L., and J.L. Soulages. 2010. Insect fat body: Energy, metabolism and regulation. Annu Rev Entomol 55: 207–255.

Blackburn, M.B., J.M. Domek, D.B. Gelman, and J.S. Hu. 2005. The broadly insecticidal *Photorhabdus luminescens* toxin complex a (Tca): Activity against the Colorado potato beetle, *Leptinotarsa decemlineata*, and sweet potato whitefly, *Bemisia tabaci*. J Insect Sci 5: 32.

Breeuwer, J.A., and J.H. Werren. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature 346: 558–560.

Bressac, C., and F. Rousset. 1993. The reproductive incompatibility system in *Drosophila simulans*: DAPI-staining analysis of the *Wolbachia* symbionts in sperm cysts. J Invertebr Pathol 61: 226–230.

Bright, M., and S. Bulgheresi. 2010. A complex journey: transmission of microbial symbionts. Nat Rev Microbiol 8: 218–230.

Brownlie, J.C., and K.N. Johnson. 2009. Symbiont-mediated protection in insect hosts. Trends Microbiol 17: 348–354.

Callaini, G., M.G. Riparbelli, and R. Dallai. 1994. The distribution of cytoplasmic bacteria in the early *Drosophila* embryo is mediated by astral microtubules. J Cell Sci 107: 673–682.

Champion de Crespigny, F.E., R.K. Butlin, and N. Wedell. 2005. Can cytoplasmic incompatibility inducing *Wolbachia* promote the evolution of mate preferences? J Evol Biol 18: 967–977.

Chanbusarakum, L.J., and D.E. Ullman. 2009. Distribution and ecology of *Frankliniella occidentalis* (Thysanoptera: Thripidae) bacterial symbionts. Environ Entomol 38: 1069–1077.

Chao, T.H., L. Ehrman, A. Permaul, R. Vincent, L. Sattaur, and D. Brandt. 2010. Male-specific cuticular compounds of six *Drosophila paulistorum* semispecies: structural identification and mating effect. J Chem Ecol 36: 933–942.

Clark, M.E., Z. Veneti, K. Bourtzis, and T.L. Karr. 2002. The distribution and proliferation of the intracellular bacteria *Wolbachia* during spermatogenesis in *Drosophila*. Mech Dev 111: 3–15.

Clark, M.E., Z. Veneti, K. Bourtzis, and T.L. Karr. 2003. *Wolbachia* distribution and cytoplasmic incompatibility during sperm development: the cyst as the basic cellular unit of CI expression. Mech Dev 120: 185–98.

Clark, M.E., C.L. Anderson, J. Cande, and T.L. Karr. 2005. Widespread prevalence of *Wolbachia* in laboratory stocks and the implications for *Drosophila* research. Genetics 170: 1667–1675.

Davidson, S.K., S.W. Allen, G.E. Lim, C.M. Anderson, and M.G. Haygood. 2001. Evidence for the biosynthesis of bryostatins by the bacterial symbiont 'Candidatus Endobugula sertula' of the bryozoan *Bugula neritina*. Appl Environ Microbiol 67: 4531–4537.

de Vries, E.J., G. Jacobs, M.W. Sabelis, S.B.J. Menken, and J.A.J. Breeuwer. 2004. Diet-dependent effects of gut bacteria on their insect host: the symbiosis of *Erwinia* sp. and western flower thrips. Proc R Soc Lond B 271: 2171–2178.

Dobson, S.L., K. Bourtzis, H.R. Braig, B.F. Jones, W. Zhou, and et al. 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem Mol Bio 29: 153–160.

Dong, J., X.W. Li, X.C. Zhang, and J.N. Feng. 2012. Sequencing and phylogenetic analysis of the *wsp* gene of *Wolbachia* in *Echinothrips americanus* Morgan. Journal of Northwest A & F University 3: 131–134.

Eleftherianos, I., J. Atri, J. Accetta, and J.C. Castillo. 2013. Endosymbiotic bacteria in insects: guardians of the immune system? Front Physiol. 4: 46.

Ferree, P.M., H.M. Frydman, J.M. Li, J. Cao, E. Wieschaus, and W. Sullivan. 2005. *Wolbachia* utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte. PLoS Pathog 1: e14.

Fuller, M.T. 1993. Spermatogenesis, pp. 71–147. In M. Bate, and A.M. Arias (eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbor Lab. Press, New York.

Frydman, H.M., J.M. Li, D.N. Robson, and E. Wieschaus. 2006. Somatic stem cell niche tropism in *Wolbachia*. Nature 441: 509–512.

Gerritsen, L.J.M., J. Georgieva, and G.L. Wiegers. 2004. Oral toxicity of bacterial toxins against thrips species. Pest Control 15: 75–80.

Gerritsen, L.J.M., J. Georgieva, and G.L. Wiegers. 2005. Oral toxicity of *Photorhabdus* toxins against thrips species. J Invertebr Pathol 88: 207–211.

Gray, S.M., and N. Banerjee. 1999. Mechanisms of arthropod transmission of plant and animal viruses. Microbio Mol Bio Rev 63: 128–148.

Guo, Y., A.A. Hoffmann, X.-Q. Xu, P.-W. Mo, H.-J. Huang, and et al. 2018. Vertical transmission of *Wolbachia* is associated with host vitellogenin in *Laodelphax striatellus*. Front Microbiol 9: 2016.

Hadfield, S.J., and J.M. Axton. 1999. Germ cells colonized by endosymbiotic bacteria. Nature 402: 482.

Hanesch, U., K.F. Fischbach, and M. Heisenberg. 1989. Neuronal architecture of the central complex in *Drosophila melanogaster*. Cell Tissue Res 257: 343–366.

Hatcher, M.J. 2000. Persistence of selfish genetic elements: population structure and conflict. Trends Ecol Evol 15: 271–277.

Herren, J.K., J.C. Paredes, F. Schüpfer, and B. Lemaitre. 2013. Vertical transmission of a *Drosophila* endosymbiont via cooption of the yolk transport and internalization machinery. mBio 4:e00532-12.

Hoffmann, A.A. 1988. Partial cytoplasmic incompatibility between two Australian populations of *Drosophila melanogaster*. Entomol Exp Appl 48: 61–67.

Jones, E.O., A. White, and M. Boots. 2007. Interference and the persistence of vertically transmitted parasites. J Theor Biol 246: 10–17.

Kaczmarczyk, A., H. Kucharczyk, M. Kucharczyk, and S. Zielińska. 2017. First report of *Wolbachia* in arrhenotokous populations of mycophagous species *Hoplothrips pedicularius* (Haliday 1836) (Thysanoptera, Phlaeothripidae). 5th Symposium on Palaearctic Thysanoptera, Cracow, Poland, 24

Kaczmarczyk, A., H. Kucharczyk, M. Kucharczyk, P. Kapusta, J. Sell, and S. Zielińska. 2018. First insight into microbiome profile of fungivorous thrips *Hoplothrips carpathicus* (Insecta: Thysanoptera) at different developmental stages: molecular evidence of *Wolbachia* endosymbiosis. Sci Rep 8:14376.

Kim, Y.K., D.R. Phillips, T. Chao, and L. Ehrman. 2004. Developmental isolation and subsequent adult behaviour of *Drosophila paulistorum*. VI. Quantitative variation in cuticular hydrocarbons. Behav Genet 34: 385–394.

Kumm, S. 2002. Reproduction, progenesis and embryogenesis of thrips (Thysanoptera, Insecta). Ph.D. dissertation. Martin-Luther-University Halle-Wittenberg, Halle.

Kumm. S., and G. Moritz. 2008. First detection of *Wolbachia* in arrhenotokous populations of thrips species (Thysanoptera: Thripidae and Phlaeothripidae) and its role in reproduction. Environ Entomol 37: 1422–1428.

Kumm S., and G. Moritz. 2012. *Wolbachia* in arrhenotokous thrips species. Julius-Kühn-Archiv 438: 125–126.

Kumm, S., R. Kranz, and G. Moritz. 2006. Mikroorganismen-gesteuerte Parthenogenese bei Thysanopteren. Mitt Dtsch Ges allg angew Ent 15: 153–156.

Landmann, F. 2019. The *Wolbachia* endosymbionts. Microbiol Spectrum 7: BAI-0018-2019.

Lane, D.J. 1991. 16s/23s RNA sequencing, pp. 115–176. In E. Stackebrandt, and M. Goodfellow (eds.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd, Baffins Lane, Chichester, West Sussex PO19 1UD, England.

Li, S.J., M.Z. Ahmed, N. Lv, P.Q. Shi, X.M. Wang, and et al. 2017. Plant-mediated horizontal transmission of *Wolbachia* between whiteflies. ISME J 11: 1019–1028.

Lively, C.M., K. Clay, M.J. Wade, and C. Fuqua. 2005. Competitive co-existence of vertically and horizontally transmitted parasites. Evol Ecol Res 7: 1183–1190.

Lopanik, N., N. Lindquist, and N. Targett. 2004. Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. Oecologia 139: 131–139.

McGraw, E.A., D.J. Merritt, J.N. Droller, and S.L. O'Neill. 2001. *Wolbachia*-mediated sperm modification is dependent on the host genotype in *Drosophila*. Proc R Soc Lond Ser B Biol Sci 268: 2565–2570.

Melnikow, E., S. Xu, J. Liu, A.J. Bell, E. Ghedin, and et al. 2013. A potential role for the interaction of *Wolbachia* surface proteins with the *Brugia malayi* glycolytic enzymes and cytoskeleton in maintenance of endosymbiosis. PLoS Negl Trop Dis 7: e2151.

Miles, P.W. 1968. Insect secretions in plants. Annu Rev Phytopathol 6: 137–164.

Miller, W.J., L. Ehrman, and D. Schneider. 2010. Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. PLoS Pathog 6e1001214.

Moran, N.A., J.P. McCutcheon, and A. Nakabachi. 2008. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42: 165–190.

Moritz, G. 1997. Structure, growth and development. In T. Lewis (ed.), Thrips as crop pests. CAB International, Oxon and New York, USA.

Moritz, G. 2006. Thripse Fransenflügler, Thysanoptera. Die neue Brehm-Bücherei, Westarp Wissenschaft, Hohenwarsleben.

Nässel, D. 1993. Neuropeptides in the insect brain: a review. Cell Tissue Res 273: 1–29.

Nehme, N.T., S. Liégeois, B. Kele, P. Giammarinaro, E. Pradel, and et al. 2007. A model of bacterial intestinal infections in *Drosophila melanogaster*. PLOS Pathog 3: e173.

Newton, I.L.G., O. Savytskyy, and K.B. Sheehan. 2015. *Wolbachia* utilize host actin for efficient maternal transmission in *Drosophila melanogaster*. PLoS Pathog 11: e1004798.

Nielsen-LeRoux, C., S. Gaudriault, N. Ramarao, D. Lereclus, and A. Givaudan. 2012. How the insect pathogen bacteria *Bacillus thuringiensis* and *Xenorhabdus/Photorhabdus* occupy their hosts. Curr Opin Microbiol 15: 220–231.

Nguyen, D.T., J.L. Morrow, R.N. Spooner-Hart, and M. Riegler. 2017. Independent cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* maintains endosymbiont coinfections in haplodiploid thrips populations. Evolution 71: 995–1008.

O'Neill, S.L., A.A. Hoffmann, and J.H. Werren. 1997. Influential passengers inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford, UK.

Peng, Y., J.E. Nielsen, J.P. Cunningham, and E.A. McGraw. 2008. *Wolbachia* infection alters olfactory-cued locomotion in *Drosophila* spp. Appl Environ Microbiol 74: 3943–3948.

Perlman, S.J., S.E. Kelly, E. Zchori-Fein, and M.S. Hunter. 2006. Cytoplasmic incompatibility and multiple symbiont infection in the ash whitefly parasitoid, *Encarsia inaron*. Biol Control 39: 474–480.

Pfarr, K.M., and A. Hoerauf. 2007. A niche for Wolbachia. Trends Parasitol 23: 5–7.

Pietri, J.E., H. DeBruhl, and W. Sullivan. 2016. The rich somatic life of *Wolbachia*. Microbiologyopen 5: 923–936.

Pintureau, B., F. Lassablière, C. Khatchadourian, and J. Daumal. 1999. Parasitoïdes oophages et symbiotes de deux thrips europeens. Ann Soc Entomol Fr 35: 416–420.

Pintureau, B., S. Grenier, B. Boleat, F. Lassabliere, A. Heddi, and C. Khatchadourian. 2000. Dynamics of *Wolbachia* populations in transfected lines of *Trichogramma*. J Invertebr Pathol 76: 20–25.

Reynolds, K.T., and A.A. Hoffmann. 2002. Male age and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally-transmitted *Wolbachia*. Genet Res 80: 79–87.

Riegler, M., M. Sidhu, W.J. Miller, and S.L. O'Neill. 2005. Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. Curr Biol 15: 1428–1433.

Riparbelli, M.G., R. Giordano, and G. Callaini. 2007. Effects of *Wolbachia* on sperm maturation and architecture in *Drosophila simulans* Riverside. Mech Dev 124: 699–714.

Russell, S.L., L. Chappell, and W. Sullivan. 2019. A symbiont's guide to the germline. Curr Top Dev Biol 135: 315–351.

Saurav, G.K., G. Daimei, V.S. Rana, S. Popli S, and R. Rajagopal. 2016. Detection and Localization of *Wolbachia* in *Thrips palmi* Karny (Thysanoptera: Thripidae). Indian J Microbiol 56: 167–171.

Serbus, L.R., and W. Sullivan. 2007. A cellular basis for *Wolbachia* recruitment to the host germline. PLoS Pathog 3:1930–1937.

Serbus, L.R., C. Casper-Lindley, F. Landmann, and W. Sullivan. 2008. The genetics and cell biology of *Wolbachia*-host interactions. Annu Rev Genet 42: 683–707.

Snook, R.R., S.Y. Cleland, M.F. Wolfner, and T.L. Karr. 2000. Offsetting effects of *Wolbachia* infection and heat shock on sperm production in *Drosophila simulans*: Analyses of fecundity, fertility and accessory gland proteins. Genetics 155: 167–178.

Shokal, U., S. Yadav, J. Atri, J. Accetta, E. Kenney, and et al. 2016. Effects of co-occurring *Wolbachia* and *Spiroplasma* endosymbionts on the *Drosophila* immune response against insect pathogenic and non-pathogenic bacteria. BMC Microbiol 16: 16.

Silva, C.P., N.R. Waterfield, P.J. Daborn, P. Dean, T. Chilver, and et al. 2002. Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. Cell Microbiol 4: 329–339.

Stouthamer, R., J.A.J. Breeuwer, R.F. Luck, and J.H. Werren. 1993. Molecular identification of microorganisms associated with parthenogenesis. Nature 361: 66–68.

Strunov, A., E. Kiseleva, and Y. Gottlieb. 2013. Spatial and temporal distribution of pathogenic *Wolbachia* strain *w*MelPop in *Drosophila melanogaster* central nervous system under different temperature conditions. J Invertebr Pathol 114: 22–30.

Strunov, A., D.I. Schneider, R. Albertson, and W.J. Miller. 2017. Restricted distribution and lateralization of mutualistic *Wolbachia* in the *Drosophila* brain. Cell Microbiol 19: e12639.

Turelli, M., and A.A. Hoffmann. 1991. Rapid spread of an inherited incompatibility factor in California *Drosophila*. Nature 353: 440–442.

Uma, G.P., A. Prabhuraj, and Y. Vimala. 2010. Bioefficacy of *Photorhabdus luminescens*, a symbiotic bacterium against *Thrips palmi* Karny (Thripidae: Thysanoptera). JBiopest 3: 458–462.

Vallet-Gely, I., B. Lemaitre, and F. Boccard. 2008. Bacterial strategies to overcome insect defences. Nat Rev Microbiol 6: 302–313.

Vavre, F., F. Fleury, J. Varaldi, P. Fouillet, and M. Bouletreau. 2000. Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: epidemiologic and evolutionary consequences. Evolution 54: 191–200.

Veneti, Z., M.E. Clark, T.L. Karr, C. Savakis, and K. Bourtzis. 2004. Heads or tails: host-parasite interactions in the Drosophila-*Wolbachia* system. Appl Environ Microbiol 70: 5366-5372.

Weisburg, W.G., S.M. Barns, D.A. Pelletier, and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697–703.

Werren, J.H., and S.L. O'Neill. 1997. The evolution of heritable symbionts, pp 1–41. In S.L. O'Neill, A.A. Hoffmann, and J.H. Werren (eds.), Influential Passengers, Oxford University Press, New York, NY, USA.

White, J.A., S.E. Kelly, S.J. Perlman, and M.S. Hunter. 2009. Cytoplasmic incompatibility in the parasitic wasp *Encarsia inaron:* disentangling the roles of *Cardinium* and *Wolbachia* symbionts. Heredity 102: 483–489.

Wijkamp, I., and D. Peters. 1993. Determination of the median latent period of two tospoviruses in *Frankliniella occidentalis*, using a novel leaf disk assay. Phytopathology 83: 986–991.

Zabalou, S., A. Apostolaki, S. Pattas, Z Veneti, C. Paraskevopoulos, and et al. 2008. Multiple rescue factors within a *Wolbachia* strain. Genetics 178: 2145–2160.

Zars, T., M. Fischer, R. Schulz, and M. Heisenberg. 2000. Localization of a short-term memory in *Drosophila*. Science 288: 672–675.

Zchori-Fein, E., R.T. Roush, and D. Rosen. 1998. Distribution of parthenogenesis-inducing symbionts in ovaries and embryos of *Aphytis* (Hymentoptera: Aphelinidae). Curr Microbiol 36: 1–8.

CHAPTER 8

Summary

Thrips are micro-insects with a high impact on the agriculture. The plant sap-sucking insects possess a rapid formation of resistances against biological and chemical components, so that thrips can be responsible for serious economic and ecological damage on ornamental and culture plants. So far, for the biological control of thrips were found assassin bugs, parasitic wasps, and a few predatory species of thrips. These control agents are mainly efficient in greenhouses. In addition to it, some thrips species, like *E*. *americanus*, are infected with one or several microorganisms, which can have a broad spectrum of effects on their hosts. For example, symbiotic microorganisms are able to affect the immune response of their hosts against natural enemies. Symbiont-insect associations may directly or indirectly influence the host biology. In this thesis, the effects and distribution patterns of endosymbionts and other bacteria were investigated in economically relevant thrips species. At this, in the centre of this investigations stood the thrips, *Echinothrips americanus*, a new potential pest in European greenhouses.

Distribution patterns of the *Wolbachia* endosymbiont during development

The *Wolbachia* distribution patterns were ascertained in the developmental stages (larvae, resting stages and adults of both sexes) of *E*. *americanus*, whereby the bacterial dispersion changes during the development of this thrips species. In the larval stages, the *Wolbachia* bacteria were distributed in the complete nervous system (supraoesophageal ganglion), in the salivary gland, in the intestinal epithelium of the midgut, in the muscular parts of the legs and in the fat body of the abdomen of both sexes. The female and male propupae showed bacterial occurrences in different neuronal tissue parts, especially in the protocerebrum (mushroom bodies), in the tritocerebrum and metathoracic ganglion. Furthermore, *Wolbachia* clusters were proven in the fat body cells of all body parts (head, thorax, abdomen) and in the developing wings. Sex-dependent bacterial distribution patterns were ascertained during the resting stages. Female propupae presented *Wolbachia* bacteria in the antennae and central complex of the protocerebrum. Male propupae showed bacterial clusters in the deutocerebrum, in the testes and accessory glands. In addition to the mentioned occurrences during resting stages, the *Wolbachia* was detected in thoracic muscles and midgut epithelium in the adult stages of both sexes. Moreover, *Wolbachia* bacteria were located in the ovaries of adult females,

particularly in the germarium, in the follicular epithelial cells and in the yolk mass of mature eggs.

Coinfection

Molecular investigations (*wsp* gene, *ftsZ* gene, 16S rDNA) determined the existence of a double infection with *Wolbachia* and *Cardinium* in *E*. *americanus*. The coinfection with that endosymbionts could be detected for all developmental stages of both sexes (from the egg to the adult). Furthermore, the maternal transmission of the bacteria was also proven by use of *in situ* hybridization technique. *Wolbachia* and *Cardinium* clusters were located in the yolk mass of mature oocytes just before oviposition. In addition, bacterial appearances of both symbionts could be ascertained in the prolarvae.

Impact of the *Cardinium* endosymbiont on the sex ratio

The impact of the *Cardinium* endosymbiont on the sex ratio of the double infected *E*. *americanus* (I_{WC} x I_{WC}, natural infection status) was investigated by crossing experiments. For the *Cardinium* symbiont a beneficial effect was detected on the male production. The analysis showed that the number of males increased significant in crossing combinations, where only one crossing partner was infected with the *Cardinium* bacterium (I_W x I_{WC}, 38.78% male amount; I_{WC} x I_W, 40.79% male amount) as compared to crosses, in which both crossing partner own a double infection (I_{WC} x I_{WC}, control group, 27.33% male amount). An embryonic mortality of fertilized eggs couldn't be detected in the tested crossing combinations (number of unhatched eggs). Also, the female production was unaffected through the symbiont-induced effect (number of females). The results of the crossing experiments pointed to a *Cardinium* strain (or strains), which can rescue his own modification, if the bacterium is present in both crossing partners. For the *Wolbachia* endosymbiont, sequence analysis showed high identity homology to the *Wolbachia pipientis* strain *w*Mel and *Wolbachia* sp. strain *w*Ri for both females and males of *E*. *americanus* (identity homology > 99%).

Distribution patterns of endosymbionts during oogenesis and embryogenesis

The distribution patterns of the endosymbionts *Wolbachia* and *Cardinium* during oogenesis and embryonic development of *E*. *americanus* and *H*. *femoralis* were determined by electron-microscopic examinations and *in situ* hybridization technique. Bacterial structures were localized in the oocytes during vitellarium in both organisms by electron microscopy. The bacteria were distributed in the cytoplasm and follicular epithelial cells of developing oocytes, and also in the yolk mass of mature eggs for the oviposition. Through *in situ* hybridization technique a characteristic distribution pattern was ascertained for the different endosymbiotic bacteria in both thrips species. *E*. *americanus* possesses a coinfection with *Wolbachia* and

Cardinium bacteria. The endosymbionts were localized during the early oogenesis (vitellarium stage) in the nucleus, the cytoplasm and in the follicular epithelial cells. In the later phase of vitellarium bacterial occurrences of both symbionts were detected in the transition area between the single oocytes (follicle cells) and in the yolk mass of vitellogenic eggs. The endosymbionts were distributed in all developmental phases of the embryogenesis. During early development, the bacteria were inhomogeneous distributed in the periphery of the yolk mass (1 d ± 12 h old egg). After the germ band elongation, the symbionts were mainly located in the developing head anlage (4 d ± 12 h old embryo). In the course of the further development the bacteria were detected in the cephalic, thoracic and abdominal segments of the embryos (7 d ± 12 old embryo). In addition, the symbionts were located in the appendages, especially in the antennae and legs, and in the amnion-serosa complex of the developing prolarva. The thrips species *H*. *femoralis* possesses an infection with the *Wolbachia* bacterium. For the *Wolbachia* symbiont, identical distribution patterns to *E*. *americanus* could be ascertained during oogenesis of this panchaetothripine, only the bacterial occurrences in the cytoplasm of oocytes and in the follicle cells between single oocytes could not be detected. During embryogenesis, the bacteria could be detected in all developmental phases. At the beginning of the embryonic development, the *Wolbachia* symbionts was distributed uneven in the yolk mass, more towards the periphery of the egg (egg 3 d ± 12 h). After the formation of the germ ligament, the bacterium was found in the later cephalic and thoracic structures of the germ anlage and also in the surrounding yolk mass of the 4 d ± 12 h old embryo. In the course of the development, the bacterium was found in the neuronal tissue, in the antennae, in the thoracic and abdominal segments and in the extraembryonic tissues (amnion-serosa complex).

The toxic impact of *Photorhabdus luminescens* on thrips

The toxic impact of the bacterium *P*. *luminescens* on the survival rate were investigated on *E*. *americanus*, *F*. *occidentalis*, *H*. *femoralis* and *T*. *tabaci*. For the analysis, all tested thripine species ingested an application solution (composed of 75 µl sucrose solution (10%), 50 µl patent blue V solution (4%) and 75 µl *P*. *luminescens* liquid culture) for 24 hours. The control group got the same solution, but without the bacteria in the grow medium. The viability was checked daily to find out if *P. luminescens* has a direct effect on the survival rate of the used thrips species. The statistical evaluations identified a significant variation on the survival rate between the *P*. *luminescens* and control group of *E*. *americanus*. Interestingly, *E*. *americanus* possesses a bacterial infection with the immunoregulating bacteria *Wolbachia* and *Cardinium*, whereby the coinfection exhibits no protective effect on the survival rate of this thrips. In contrast, the other tested thrips species show no significant difference in probability of survival between the group with *P*. *luminescens* application and the control group. For the possible

localization of this bacterium within the thrips body parts, an *in situ* hybridization technique was used (16S rDNA, primers fD1/1492r). In all thrips species bacterial DNA could prevalent detected in the intestinal epithelium of the gut tract (mainly in the midgut), but also in the thoracic muscles, gonads, and neuronal tissue of the females.

Conclusion

The main purpose of this work was to investigate the importance of endosymbiont-thrips-interactions. An important point was the determination of the symbiont-dependent distribution patterns in ecological relevant thrips species. By means of *in situ* hybridization technique, a fundamental overview about bacterial distribution, especially for *Wolbachia* bacteria, could be documented during the development of thrips. A further objective was the analysis of endosymbiont and/or bacteria effects. The current thesis ascertained endosymbiont-dependent regulation patterns on the sex ratio in the naturally double infected *E*. *americanus*. Moreover, survival analyses to the toxic impact of the entomopathogenic bacterium *P*. *luminescens* showed that the influence of the microorganism is dependent on the target insect. Finally, this work ascertains a variety of interesting association aspects in the endosymbiont-thrips ecosystem and offers potential for further research.

Zusammenfassung

Thripse sind Mikroinsekten mit einem hohen Einfluss auf die Landwirtschaft. Die pflanzensaft-saugenden Insekten weisen eine schnelle Resistenzbildung gegen biologische und chemische Komponenten auf, sodass Thripse für schwerwiegende ökonomische und ökologische Schäden an Zier- und Kulturpflanzen verantwortlich sein können. Zur biologischen Bekämpfung der Thripse wurden bisher Raubwanzen, parasitäre Wespen und einige wenige räuberische Thrips-Arten gefunden. Diese Gegenspieler sind vor allem in Gewächshäusern effizient. Darüber hinaus sind einige Thrips-Arten, wie *E*. *americanus*, mit einem oder mehreren Mikroorganismen infiziert, welche ein breites Spektrum an Effekten auf ihre Wirte haben können. Beispielsweise sind symbiontische Mikroorganismen in der Lage die Immunantwort ihrer Wirte gegen natürliche Feinde zu beeinflussen. Symbiont-Insekt-Assoziationen können direkt oder indirekt auf die Wirtsbiologie Einfluss nehmen. In dieser Arbeit wurden die Auswirkungen und Verteilungsmuster von Endosymbionten und anderen Bakterien in ökonomisch relevanten Thrips-Arten untersucht. Im Mittelpunkt der Untersuchungen stand hierbei der Thrips, *Echinothrips americanus*, ein neuer potenzieller Schädling in den europäischen Gewächshäusern.

Verteilungsmuster des *Wolbachia*-Endosymbionten während der Entwicklung

Die Verteilungsmuster der Wolbachien wurden in den Entwicklungsstadien (Larven, Ruhestadien und Adulten beider Geschlechter) von *E*. *americanus* ermittelt, wobei sich die bakterielle Verteilung während der Entwicklung dieser Thrips-Art verändert. In den larvalen Stadien waren die *Wolbachia*-Bakterien im gesamten Nervensystem (Supraösophagealganglion/ Oberschlundganglion), in der Speicheldrüse, in dem Darmepithel des Mitteldarms, in den muskulären Teilen der Beine und im Fettkörper des Abdomens beider Geschlechter verteilt. Die weiblichen und männlichen Propuppen zeigten bakterielle Vorkommen in verschiedenen neuronalen Gewebeabschnitten, speziell in dem Protocerebrum (Pilzkörper), in dem Tritocerebrum und Metathorakalganglion. Darüber hinaus wurden Wolbachien Ansammlungen in den Fettkörperzellen aller Körperteile (Kopf, Thorax, Abdomen) und in den sich entwickelnden Flügeln nachgewiesen. In den Ruhestadien wurden geschlechtsabhängige bakterielle Verteilungsmuster ermittelt. Weibliche Propuppen wiesen *Wolbachia*-Bakterien in den Antennen und dem Zentralkörper des Protocerebrums auf. Männliche Propuppen zeigten Bakteriencluster im Deutocerebrum, in den Testis (Hoden) und den akzessorischen Drüsen. Zusätzlich zu den genannten Vorkommen während der Ruhestadien wurden Wolbachien in der Thoraxmuskulatur und im Mitteldarmepithel, beider

Geschlechter der adulten Stadien, nachgewiesen. Darüber hinaus befanden sich *Wolbachia*-Bakterien in den Ovarien der adulten Weibchen, insbesondere im Germarium, in den follikulären Epithelzellen und in der Dottermasse von reifen Eiern.

Koinfektion

Molekulare Untersuchungen (*wsp* Gen, *ftsZ* Gen, 16S rDNA) stellten die Existenz einer Doppelinfektion mit *Wolbachia*- und *Cardinium*-Bakterien in *E*. *americanus* fest. Die Koinfektion mit diesen Endosymbionten konnte für alle Entwicklungsstadien beider Geschlechter (vom Ei bis zum Adulten) nachgewiesen werden. Weiterhin wurde die mütterliche (maternale) Übertragung der Bakterien auch mittels der *In-situ*-Hybridisierungstechnik nachgewiesen. *Wolbachia*- und *Cardinium*- Ansammlungen wurden in der Dottermasse der reifen Oozyten kurz vor der Eiablage lokalisiert. Zudem konnten bakterielle Nachweise beider Symbionten in den Prolarven ermittelt werden.

Auswirkung des *Cardinium*-Endosymbionten auf das Geschlechterverhältnis

Die Auswirkung des *Cardinium*-Endosymbionten auf das Geschlechterverhältnis des doppelt infizierten *E. americanus* (I_{WC} x I_{WC}, natürlicher Infektionsstatus) wurde anhand von Kreuzungsexperimenten untersucht. Für den *Cardinium*-Symbionten wurde eine förderliche Wirkung auf die Männchen Produktion nachgewiesen. Die Analyse zeigte, dass die Anzahl der Männchen signifikant ansteigt in Kreuzungskombinationen, wo nur ein Kreuzungspartner mit dem *Cardinium*-Bakterium infiziert ist (I_W x I_{WC}, 38,78 % Männchen-Anteil; I_{WC} x I_W, 40,79 % Männchen-Anteil), im Vergleich zu Kreuzungen, in denen beide Kreuzungspartner eine Doppelinfektion besitzen (I_{WC} x I_{WC}, Kontrollgruppe, 27,33 % Männchen-Anteil). In den getesteten Kreuzungskombinationen konnte keine embryonale Mortalität von befruchteten Eiern nachgewiesen werden (Anzahl der ungeschlüpften Eier). Ebenso unbeeinflusst von dem induzierten Effekt des Symbionten war die Produktion der Weibchen (Anzahl der Weibchen). Die Ergebnisse der Kreuzungsexperimente deuten auf einen *Cardinium*-Stamm (oder Stämme) hin, welcher seine eigene Modifikation aufheben kann, wenn das Bakterium in beiden Kreuzungspartnern vorhanden ist. Für den *Wolbachia*-Endosymbionten zeigten Sequenzanalysen eine hohe Identitätshomologie zu dem *Wolbachia pipientis* Stamm *w*Mel und *Wolbachia* sp. Stamm *w*Ri für die Weibchen und Männchen von *E*. *americanus* (Identitätshomologie > 99 %).

Verteilungsmuster von Endosymbionten während der Oogenese und Embryogenese

Die Verteilungsmuster der *Wolbachia*- und *Cardinium*-Endosymbionten während der Oogenese und Embryonalentwicklung, von *E*. *americanus* und *H*. *femoralis*, wurden durch elektronenmikroskopische Untersuchungen und der *In-situ*-Hybridisierungstechnik ermittelt. Bakterienstrukturen wurden während des Vitellariums in den Oozyten (Eizellen) beider Organismen, mittels Elektronenmikroskopie, lokalisiert. Die Bakterien waren im Zytoplasma und in den Follikelepithelzellen, der sich entwickelnden Oozyten, sowie in der Dottermasse reifer Oozyten für die Eiablage, verteilt. Mittels *In-situ*-Hybridisierungstechnik wurde ein charakteristisches Verteilungsmuster für die verschiedenen endosymbiotischen Bakterien in beiden Thrips-Arten ermittelt. *E*. *americanus* besitzt eine Koinfektion mit *Wolbachia*- und *Cardinium*-Bakterien. Die Endosymbionten wurden während der frühen Oogenese (im Vitellarium-Stadium) im Zellkern, dem Zytoplasma und den Follikelepithelzellen lokalisiert. In der späteren Phase des Vitellariums wurden bakterielle Nachweise beider Symbionten, im Übergangsbereich zwischen den einzelnen Oozyten (Follikelzellen) und in der Dottermasse vitellogener Eier, nachgewiesen. Die Endosymbionten waren in allen Entwicklungsphasen der Embryogenese verteilt. Während der frühen Entwicklung waren die Bakterien inhomogen in der Peripherie der Dottermasse verteilt (1 d ± 12 h altes Ei). Nach der Keimband-Elongation (Keimbandverlängerung) waren die Symbionten vorwiegend in der sich entwickelnden Kopfanlage lokalisiert (4 d ± 12 h alter Embryo). Im Verlauf der weiteren Entwicklung wurden die Bakterien in den cephalen, thorakalen und abdominalen Segmenten des Embryos nachgewiesen (7 d ± 12 h alter Embryo). Zudem wurden die Symbionten in den Gliedmaßen, speziell in den Antennen und Beinen, und in dem Amnion-Serosa-Komplex der sich entwickelnden Erstlarve lokalisiert. Die Thrips-Art *H*. *femoralis* besitzt eine Infektion mit dem *Wolbachia*-Bakterium. Für den *Wolbachia*-Symbionten konnten während der Oogenese dieses Panchaetothripinae identische Verteilungsmuster wie bei *E*. *americanus* festgestellt werden, nur die bakteriellen Vorkommen im Zytoplasma der Oozyten und in den Follikelzellen zwischen einzelnen Oozyten (Übergangsbereich) konnten nicht nachgewiesen werden. Während der Embryogenese konnten die Bakterien in allen Entwicklungsphasen nachgewiesen werden. Zu Beginn der Embryonalentwicklung waren die *Wolbachia*-Symbionten ungleichmäßig in der Dottermasse, eher in Richtung der Peripherie des Eies, verteilt (Ei 3 d ± 12 h). Nach der Ausbildung des Keimbands wurde das Bakterium in den späteren cephalen- und thorakalen-Strukturen der Keimanlage sowie in der umgebenden Dottermasse des 4 d ± 12 h alten Embryos gefunden. Im Verlauf der Entwicklung wurde das Bakterium in den neuronalen Geweben, in den Antennen, in den thorakalen und abdominalen Segmenten und in den extraembryonalen Geweben (Amnion-Serosa-Komplex) gefunden.

Die toxische Wirkung von *Photorhabdus luminescens* auf Thripse

Der toxische Einfluss des entomopathogenen Bakteriums *P*. *luminescens* auf die Überlebensrate wurde an den Weibchen von *E*. *americanus*, *F*. *occidentalis*, *H*. *femoralis* und *T*. *tabaci* untersucht. Für die Analyse nahmen alle getesteten Thripidae-Arten eine Applikationslösung (bestehend aus 75 µl Saccharose-Lösung (10 %), 50 µl Patentblau V-Lösung (4 %) und 75 µl *P*. *luminescens* Flüssigkultur) für 24 h auf. Die Kontrollgruppe erhielt die gleiche Lösung, aber ohne Bakterien im Wachstumsmedium. Die Lebensfähigkeit wurde täglich überprüft, um herauszufinden, ob *P*. *luminescens* einen direkten Einfluss auf die Überlebensrate der verwendeten Thrips-Arten hat. Die statistische Auswertung identifizierte einen signifikanten Unterschied zwischen der Überlebensrate der *P*. *luminescens* und Kontrollgruppe von *E*. *americanus*. Interessanterweise besitzt *E*. *americanus* eine bakterielle Infektion mit den immunregulierenden *Wolbachia*- und *Cardinium*-Bakterien, wobei die Koinfektion keinen schützenden Effekt auf die Überlebensrate des Thrips hat. Im Gegensatz dazu zeigen die anderen getesteten Thrips-Arten keinen signifikanten Unterschied in der Überlebenswahrscheinlichkeit zwischen der Gruppe mit *P*. *luminescens*-Anwendung und der Kontrollgruppe. Zur möglichen Lokalisierung des Bakteriums innerhalb der Thrips-Körperteile wurde die Technik der *In-situ*-Hybridisierung verwendet (16S rDNA, Primer fD1/1492r). Bei allen Thrips-Arten konnte bakterielle DNA überwiegend in den Epithelien des Darmtraktes (hauptsächlich im Mitteldarm), aber auch in den thorakalen Muskeln, den Gonaden und im neuronalen Gewebe der Weibchen nachgewiesen werden.

Fazit

Das Hauptziel dieser Arbeit war es, die Bedeutung von Endosymbionten-Thrips-Interaktionen zu untersuchen. Ein wichtiger Punkt war die Bestimmung der Symbiont-abhängigen Verteilungsmuster in ökonomisch relevanten Thrips-Arten. Mittels der *In-situ*-Hybridisierungs-technik konnte ein grundlegender Überblick über die bakterielle Verteilung, insbesondere für *Wolbachia*-Bakterien, während der Thrips-Entwicklung dokumentiert werden. Ein weiteres Ziel war die Analyse der Endosymbionten- und/oder Bakterien-Effekte. In der vorliegenden Arbeit wurden die Endosymbionten-abhängigen Regulationsmuster auf das Geschlechterverhältnis im natürlicherweise doppelt infizierten *E*. *americanus* ermittelt. Darüber hinaus zeigten Überlebensanalysen zur toxischen Wirkung des entomopathogenen Bakteriums *P*. *luminescens*, dass der Einfluss des Mikroorganismus abhängig vom Zielinsekt ist. Schließlich stellt diese Arbeit eine Vielzahl interessanter Assoziationsaspekte im Ökosystem Endosymbionten-Thrips fest und bietet Potenzial für weitere Forschung.

SUPPLEMENTARY MATERIAL DIRECTORY

Additional methods, preparation information of used chemicals and solutions, time schedules for preparation procedures and further results are presented in the supplementary material in the attached CD-ROM. A PDF version of the whole thesis is also contained. The numbering of the single chapters is conform to the chapter-numbers in the thesis.

PDF file dissertation Julia Chuttke

CHAPTER S2 Distribution patterns of the *Wolbachia* endosymbiont during development

- Materials and methods
  - Instruction on *Wolbachia* detection in prepared tissue and body parts
  - Table of preparation of the used chemicals
  - Table of deparaffinization procedure for histological sections
- Results
  - Figure of *in situ* hybridization findings in the developmental stages of *F*. *occidentalis*
  - Table of *Wolbachia* detection (PCR results) in the prepared tissue and body parts of the *E*. *americanus* adults
  - Table of *Wolbachia* findings in the developmental stages of both sexes in *E*. *americanus*

CHAPTER S3 Coinfection

- Materials and methods
  - Table of used primers for the detection of *Wolbachia* and *Cardinium*
- Results
  - Table of total endosymbiont findings of all tested primers in the developmental stages of *E*. *americanus* and *F*. *occidentalis*
  - Table of *Wolbachia* and *Cardinium* infection levels in the developmental stages of *E*. *americanus*

ACKNOWLEDGEMENTS

I would like to thank Prof. Dr. habil. Gerald Moritz for the friendly supply of this interesting topic and for allowing the possibility to make my dissertation in his department. In addition, I express thanks for helpful support and stimulating discussions, which was an essential contribution to the success of this work. Particularly, I am thankful for granting scientific open spaces and the opportunity to present my research work in conferences.

A special thank goes to the entire working group of Developmental biology for the pleasant working atmosphere and active support in preparing the current work. PD Dr. habil. Gunther Tschuch, I thank for the technical support in the laboratory. I thank Dr. Stephanie Krüger for her always friendly and unreserved willingness to discuss various issues in the work. In addition to that, I also thank her for revisions and many constructive comments to the dissertation. Furthermore, I would like to thank Marcus Jilge for his help in the laboratory and the extremely pleasant get-together in the office. Moreover, Renate Kranz and Angelika Steller, I thank for their support in both the practical and written part of the current work.

For her participation in chapter 2, I would like to thank Franziska Fahlteich. Additionally, I thank Claudia Köcher for the involvement in chapter 6. Both are co-authors of the manuscripts resulting from the chapters.

My thanks also goes to Dr. Sandra Kumm for her engagement in reviewing this work and for the valuable comments to single chapters.

Sincere thanks to Sina Cassau for her comments on the English translation of this work and the attached appendices.

For the financial participation of this dissertation, I thank the woman's promotion of the Martin-Luther-University Halle-Wittenberg.

A very special thanks goes to my family, who made my degree possible and also always supported me and stood lovingly at my side during this dissertation. I would especially like to thank my mother and my sister for the shown confidence, the continuous motivation and the support at any time.

DANKSAGUNG

Herrn Prof. Dr. habil. Gerald Moritz möchte ich herzlich für die freundliche Überlassung des interessanten Themas und die Möglichkeit, meine Dissertation in seinem Fachbereich anfertigen zu dürfen, danken. Darüber hinaus bedanke ich mich für die hilfreiche Unterstützung und anregenden Diskussionen, was maßgeblich zum Gelingen dieser Arbeit beigetragen hat. Besonders bedanken möchte ich mich auch für die Gewährung wissenschaftlicher Freiräume und die Gelegenheit, meine Forschungsarbeit auf Tagungen vorzustellen.

Ein besonderer Dank geht an die gesamte Arbeitsgruppe der Entwicklungsbiologie für die angenehme Arbeitsatmosphäre und tatkräftige Unterstützung bei der Anfertigung der vorliegenden Arbeit. Bei Herrn PD Dr. habil. Gunther Tschuch möchte ich mich für die technische Unterstützung im Labor bedanken. Frau Dr. Stephanie Krüger danke ich für ihre immer freundliche und uneingeschränkte Diskussionsbereitschaft zu verschiedenen Fragestellungen dieser Arbeit. Zudem bedanke ich mich bei ihr für die Durchsicht und für die vielen konstruktiven Anmerkungen zur Dissertation. Im Weiteren möchte ich mich bei Herrn Marcus Jilge für die Hilfe im Labor und das äußerst angenehme Beisammensein im Büro bedanken. Außerdem danke ich Frau Renate Kranz und Frau Angelika Steller für ihre Unterstützung sowohl im praktischen als auch schriftlichen Teil der vorliegenden Arbeit.

Für ihre Mitwirkung an dem Kapitel 2 möchte ich Frau Franziska Fahlteich danken. Des Weiteren bedanke ich mich bei Frau Claudia Köcher für die Beteiligung an Kapitel 6. Beide sind Koautoren der aus den Kapiteln resultierenden Manuskripte.

Mein Dank geht ebenso an Frau Dr. Sandra Kumm für ihr Engagement bei der Durchsicht dieser Arbeit und für die wertvollen Anmerkungen zu einzelnen Kapiteln.

Frau Sina Cassau danke ich ganz herzlich für ihre Unterstützung bei der Englisch-Korrektur dieser Arbeit und der dazugehörigen Anhänge.

Für die finanzielle Unterstützung der Dissertation danke ich der Frauenförderung der Martin-Luther-Universität Halle-Wittenberg.

Ein ganz besonderer Dank geht an meine Familie, die mir das Studium ermöglicht hat und mir auch während der Anfertigung der Dissertation immer unterstützend und liebevoll zur Seite standen. Insbesondere möchte ich mich bei meiner Mutti und meiner Schwester für das entgegengebrachte Vertrauen, die stetige Motivation und die Unterstützung zu jeder Zeit bedanken.

CURRICULUM VITAE

Julia Chuttke
MSc Biology
born on October 6th, 1989 in Cottbus

EDUCATION

04/2013–04/2015 Master of Science, Biology, Martin Luther University Halle-Wittenberg
- » Master thesis: “Histological and molecular detection of *Wolbachia* in the developmental stages of the haplodiploid thripine *Echinothrips americanus*”
- » Original title: „Histologische und molekulare Nachweise von Wolbachien in allen ontogenetischen Stadien des haplo-diploiden Thripiden *Echinothrips americanus*“

10/2009–10/2012 Bachelor of Science, Biology, University of Hamburg
- » Bachelor thesis: “Investigations to the protein expression on the three-spine stickleback (*Gasterosteus aculeatus*) under hypoxia”
- » Original title „Untersuchungen zur Proteinexpression beim Dreistachligen Stichling (*Gasterosteus aculeatus*) unter Hypoxie“

08/2002–06/2009 Theodor-Fontane-Schule, Cottbus
- » A-level

PROFESSIONAL EXPERIENCE

08/2015–07/2020 Research assistant, Martin Luther University Halle-Wittenberg, Institute of Biology, Research group Developmental Biology

Since 01/2021 Research project assistant, IADP at the Martin Luther University Halle-Wittenberg

ADVANCED TRAINING

01/2020 University didactic qualification measure: “Successful teaching – towards the didactics of teaching and learning processes at the university”,
Martin Luther University Halle-Wittenberg

06/2017 Mentoring- und Coachingproject MeCoSa 4.0,
coordinating position gender research & equal opportunity Saxony-Anhalt

02/2014 Qualification certificate “animal protection, experimental animal science and animal experimentation”,
Martin Luther University Halle-Wittenberg

FUNDINGS

2016–2019	Woman's promotion of Martin Luther University Halle-Wittenberg

ATTENDANCE AT CONFERENCES

03/2019	DGaaE conference 2019, Halle Talk: ”Impacts of the nematode-symbiont bacterium *Photorhabdus luminescens* on the survival rate of *Echinothrips americanus* MORGAN 1913 (Thysanoptera, Thripidae)”
09/2018	61st German plant protection conference, Hohenheim Poster: ”Nachweis einer Koinfektion beim Fransenflügler *Echinothrips americanus* (Thripidae, Thysanoptera)“
09/2017	5th Symposium on palaearctic Thysanoptera, Cracow Talk: “Detection of *Wolbachia* in the ontogenetic stages of *Echinothrips americanus* MORGAN 1913 (Thysanoptera, Thripidae)“
03/2017	DGaaE conference 2017, Freising Talk: “Detection of *Wolbachia* in the arrhenotokous thrips *Echinothrips americanus* (MORGAN, 1913)”
09/2016	60th German plant protection conference, Halle Talk: “Nachweis von Wolbachien beim Fransenflügler *Echinothrips americanus* (Thripidae, Thysanoptera)„

TEACHING

WS 2015/2016– WS 2017/2018	Practical course developmental biology – embryogenesis of chicken
SoSe 2016–2020	Project module developmental biology – participation in the practical implementation
WS 2018/2019– WS 2019/2020	Practical course developmental biology – embryogenesis of insects
SoSe 2018– WS 2019/2020	Seminar course developmental and human biology – senses

Halle (Saale), 18th February 2021

STATEMENT OF AUTHORSHIP

I declare under oath that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.
Further, I declare that all information given is accurate and complete. The thesis has not been used previously at this or any other university in order to achieve an academic degree.

EIGENSTÄNDIGKEITSERKLÄRUNG

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.
Weiterhin erkläre ich, die Angaben wahrheitsgemäß gemacht und die wissenschaftliche Arbeit an keiner anderen wissenschaftlichen Einrichtung zur Erlangung eines akademischen Grades eingereicht zu haben.

Julia Chuttke Halle (Saale), 18th February 2021

CHAPTER S2

Detection of the *Wolbachia* distribution in the developmental stages of *Echinothrips americanus* (Thysanoptera: Thripidae)

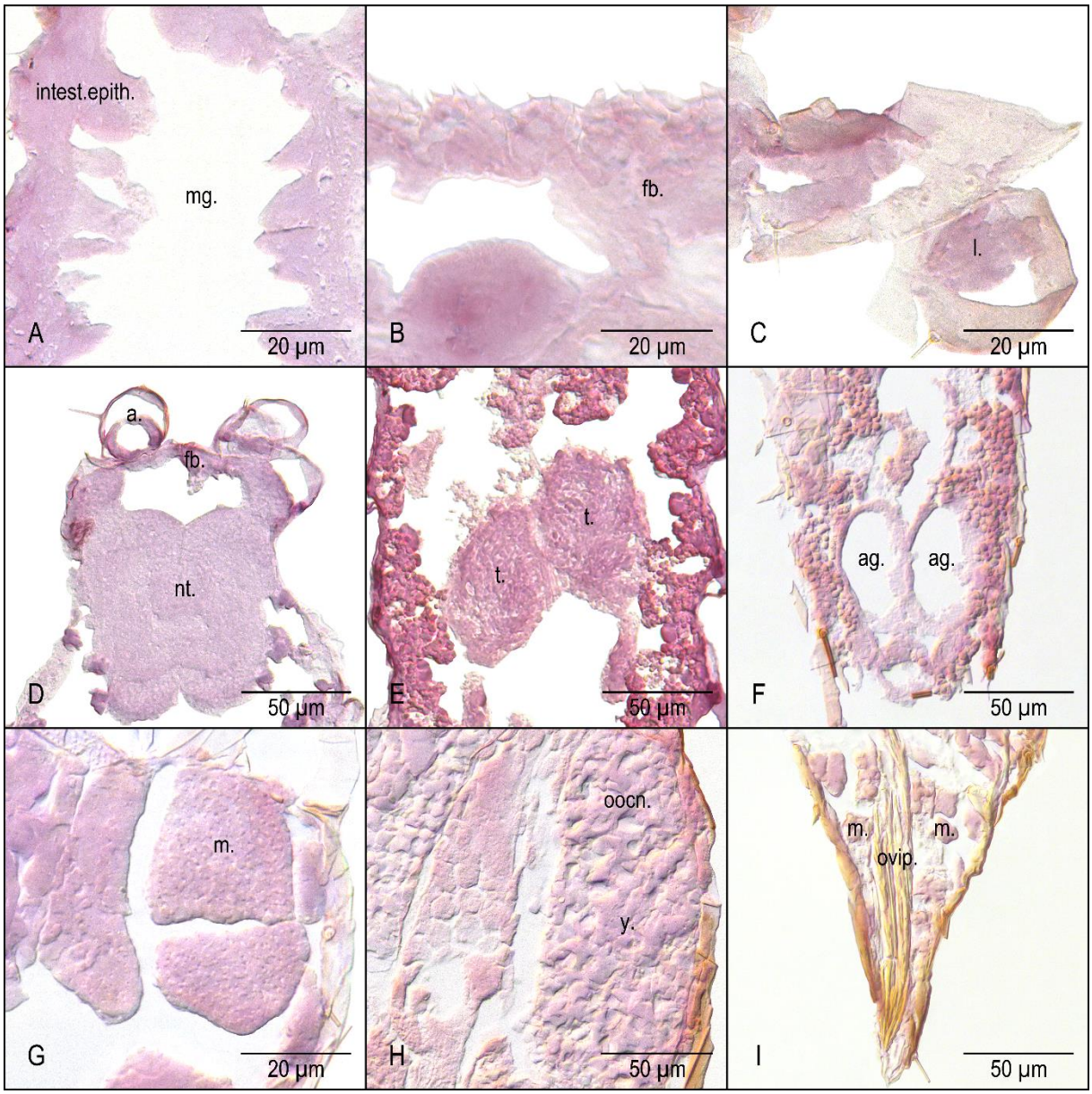
S2 Suppl. method: *Wolbachia* detection in prepared tissue and body parts

The *Wolbachia* presence in tissue and body parts (head, thorax, abdomen, intestinal system, ovaries, testes) of *E*. *americanus* was verified by the *wsp* gene (primers: 81F/691R, Zhou et al. 1998) detection. Altogether, 10 adult thrips of each sex were investigated on the occurrence of this symbiont.

In the first step, the thrips were surface-sterilized after the method of de Vries et al. 2001. Each thrips was sterilized through 70% ethanol for 60 s, 5% $NaClO_3$ for 60 s and a rinsing for three times in sterile water.

For the DNA extraction, a modified version of the “100 fly method” after Roberts (1998) was used (described in Kumm et al. 2006). The prepared tissue and body parts of each thrips was crushed in liquid nitrogen, homogenized in 100 µl extraction buffer (0.2 M Sucrose, 0.1 M Tris, 0.1 M NaCl, 0.05 M EDTA, 0.5% SDS, pH 9.2) and then incubated for 15 min at 70 °C. Following this, 15 µl of ice-cold 8 M potassium acetate was added, mixed and incubated for 15 min on ice. After the centrifugation (15 min at 13200 rpm), the supernatant (90 µl) was transferred in a new tube with 2.5 vol. ethanol and 0.1 vol. 3 M sodium acetate (4 °C) and vigorously mixed. Subsequently, the DNA was precipitated at -80 °C for 45 min. After this step, the DNA was sedimented by centrifugation at 13200 rpm (15 min) and the supernatant was discarded. The sediment was washed with 70% ethanol by centrifugation (13200 rpm) for 5 min. Afterwards the supernatant was discarded again and the sediment dried at 45 °C for 5 min. Finally, the sediment was dissolved in 16 µl ddH_2O for 10 min at 45 °C.

For *Wolbachia* detection, primers for the *wsp* gene (81F/691R, Zhou et al. 1998) were used. The PCR reaction mixture comprised 5 µl 10x reaction buffer, 4 µl $MgCl_2$ (25 mM), 0.4 µl dNTPs (25 mM), 1.5 µl of each primer (20 pmol/µl), 0.2 µl *Taq* polymerase and 2 µl of the template DNA in a total volume of 50 µl. Following program conditions were utilized: 3min at 95 °C initial denaturation, 35 cycles 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C and a last elongation step 5 min at 72 °C. The PCR products were verified through a 2% agarose gel, visualized with the gel documentation system DIAS-III from SERVA and documented with GelScan V6.0.



S2 Fig. 1. *In situ* hybridization investigations in both sexes of selected developmental stages (frontal sections of first instar larva, propupa and adult) of *F. occidentalis*. *F. occidentalis* used as a control, because this thrips species is not infected with *Wolbachia*. Fig. 1A–C first instar larva: (A) midgut (female), (B) fat body (male), (C) femur and tibia (male), Fig. 1D–F propupa: (D) scape, pedicellus and parts of protocerebrum (female), (E) testes (male), (F) accessory glands (male), Fig. 1G–I adult: (G) oscillatoric flight muscles, pterothorax (male), (H) first part of midgut and vitellogenic egg (female), (I) basal structures of ovipositor valves (female). (a.– antenna, ag.– accessory gland, e.– extremity, fb.– fat body, m.– muscles, mg.– midgut, nt.– neuronal tissue, ovip.– ovipositor, t.– testis, y.– yolk).

S2 Tab. 1. Results of the preliminary investigations for the *Wolbachia* detection (*wsp* gene) in prepared tissue and body parts of the female and male adults of *E*. *americanus*. The gene detection was realized after the description in S2 Suppl. method.

Detection of *Wolbachia* in female tissue and body parts	**Detection of *Wolbachia* in male tissue and body parts**
Abdomen	Abdomen
Head	Head
Intestinal system	Intestinal system
Ovaries (Ovarioles, vitellogenic egg)	Testes
Thorax	Thorax

S2 Tab. 2. Preparation of the used chemicals.

Chemicals	Preparation of chemicals
Anti-digoxigenin antibody (200 µg/ml)	▪ 1 : 250 diluted in TRIS-HCl/NaCl buffer
Anti-digoxigenin-AP Fab fragments 200 µl (150 U)	▪ 1 : 5000 diluted in TRIS-HCl/NaCl buffer
Blocking buffer	▪ 50 mM TRIS ▪ 300 mM NaCl ▪ 1% albumin fraction V (Carl Roth GmbH + Co. KG, Germany)
5-bromo-4-chloroindolyl phosphate (BCIP)	▪ 50 mg/ml BCIP dissolved in 100% dimethylformamide (DMF)
2.5% glutaraldehyde	▪ 25% glutaraldehyde diluted in Sorensen's phosphate buffer (pH 7.4)
Nitro blue tetrazolium salt (NBT)	▪ 75 mg/ml NBT dissolved in 70% dimethylformamide (DMF)
4% paraformaldehyde	▪ 2 g PFA dissolved in 10 ml ddH_2O ▪ Warm up solution to 65 °C while stirring ▪ Add NaOH dropwise until the solution is clear, afterwards filter ▪ 10 ml PFA added to 40 ml 0.1 M Sorensen's phosphate buffer (pH 7.2)
Proteinase K	▪ Stock solution 10 mg dissolved in 1 ml ddH_2O ▪ Stored at -20 °C
Sorensen's phosphate buffer, pH 7.2	▪ 36 ml of 0.2 M Na_2HPO_4 buffer ▪ 14 ml of 0.2 M NaH_2PO_4 buffer ▪ Added to 100 ml ddH_2O
Substrate solution	▪ 4.5 µl NBT ▪ 3.5 µl BCIP ▪ Diluted in TRIS-HCl/NaCl/$MgCl_2$ buffer
TRIS-HCl/$CaCl_2$ buffer, pH 7.6	▪ 20 mM TRIS ▪ 2 mM $CaCl_2$
TRIS-HCl/NaCl buffer, pH 7.6	▪ 20 mM TRIS ▪ 300 mM NaCl
TRIS-HCl/NaCl/$MgCl_2$ buffer, pH 9.5	▪ 20 mM TRIS-HCl ▪ 300 mM NaCl ▪ 50 mM $MgCl_2$

S2 Tab. 3. Deparaffinization of histological sections. The deparaffinization took place after an overnight drying step at 32 °C.

Chemicals	Time conditions
Xylene	2 min
Isopropyl alcohol	2 min
100%, 90%, 85%, 75%, 50%, 30% ethanol	Each 1 min

S2 Tab. 4. Summary of infected *Wolbachia* tissues in selected developmental stages (first instar larva, propupa, adult) of both sexes in *E*. *americanus*.

Developmental stage	Detection of *Wolbachia* in females	Detection of *Wolbachia* in males
First instar larva	Fat body of abdomen	Fat body of abdomen
	Midgut epithelium	Midgut epithelium
	Nervous system (complete supraoesophageal ganglion) Salivary gland	Nervous system (complete supraoesophageal ganglion) Salivary gland
	Leg/extremity	Leg/extremity
Propupa		Accessory glands
	Antennae (scape-pedicel complex)	
	Fat body of head, thorax and abdomen	Fat body of head, thorax and abdomen
	Nervous system: protocerebrum (mushroom bodies and central body), tritocerebrum, metathoracic ganglion	Nervous system: protocerebrum (mushroom bodies), deuto- (antennal lobes) and tritocerebrum, metathoracic ganglion Testes
	Wings	Wings
Adult		Accessory glands
	Fat body of head, thorax and abdomen	Fat body of head, thorax and abdomen
	Midgut epithelium	Midgut epithelium
	Muscles	Muscles
	Nervous system: protocerebrum (mushroom bodies and central complex), tritocerebrum, metathoracic ganglion Ovaries (germarium, follicular epithelial cells, yolk)	Nervous system: protocerebrum (mushroom bodies), deuto- (antennal lobes) and tritocerebrum, metathoracic ganglion
		Testes
	Wings	Wings

S2 Supplementary references

de Vries, E.J., G. Jacobs, and J.A. Breeuwer. 2001. Growth and transmission of gut bacteria in the Western flower thrips, *Frankliniella occidentalis*. J Invertebr Pathol 77: 129–137.

Kumm, S., R. Kranz, and G. Moritz. 2006. Mikroorganismen-gesteuerte Parthenogenese bei Thysanopteren. Mitt Dtsch Ges allg angew Ent 15: 153–156.

Zhou, W., F. Rousset, and S. O'Neill. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc Lond B 265: 509–515.

CHAPTER S3

Evidence of a coinfection with *Cardinium* and *Wolbachia* in the arrhenotokous thrips *Echinothrips americanus* (Thysanoptera: Thripidae

S3 Tab. 1. Used primers for the detection of *Wolbachia* and *Cardinium*.

Primer name	Primer	Sequence (5'→3')	References
16S rRNA	99F	TTGTAGCCTGCTATGGTATAACT	O'Neill et al. 1992
(*Wolbachia*)	994R	GAATAGGTATGATTTTCATGT	
ftsZ	494F	GGACCGGATCCGTATGCCGATTGCAGAGCTTG	Holden et al. 1993
(*Wolbachia*)	1262R	GGACCGAATTCGCCATGAGTATTCACTTGGCT	
wsp	81F	TGGTCCAATAAGTGATGAAGAAAC	Zhou et al. 1998
(*Wolbachia*)	691R	AAA AAT TAA ACG CTA CTC CA	
16S rDNA (*Cardinium*)	ChF	TACTGTAAGAATAAGCACCGGC	Zchori-Fein & Perlman 2004
	ChR	GTGGATCACTTAACGCTTTCG	
16S rDNA (*Cardinium*)	CLO-f1	GGAACCTTACCTGGGCTAGAATGTATT	Gotoh et al. 2007
	CLO-r1	GCCACTGTCTTCAAGCTCTACCAAC	

S3 Tab. 2. Presence of *Wolbachia* and *Cardinium* in the developmental stages (female and male) of *E*. *americanus* (infected species) and *Frankliniella occidentalis* (uninfected species). (+ Evidence of the endosymbiont; - No evidence of the endosymbiont).

Thrips species	***Wolbachia* *wsp* gene** 81F/691R	***Wolbachia* *ftsZ* gene** 494F/1262R	***Wolbachia* 16S rRNA** 99F/994R	***Cardinium* 16S rDNA** ChF/ChR	***Cardinium* 16S rDNA** COL-f1/CLO-r1
E. americanus					
Adult ♂	+	+	+	-	+
Pupa ♂	+	+	+	-	+
Propupa ♂	+	+	+	-	+
Second instar larva ♂	+	+	+	-	+
First instar larva ♂	+	+	+	-	+
Egg ♂	+	+	+	+	+
Adult ♀	+	+	+	-	+
Pupa ♀	+	+	+	-	+
Propupa ♀	+	+	+	+	+
Second instar larva ♀	+	+	+	+	+
First instar larva ♀	+	+	+	-	+
Egg ♀	+	+	+	-	+
F. occidentalis					
Adult ♂	-	-	-	-	-
Pupa ♂	-	-	-	-	-
Propupa ♂	-	-	-	-	-
Second instar larva ♂	-	-	-	-	-
First instar larva ♂	-	-	-	-	-
Egg ♂	-	-	-	-	-
Adult ♀	-	-	-	-	-
Pupa ♀	-	-	-	-	-
Propupa ♀	-	-	-	-	-
Second instar larva ♀	-	-	-	-	-
First instar larva ♀	-	-	-	-	-
Egg ♀	-	-	-	-	-

S3 Tab. 3. *Wolbachia* and *Cardinium* infection levels in the developmental stages of *E*. *americanus*. To determine the infection level in each developmental stage, 20 samples of eggs, first instar larvae, second instar larvae, propupae, pupae and adults of both sexes were investigated for the presence of both symbionts. Rearing conditions of the thrips stages described under 3.3.1. DNA extraction, gene amplification and agarose gel electrophoresis took place as described under 3.3.2.

Developmental stage	***Wolbachia* *ftsZ* gene** 494F/1262R	***Cardinium* 16S rDNA** COL-f1/CLO-r1
Adult ♂	90%	90%
Pupa ♂	90%	90%
Propupa ♂	90%	70%
Second instar larva ♂	90%	80%
First instar larva ♂	90%	90%
Egg ♂	90%	90%
Adult ♀	90%	90%
Pupa ♀	100%	70%
Propupa ♀	100%	70%
Second instar larva ♀	70%	70%
First instar larva ♀	80%	80%
Egg ♀	80%	90%

S3 Supplementary references

Gotoh, T., H. Noda, and S. Ito. 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. Heredity 98: 13–20.

Holden, P.R., J.F.Y. Brookfield, and P. Jones. 1993. Cloning and characterization of an *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. Mol Gen Genet 240: 213–220.

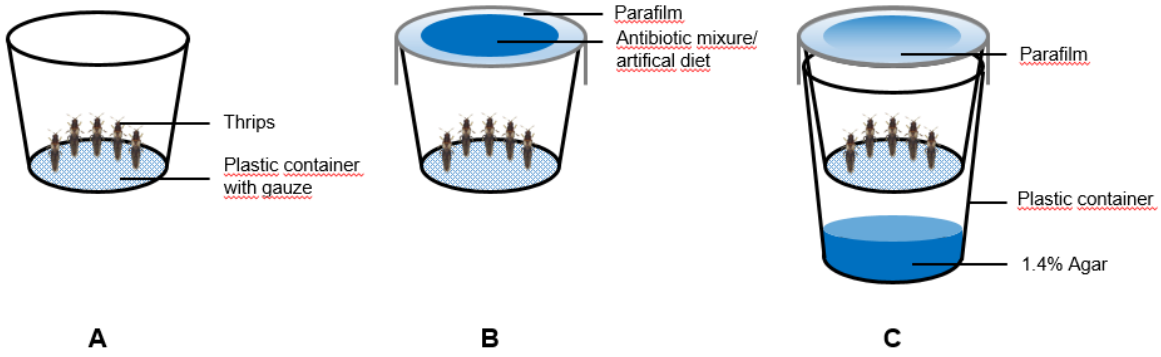
O'Neill, S.L., R. Giordano, A.M.E. Colbert, T.L. Karr, and H.M. Robertson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc Natl Acad Sci USA 89: 2699–2702.

Zchori-Fein, E., and S.J. Perlman. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. Mol Ecol 13: 2009–2016.

Zhou, W., F. Rousset, and S. O'Neill. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc Lond B 265: 509–515.

CHAPTER S4

Are *Wolbachia* and/or *Cardinium* infections responsible for a cytoplasmic incompatibility in *Echinothrips americanus*? Investigation to the impact of the endosymbiont *Cardinium* on the male sex ratio



S4 Fig. 1. Application system for antibiotic mixture (antibiotic agent and artifical diet) or artifical diet for thrips. (A) Plastic container with gauze on one side and thrips inside. (B) Parafilm closed plastic container with the applied antibiotic mixture or artificial diet. (C) Complete application system composed of an enclosed solution (by a layer of parafilm) and a transfer on a second plastic container with a layer on agar on the bottom.

S4 Tab. 1. Used antibiotics and their preparation conditions.

Used antibiotics	Preparation of antibiotics
Ampicillin sodium salt ≥97% BioScience Grade for molecular biology, K029.1, Carl Roth GmbH + Co. KG, Germany	50 mg ampicillin were dissolved in 1 ml of ddH_2O
Tetracycline hydrochlorid ≥95% (European Pharmacopoeia HPLC assay), T3383, Sigma Aldrich, USA	50 mg tetracycline were dissolved in 1 ml of 35% ethanol
Rifampicin ≥ 90% for biochemistry, K029.1, Carl Roth GmbH + Co. KG, Germany	15 mg rifampicin were dissolved in 1 ml of 100% methanol

S4 Tab. 2. Ingredients of the artificial diet (amount per 100 ml) after Jilge 2016.

Ingredients	Amount	Manufacturer
Lecithin ≥ 97% made from soybeans	120 mg	Carl Roth GmbH + Co. KG, Germany
Cholesterol	20 mg	Carl Roth GmbH + Co. KG, Germany
Tween80	100 mg	Serva Feinbiochemica, Germany
Linoleic acid methyl ester	100 mg	Carl Roth GmbH + Co. KG, Germany
Casein hydrolysate standard	3 g	Carl Roth GmbH + Co. KG, Germany
Wesson's salt mixture	2 g	MP Biomedicals, LLC, France
Yeast extract	2 g	Carl Roth GmbH + Co. KG, Germany
Choline chloride	100 mg	Sigma-Aldrich, USA
Meso-Inositol	100 mg	Carl Roth GmbH + Co. KG, Germany
Pollen	1 g	Naturprodukte Lembcke GbR, Germany
Wheat germ buds	1 g	Dr. Grandel GmbH, Germany
Ascorbic acid	1 g	Carl Roth GmbH + Co. KG, Germany
Honey	11.4 g	Honigland GmbH, Germany
Patent blue V sodium salt	4 g	Sigma-Aldrich, USA
pH	6	

S4 Tab. 3. Sequencing results for the PCR products.

Gene	Primer	*E. americanus*	Sequencing result
16S rRNA	99F	-female-	AAGTGAATCTACCTAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACG CCCTACGGGGGAAAAATTTATTGCTATTAGATGAGCCTATATTAGATTAGCTAGTTGGTG GAGTAATAGCCTACCAAGGCAATGATCTATAGCTGATCTGAGAGGATGATCAGCCACACT GGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG GCGAAAGCCTGATCCAGCCATGCCGCATGAGTGAAGAAGGCCTTTGGGTTGTAAAGCTCT TTTAGTGAGGAAGATAATGACGGTACTCACAGAAGAAGTCCTGGCTAACTCCGTGCCAGC AGCCGCGGTAATACGGAGAGGGCTAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGT AGGCGGATTAGTAAGTTAAAAGTGAAATCCCAAGGCTCAACCTTGGAATTGCTTTTAAAA CTGCTAATCTAGAGATTGAAAGAGGATAGAGGAATTCCTAGTGTAGAGGTGAAATTCGTA AATATTAGGAGGAACACCAGTGGCGAAGGCGTCTATCTGGTTCAAATCTGACGCTGAGGC GCGAAGGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGA ATGTTAAATATGGGAAGTTTTACTTTCTGTATTACAGCTAACGCGTTAAACATTCCGCCT GGGGACTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTG GAGCATGTGGTTTAATTCGATGCAACGCGAAAACCTTACACTCCTGCTACAAAA Product length: 834 bp
	99F	-male-	TGTATAGGAATCTACCTAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTAT ACGCCCTACGGGGGAAAAATTTATTGCTATTAGATGAGCCTATATTAGATTAGCTAGTTG GTGGAGTAATAGCCTACCAAGGCAATGATCTATAGCTGATCTGAGAGGATGATCAGCCAC ACTGGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAA TGGGCGAAAGCCTGATCCAGCCATGCCGCATGAGTGAAGAAGGCCTTTGGGTTGTAAAGC TCTTTTAGTGAGGAAGATAATGACGGTACTCACAGAAGAAGTCCTGGCTAACTCCGTGCC AGCAGCCGCGGTAATACGGAGAGGGCTAGCGTTATTCGGAATTATTGGGCGTAAAGGGCG CGTAGGCGGATTAGTAAGTTAAAAGTGAAATCCCAAGGCTCAACCTTGGAATTGCTTTTA AAACTGCTAATCTAGAGATTGAAAGAGGATAGAGGAATTCCTAGTGTAGAGGTGAAATTC GTAAATATTAGGAGGAACACCAGTGGCGAAGGCGTCTATCTGGTTCAAATCTGACGCTGA GGCGCGAAGGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGA TGAATGTTAAATATGGGAAGTTTTACTTTCTGTATTACAGCTAACGCGTTAAACATTCCG CCTGGGGACTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCG GTGGAGCATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCACTCCTGACATAA Product length: 837 bp
	994R	-male-	AACGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTT TGAGTTTTAATCTTGCGACCGTAGTCCCCAGGCGGAATGTTTAACGCGTTAGCTGTAATA CAGAAAGTAAAACTTCCCATATTTAACATTCATCGTTTACAGCGTGGACTACCAGGGTAT CTAATCCTGTTTGCTCCCCACGCCTTCGCGCCTCAGCGTCAGATTTGAACCAGATAGACG CCTTCGCCACTGGTGTTCCTCCTAATATTTACGAATTTCACCTCTACACTAGGAATTCCT CTATCCTCTTTCAATCTCTAGATTAGCAGTTTTAAAAGCAATTCCAAGGTTGAGCCTTGG GATTTCACTTTTAACTTACTAATCCGCCTACGCGCCCTTTACGCCCAATAATTCCGAATA ACGCTAGCCCTCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCAGGACTTCTTCTG TGAGTACCGTCATTATCTTCCTCACTAAAAGAGCTTTACAACCCAAAGGCCTTCTTCACT CATGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCC CGTAGGAGTCTGGACCGTATCTCAGTTCCAGTGTGGCTGATCATCCTCTCAGATCAGCTA TAGATCATTGCCTTGGTAGGCTATTACTCCACCAACTAGCTAATCTAATATAGGCTCATC TAATAGCAATAAATTTTTCCCCCGTAGGGCGTATACGGTATTAGTTGCCGTTTCCAACAA TTATTCCGTACTACTAGGTAGATTCCTATACATTACTCACCCGTCTGCCACTAGTTAAA Product length: 839 bp
wsp	81F	-female-	ACTTTACCATTAAAACCATCTTTTATAGCTGGTGGTGGTGCATTTGGTTACAAAATGGAC GACATCAGGGTTGATGTTGAAGGAGTTTATTCATACCTAAACAAAAATGATGTTAAAGAT GTAACATTTGACCCAGCAAATACTATTGCAGACAGTGTAACAGCAATTTCAAGATTAGTG AACGTGTATTACGATATAGCAATTGAAGATATGCCTATCACTCCATACATTGGTGTTGGT GTTGGTGCAGCGTATATTAGCACTCCTTTGGAACCCGCTGTGAATGATCAAAAAAGTAAA TTTGGTTTTGCTGGTCAAGTAAAAGCTGGTGTTAGTTATGATGTAACTCCAGAAGTCAAA CTTTATGCTGGAGCTCGTTATTTCGGTTCTTATGGTGCTAATTTTGATGGAAAAAAAACA GATCCTAAAGATTCAACCAGACAGGTTACTGATGCAGGCGCATACAAAGTTCTTTACAGC ACTGTTGGTGCAGAAGCTGGAGTACGTTTAAATTTTTAA Product length: 519 bp
	81F	-male-	AGTTCGTTTGCATACAACGGTGAATTTTTACCTCTTTTCACAAAAGTTGATGGTATTACC TATAAGAAAGACAAGAGTGATTACAGTCCATTAAAACCATCTTTTATAGCTGGTGGTGGT GCATTTGGTTACAAAATGGACGACATCAGGGTTGATGTTGAAGGAGTTTATTCATACCTA AACAAAAATGATGTTAAAGATGTAACATTTGACCCAGCAAATACTATTGCAGACAGTGTA ACAGCAATTTCAAGATTAGTGAACGTGTATTACGATATAGCAATTGAAGATATGCCTATC ACTCCATACATTGGTGTTGGTGTTGGTGCAGCGTATATTAGCACTCCTTTGGAACCCGCT GTGAATGATCAAAAAAGTAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGTGTTAGTTAT GATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGTTATTTCGGTTCTTATGGTGCT AATTTTGATGGAAAAAAAACAGATCCTAAAGATTCAACCAGACAGGTTACTGATGCAGGC GCATACAAAGTTCTTTACAGCACTGTTGGTGCAGAAGCTGGAGTAGTGTT Product length: 590 bp
	691R	-female-	TCGGAATTAGCACCATAAGAACCGCAAATAACGAGCTCCAGCATAAAGTTTGACTTCTGG AGTTACATCATAACTAACACCAGCTTTTACTTGACCAGCAAAACCAAATTTACTTTTTTG ATCATTCACAGCGGGTTCCAAAGGAGTGCTAATATACGCTGCACCAACACCAACACCAAT GTATGGAGTGATAGGCATATCTTCAATTGCTATATCGTAATACACGTTCACTAATCCTGA AATTGCTGTTACACTGTCTGCAATAGTATTTGCTGGGTCAAATGTTACATCTTTAACATC ATTTTTGTTTAGGTATGAATAAACTCCTTCAACATCAACCCTGATGTCGTCCATTTTGTA ACCAAATGCACCACCACCAGCTATAAAAGATGGTTTTAATGGACTGTAATCACTCTTGTC TTTCTTATAGGTAATACCATCAACTTTTGTGAAAAGAGGTAAAAATTCACCGTTGTATTG CAAACGAACGTAGTAGCTAGTTTCTTCATCATAATTTG Product length: 518 bp

S4 Tab. 3 – Continued

Gene	Primer	*E. americanus*	Sequencing result
wsp	691R	-male-	GACTTACTTTACATTTTTTTGAATCCTTTCGCACCTGCTGCAGCACCTGGTCCGGTGTAT GAACCCTGATTGCTTATTTTTTTTCCTCCTCACCTCTCCTCGACCCCAAATAATCCCCTG CATCATTAACTACACTGCCCGGACTCACCATCAACTGGCCACTGCAAAACTTGGACTTTT ACCCCAATTTACTTTTTTGATCATTCACAGCGGGTTCCAAAGGAGTGCTAATATACGCTG CACCCACACCACCACCATTATATGGAGTGATAGGCATATCTTCAATTGCTATATCGTAAT ACACTTTCACTAATCCTGAAATTGCTGTTACACTGACTGCAATAATATTTGCTGGGTCAA ATGTTACATCTTTAACATCATTTTTGTTTAGGTATGAATAAACTCCTTCAACATCAACCC TGATGTCGTCCATTTTGTAACCAAATGCTCCACCACCAGCTATAAAAGATGGTTTTAATG GACTGTAATCACTCTTGTCTTTCTTATATTTAATACCATCAACTTTTGTGAAAAGAGGTA AAAATTCACCGTTGTATTGCAAACCAACGTAATAGCTAGTTCCTTCATCACTTATTGTAT TTTATTTTAACGGTTAGTTTCAAGATTTCTCCCGACGATATCGTAGTTTTAAGCATCCAC CTACTCGAGAGAAACCAATGTGTAAACAGGATTGACGTTATACACCCAAACTATTACTTC GTACAAGCCACGACCACTATCCAGGCACACATCCCACTGGTTCT Product length: 764 bp
ftsZ	494F	-female-	GTAATTCCAAATCAGAATTTATTTAGAATTGCAAATGAAAAAACTACATTTTCTGATGCA TTTAAACTTGCTGATAATGTTCTGCACATTGGCATCAGAGGAGTAACTGACTTGATGGTC ATGCCAGGGCTTATCAATCTTGACTTCGCTGATATAGAAACAGTAATGAGCGAGATGGGC AAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAAGATAGAGCAATTAGTGCTGCAGAG GCTGCAATATCTAATCCATTGCTTGATAATGTATCAATGAAAGGTGCGCAAGGAATATTA ATTAACATTACTGGTGGCGGAGATATGACTCTGTTTGAAGTTGATGCTGCAGCCAATAGA GTGCGTGAAGAAGTAGATGAAAATGCAAATATAATATTTGGTGCTACTTTTGATCAAGCG ATGGAGGGAAGAGTTAGAGTTTCTGTTCTTGCAACTGGCATTGATGGTCGCAATAATAAA TCAGAAACTTCACCTATAAGTCAGAGCGAAGACTCAGAGAAAGAGAAATTTAAGTGGCCA TATAGTCAAAGTGAAAGTACACAAGACAAAACACTGGAAACAAAACCAGCTGAACAGGTA AGCGAAGGAGCTAAGTGGGGCAGCAATATCTATGATATACCAGCTTACTTAAGAAGAAAA AAATAATGCAATTTTGGCTACTTAAGTCAGAGCCATAAAC Product length: 700 bp
	494F	-male-	CTTATTGTCATTCCAAATCAGAATTTATTTAGAATTGCAAATGAAAAAACTACATTTTCT GATGCATTTAAACTTGCTGATAATGTTCTGCACATTGGCATCAGAGGAGTAACTGACTTG ATGGTCATGCCAGGGCTTATCAATCTTGACTTCGCTGATATAGAAACAGTAATGAGCGAG ATGGGCAAAGCGATGATCGGCACCGGAGAGGCAGAAGGAGAAGATAGAGCAATTAGTGCT GCAGAGGCTGCAATATCTAATCCATTGCTTGATAATGTATCAATGAAAGGTGCGCAAGGA ATATTAATTAACATTACTGGTGGCGGAGATATGACTCTGTTTGAAGTTGATGCTGCAGCC AATAGAGTGCGTGAAGAAGTAGATGAAAATGCAAATATAATATTTGGTGCTACTTTTGAT CAAGCGATGGAGGGAAGAGTTAGAGTTTCTGTTCTTGCAACTGGCATTGATGGTCGCAAT AATAAATCAGAAACTTCACCTATAAGTCAGAGCGAAGACTCAGAGAAAGAGAAATTTAAG TGGCCATATAGTCAAAGTGAAAGTACACAAGACAAAACACTGGAAACAAAACCAGCTGAA CAGGTAAGCGAAGGAGCTAAGTGGGGCAGCAATATCTATGATATACCAGCTTACTTAAGA AGAAAAAAATAATGCAATTTTGGCTACTTAAGTCAGAGCCATACACCC Product length: 708 bp
	1262R	-female-	TACCTGAAGTAAGCTGGTATATCATAGATATTGCTGCCCCACTTAGCTCCTTCGCTTACC TGTTCAGCTGGTTTTGTTTCCAGTGTTTTGTCTTGTGTACTTTCACTTTGACTATATGGC CACTTAAATTTCTCTTTCTCTGAGTCTTCGCTCTGACTTATAGGTGAAGTTTCTGATTTA TTATTGCGACCATCAATGCCAGTTGCAAGAACAGAAACTCTAACTCTTCCCTCCATCGCT TGATCAAAAGTAGCACCAAATATTATATTTGCATTTTCATCTACTTCTTCACGCACTCTA TTGGCTGCAGCATCAACTTCAAACAGAGTCATATCTCCGCCACCAGTAATGTTAATTAAT ATTCCTTGCGCACCTTTCATTGATACATTATCAAGCAATGGATTAGATATTGCAGCCTCT GCAGCACTAATTGCTCTATCTTCTCCTTCTGCCTCTCCGGTGCCGATCATCGCTTTGCCC ATCTCGCTCATTACTGTTTCTATATCAGCGAAGTCAAGATTGATAAGCCCTGGCATGACC ATCAAGTCAGTTACTCCTCTGATGCCAATGTGCAGAACATTATCAGCAAGTTTAAATGCA TCAGAAAATGTAGTTTTTTCATTTGCAATTCTAAATAAATTCTGATTTGGAATGACAATA AGTGTATCCACGTATTTTTGCAGTTCTTCAAGTCCAAGCTCTGA Product length: 704 bp
	1262R	-male-	ATTGCTGCCCCACTTAGCTCCTTCGCTTACCTGTTCAGCTGGTTTTGTTTCCAGTGTTTT GTCTTGTGTACTTTCACTTTGACTATATGGCCACTTAAATTTCTCTTTCTCTGAGTCTTC GCTCTGACTTATAGGTGAAGTTTCTGATTTATTATTGCGACCATCAATGCCAGTTGCAAG AACAGAAACTCTAACTCTTCCCTCCATCGCTTGATCAAAAGTAGCACCAAATATTATATT TGCATTTTCATCTACTTCTTCACGCACTCTATTGGCTGCAGCATCAACTTCAAACAGAGT CATATCTCCGCCACCAGTAATGTTAATTAATATTCCTTGCGCACCTTTCATTGATACATT ATCAAGCAATGGATTAGATATTGCAGCCTCTGCAGCACTAATTGCTCTATCTTCTCCTTC TGCCTCTCCGGTGCCGATCATCGCTTTGCCCATCTCGCTCATTACTGTTTCTATATCAGC GAAGTCAAGATTGATAAGCCCTGGCATGACCATCAAGTCAGTTACTCCTCTGATGCCAAT GTGCAGAACATTATCAGCAAGTTTAAATGCATCAGAAAATGTAGTTTTTTCATTTGCAAT TCTAAATAAATTCTGATTTGGAATGACAATAAGTGTATCCACGTATTTTTGCAGTTCTTC AAGTCCAAGCTCTG Product length: 674 bp

S4 Tab. 3 – Continued

Gene	Primer	*E. americanus*	Sequencing result
16S rRNA	CLO-f2	-female-	ACCTTCGGTAAACTTGTCACCTGCAGTTATAGATTGTTTTGTGTTATCAATGTTGCGCAA TAAGTATAATGTGATGCCAACTAACATTGGCGCGAGTGCAAGCAGAAAGATGTATTTTTT CATGGTAATATTCTGAATAGTTATATATTTATACATTAATGCTTTTGATTTGCAAAAACA TATTGACATAGTGATTATATCACATAATAATATTCTTAAAGTTGTTTGAATAGTTGGAGA TTAAAATATTTCTAAAGTAGAAATAAAGACAGCAGTATTAAGCTAAATTATTTTTTGTCG GATTTATCCGATGTACCCTCAACCTTTTATAGGTTGAAGTCTAAATTTGAGAGTTTGATC CTAGCTCAGAATGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGAGTTATATT GTAGCTTGCTATGGTATAACTTAGTGGCAGACGGGTGAGTAATGTATAGGAATCTACCTA GTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCCTACGGGGGAAAA ATTTATTGCTATTAGATGAGCCTATATTAGATTAGCTAGTTGGTGGAGTAATAGCCTACC AAGGCAATGATCTATAGCTGATCTGAGAGGATGATCAGCCACACTGGAACTGAGATACGG TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCC AGCCATGCCGCATGAGTGAAGAAGGCCTTTGGGTTGTAAAGCTCTTTTAGTGAGGAAGAT AATGACGGTACTCACAGAAGAAGTCCTGGCTAACTCCGTGCCAGCAGCCGCGGTAATACG GAGAGGGCTAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGTAGGCGGATTAGTAAG TTAAAAGTGAAATCCCAAGGCTCAACCTTGGAATTGCTTTTAAAACTGCTAATCTAGAGA TTGAAAGAGGATAGAGGAATTCCTAGTGTAGAGGTGAAATTCGTAAATATTAGGAGGAAC Product length: 1020 bp
	rP2	-female-	GAACCACTTTAATAACTCCCTCCTTGCGGTTAGGTCGTTAGCTTCGAGTGAAACCAATTC CCATGGCGTGACGGGCAGTGTGTACAAGACCCGAGAACGTATTCACCGTGGCGTGCTGAT CCACGATTACTAGCGATTCCAACTTCATGCACTCGAGTTGCAGAGTACAATCCGAACTGA GATGGCTTTTAAGGGATTAGCTTAGCCTCGCGACTTTGCAGCCCATTGTAGCCACCATTG TAGCACGTGTGTAGCCCACTCCATAAGGGCCATGATGACTTGACATCATCCCCACCTTCC TCCAGTTTATCACTGGCAGTTTCCTTAAAGTCCCCAGCATTACCTGATGGTAACTAAGGA TGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGC CATGCAACACCTGTGTGAAACCCGGCCGAACCGACCCTATCCCTTCGAATAGGTATAATT TCCATGTCAAGGAGTGGTAAGGTTTTTCGCGTTGCATCGAATTAAACCACATGCTCCACC GCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTAGTCCCCAGGC GGAATGTTTAACGCGTTAGCTGTAATACAGAAAGTAAAACTTCCCATATTTAACATTCAT CGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCCTTCGCGCCT CAGCGTCAGATTTGAACCAGATAGACGCCTTCGCCACTGGTGTTCCTCCTAATATTTACG AATTTCACCTCTACACTAGGAATTCCTCTATCCTCTTTCAATCTCTAGATTAGCAGTTTT AAAAGCAATTCCAAGGTTGAGCCTTGGGATTTCACTTTTAACTTACTAATCCGCCTACGC GCCCTTTACGCCCAATAATTCCGAATAACGCTAGCCCTCTCCGTATTACCGCGGCTGCTG GCACGGAGTTAGCCAGGACTTCTTCTGTGAGTACCGTCATTATCTTCCTCACTAAAAG Product length: 1018 bp

S4 Tab. 4. Total results for the *Cardinium* effect in the co-infected *E*. *americanus* for the crossed combinations over an 8 d period. Data for each trait are mean ± SE. Percentage dates for the unhatched eggs and sex ratio male were calculated from mean values. Not normal distributed data were transformed via extraction of square root, so that a one-way ANOVA and pairwise compared with Tukey's post hoc test ($p < 0.05$) could be performed. Nonparametric test (Kruskal-Wallis) was used for not normal distributed data even after transformation (unhatched eggs). (I_C –*Cardinium* infected organism, I_W – *Wolbachia* infected organism, I_{WC} – co-infected with *Wolbachia* and *Cardinium*, U – uninfected organism).

Crosses f x m	N	Number of eggs	Unhatched eggs	Sex ratio male	Male offspring	Female offspring	Total offspring	Sex ratio male in %	Unhatched eggs in %
U x U	3	24.33 ± 5.46	16.00 ± 3.21	0.05 ± 0.05	1.00 ± 1.00	6.00 ± 6.00	7.00 ± 7.00	4.76 ± 4.76	65.75 ± 13.21
U x I_W	3	19.67 ± 3.71	4.33 ± 2.96	0.26 ± 0.09	3.33 ± 1.20	11.00 ± 5.51	14.33 ± 6.36	25.75 ± 9.43	22.03 ± 15.06
I_W x U	3	16.00 ± 4.00	0.00 ± 0.00	0.41 ± 0.01	6.50 ± 1.50	9.50 ± 2.50	16.00 ± 4.00	40.83 ± 0.83	0.00 ± 0.00
I_W x I_W	38	23.58 ± 1.16	0.61 ± 0.16	0.34 ± 0.02	6.97 ± 0.55	14.29 ± 1.05	21.26 ± 1.26	33.73 ± 2.31	2.57 ± 0.67
I_{WC} x U	2	22.50 ± 1.50	0.50 ± 0.50	0.34 ± 0.01	6.50 ± 0.50	12.50 ± 1.50	19.00 ± 2.00	34.31 ± 0.98	2.22 ± 2.22
I_{WC} x I_{WC}	33	26.30 ± 1.61	0.76 ± 0.29	0.27 ± 0.02	5.88 ± 0.54	16.97 ± 1.58	22.85 ± 1.87	27.33 ± 1.85	2.88 ± 1.10
I_{WC} x I_W	13	20.77 ± 2.80	0.46 ± 0.27	0.41 ± 0.04	6.69 ± 1.28	11.46 ± 2.25	18.15 ± 2.99	40.79 ± 4.44	2.22 ± 1.29
I_{WC} x I_C	2	18.00 ± 2.00	0.50 ± 0.50	0.25 ± 0.00	3.50 ± 1.50	10.50 ± 4.50	14.00 ± 6.00	25.00 ± 0.00	2.78 ± 2.78
I_W x I_{WC}	12	22.33 ± 2.36	1.17 ± 0.34	0.39 ± 0.03	7.67 ± 0.74	13.00 ± 1.90	20.67 ± 2.29	38.78 ± 3.09	5.22 ± 1.54
I_C x I_{WC}	3	18.33 ± 3.38	0.67 ± 0.67	0.42 ± 0.10	5.33 ± 0.88	9.33 ± 4.48	14.67 ± 5.24	42.29 ± 10.39	3.64 ± 3.64

S4 Tab. 5. Data base searches by BLAST® in NCBI of the sequencing results. Highest homologous sequences for each sequencing product.

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
16S rRNA	99F	-female-	*Wolbachia pipientis* strain *w*Irr chromosome, complete genome	1507	1507	98%	0.0	99.76%	CP037426.1
			Wolbachia endosymbiont of *Drosophila ananassae* strain W2.1 chromosome, complete genome	1507	1507	98%	0.0	99.76%	CP042904.1
			Wolbachia pipientis strain *w*Mel_ZH26 chromosome, complete genome	1507	1507	98%	0.0	99.76%	CP042445.1
			Wolbachia pipientis strain *w*Mel_I23 chromosome, complete genome	1507	1507	98%	0.0	99.76%	CP042444.1
			Wolbachia pipientis strain *w*Mel_N25 chromosome, complete genome	1507	1507	98%	0.0	99.76%	CP042446.1
			Wolbachia endosymbiont of *Carposina sasakii* isolate *w*CauA chromosome, complete genome	1507	1507	98%	0.0	99.76%	CP041215.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopMTK02_BA01	1507	1507	98%	0.0	99.76%	LC370586.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopMTK01_BA01	1507	1507	98%	0.0	99.76%	LC370585.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopKSD03_BA01	1507	1507	98%	0.0	99.76%	LC370584.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopTKB03_BW01	1507	1507	98%	0.0	99.76%	LC370581.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopTKB02_OW01	1507	1507	98%	0.0	99.76%	LC370579.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopMTK02_AW	1507	1507	98%	0.0	99.76%	LC370785.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopMTK01_AW	1507	1507	98%	0.0	99.76%	LC370784.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopKSD03_AW	1507	1507	98%	0.0	99.76%	LC370783.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopTKB03_AW	1507	1507	98%	0.0	99.76%	LC370780.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopTKB02_AW	1507	1507	98%	0.0	99.76%	LC370779.1
			Uncultured *Wolbachia* sp. clone P4 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	KY558892.1
			Wolbachia endosymbiont of *Drosophila incompta* strain *w*Inc_Cu genome	1507	1507	98%	0.0	99.76%	CP011148.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	KT319093.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	KT319090.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	KT319088.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
16S rRNA	99F	-female-	*Wolbachia pipientis* clone 1 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	KT319087.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	KT319086.1
			Wolbachia endosymbiont of *Drosophila simulans w*Au genome assembly, chromosome: 1	1507	1507	98%	0.0	99.76%	LK055284.1
			Wolbachia endosymbiont of *Kleidocerys resedae* clone KrWlbOkn1 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	JQ726769.1
			Wolbachia secondary endosymbiont of *Koreoculio minutissimus* gene for 16S ribosomal RNA, partial sequence, clone: P47_3	1507	1507	98%	0.0	99.76%	AB604663.1
			Wolbachia secondary endosymbiont of *Archarius roelofsi* gene for 16S ribosomal RNA, partial sequence, clone: P11_4	1507	1507	98%	0.0	99.76%	AB604662.1
			Wolbachia secondary endosymbiont of *Curculio hachijoensis* gene for 16S ribosomal RNA, partial sequence, clone: P12_7	1507	1507	98%	0.0	99.76%	AB604661.1
			Wolbachia sp. *w*Ri, complete genome	1507	1507	98%	0.0	99.76%	CP001391.1
			Wolbachia endosymbiont of *Ephestia kuehniella* gene for 16S ribosomal RNA, partial sequence, strain: *w*Kue	1507	1507	98%	0.0	99.76%	AB360384.1
			Wolbachia pipientis strain EW-p 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	EU096232.1
			Wolbachia endosymbiont of *Drosophila ananassae* strain *w*Ri 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	DQ412084.1
			Wolbachia endosymbiont of *Drosophila melanogaster* strain *w*Mel 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	DQ412083.1
			Wolbachia sp. Dcris16SWol 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	AY007550.1
			Wolbachia sp. Ablan16SWol 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	AY007548.1
			Wolbachia sp. Dlem16SWol 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	99.76%	AY007547.1
			Wolbachia endosymbiont of *Drosophila melanogaster*, complete genome	1507	1507	98%	0.0	99.76%	AE017196.1
			Wolbachia pipientis partial 16S rRNA gene, strain hr1/dsz	1507	1507	98%	0.0	99.76%	AJ306308.1
			Wolbachia sp. DNA for 16S ribosomal RNA (*A.diaspidis* as host)	1507	1507	98%	0.0	99.76%	X87407.1
	99F	-male-	*Wolbachia pipientis* strain *w*Irr chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP037426.1
			Wolbachia endosymbiont of *Drosophila ananassae* strain W2.1 chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP042904.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
16S rRNA	99F	-male-	*Wolbachia pipientis* strain *w*Mel_ZH26 chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP042445.1
			Wolbachia pipientis strain *w*Mel_I23 chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP042444.1
			Wolbachia pipientis strain *w*Mel_N25 chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP042446.1
			Wolbachia endosymbiont of *Carposina sasakii* isolate *w*CauA chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP041215.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopMTK02_BA01	1500	1500	99%	0.0	99.88%	LC370586.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopMTK01_BA01	1500	1500	99%	0.0	99.88%	LC370585.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopKSD03_BA01	1500	1500	99%	0.0	99.88%	LC370584.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopTKB03_BW01	1500	1500	99%	0.0	99.88%	LC370581.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopTKB02_OW01	1500	1500	99%	0.0	99.88%	LC370579.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopMTK02_AW	1500	1500	99%	0.0	99.88%	LC370785.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopMTK01_AW	1500	1500	99%	0.0	99.88%	LC370784.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopKSD03_AW	1500	1500	99%	0.0	99.88%	LC370783.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopTKB03_AW	1500	1500	99%	0.0	99.88%	LC370780.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopTKB02_AW	1500	1500	99%	0.0	99.88%	LC370779.1
			Wolbachia endosymbiont of *Drosophila incompta* strain *w*Inc_Cu genome	1500	1500	99%	0.0	99.88%	CP011148.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319093.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319090.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319088.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319087.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319086.1
			Wolbachia endosymbiont of *Drosophila simulans w*Au genome assembly, chromosome: 1	1500	1500	99%	0.0	99.88%	LK055284.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
16S rRNA	99F	-male-	*Wolbachia* endosymbiont of *Kleidocerys resedae* clone KrWlbOkn1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	JQ726769.1
			Wolbachia endosymbiont of *Kleidocerys resedae* clone KrWlbOkn1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	JQ726769.1
			Wolbachia secondary endosymbiont of *Koreoculio minutissimus* gene for 16S ribosomal RNA, partial sequence, clone: P47_3	1500	1500	99%	0.0	99.88%	AB604663.1
			Wolbachia secondary endosymbiont of *Archarius roelofsi* gene for 16S ribosomal RNA, partial sequence, clone: P11_4	1500	1500	99%	0.0	99.88%	AB604662.1
			Wolbachia secondary endosymbiont of *Curculio hachijoensis* gene for 16S ribosomal RNA, partial sequence, clone: P12_7	1500	1500	99%	0.0	99.88%	AB604661.1
			Wolbachia sp. *w*Ri, complete genome	1500	1500	99%	0.0	99.88%	CP001391.1
			Wolbachia pipientis strain EW-p 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	EU096232.1
			Wolbachia endosymbiont of *Drosophila ananassae* strain *w*Ri 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	DQ412084.1
			Wolbachia endosymbiont of *Drosophila melanogaster* strain *w*Mel 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	DQ412083.1
			Wolbachia sp. Dcris16SWol 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	AY007550.1
			Wolbachia sp. Ablan16SWol 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	AY007548.1
			Wolbachia sp. Dlem16SWol 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	AY007547.1
			Wolbachia endosymbiont of *Drosophila melanogaster*, complete genome	1500	1500	99%	0.0	99.88%	AE017196.1
			Wolbachia pipientis partial 16S rRNA gene, strain hr1/dsz	1500	1500	99%	0.0	99.88%	AJ306308.1
			Wolbachia sp. DNA for 16S ribosomal RNA (*A.diaspidis* as host)	1500	1500	99%	0.0	99.88%	X87407.1
	994R	-male-	*Wolbachia pipientis* strain *w*Irr chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP037426.1
			Wolbachia endosymbiont of *Drosophila ananassae* strain W2.1 chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP042904.1
			Wolbachia pipientis strain *w*Mel_ZH26 chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP042445.1
			Wolbachia pipientis strain *w*Mel_I23 chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP042444.1
			Wolbachia pipientis strain *w*Mel_N25 chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP042446.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
16S rRNA	994R	-male-	*Wolbachia* endosymbiont of *Carposina sasakii* isolate *w*CauA chromosome, complete genome	1500	1500	99%	0.0	99.88%	CP041215.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopMTK02_BA01	1500	1500	99%	0.0	99.88%	LC370586.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopMTK01_BA01	1500	1500	99%	0.0	99.88%	LC370585.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopKSD03_BA01	1500	1500	99%	0.0	99.88%	LC370584.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopTKB03_BW01	1500	1500	99%	0.0	99.88%	LC370581.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, clone: MopTKB02_OW01	1500	1500	99%	0.0	99.88%	LC370579.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopMTK02_AW	1500	1500	99%	0.0	99.88%	LC370785.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopMTK01_AW	1500	1500	99%	0.0	99.88%	LC370784.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopKSD03_AW	1500	1500	99%	0.0	99.88%	LC370783.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopTKB03_AW	1500	1500	99%	0.0	99.88%	LC370780.1
			Wolbachia sp. gene for 16S rRNA, partial sequence, strain: MopTKB02_AW	1500	1500	99%	0.0	99.88%	LC370779.1
			Wolbachia endosymbiont of *Drosophila incompta* strain *w*Inc_Cu genome	1500	1500	99%	0.0	99.88%	CP011148.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319093.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319090.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319088.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319087.1
			Wolbachia pipientis clone 1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	KT319086.1
			Wolbachia endosymbiont of *Drosophila simulans w*Au genome assembly, chromosome: 1	1500	1500	99%	0.0	99.88%	LK055284.1
			Wolbachia endosymbiont of *Kleidocerys resedae* clone KrWlbOkn1 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	JQ726769.1
			Wolbachia secondary endosymbiont of *Koreoculio minutissimus* gene for 16S ribosomal RNA, partial sequence, clone: P47_3	1500	1500	99%	0.0	99.88%	AB604663.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
16S rRNA	994R	-male-	*Wolbachia* secondary endosymbiont of *Archarius roelofsi* gene for 16S ribosomal RNA, partial sequence, clone: P11_4	1500	1500	99%	0.0	99.88%	AB604662.1
			Wolbachia secondary endosymbiont of *Curculio hachijoensis* gene for 16S ribosomal RNA, partial sequence, clone: P12_7	1500	1500	99%	0.0	99.88%	AB604661.1
			Wolbachia sp. *w*Ri, complete genome	1500	1500	99%	0.0	99.88%	CP001391.1
			Wolbachia pipientis strain EW-p 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	EU096232.1
			Wolbachia endosymbiont of *Drosophila ananassae* strain *w*Ri 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	DQ412084.1
			Wolbachia endosymbiont of *Drosophila melanogaster* strain *w*Mel 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	DQ412083.1
			Wolbachia sp. Dcris16SWol 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	AY007550.1
			Wolbachia sp. Ablan16SWol 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	AY007548.1
			Wolbachia sp. Dlem16SWol 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	99.88%	AY007547.1
			Wolbachia endosymbiont of *Drosophila melanogaster*, complete genome	1500	1500	99%	0.0	99.88%	AE017196.1
wsp	81F	-female-	*Wolbachia* endosymbiont of *Anastrepha* sp. 2 outer surface protein precursor (*wsp*) gene, partial cds	931	931	98%	0.0	99.42%	EU116316.1
			Wolbachia endosymbiont of *Anastrepha striata* outer surface protein precursor (*wsp*) gene, partial cds	929	929	98%	0.0	99.42%	EU116328.1
			Wolbachia endosymbiont of *Anastrepha grandis* outer surface protein precursor (*wsp*) gene, partial cds	929	929	98%	0.0	99.42%	EU116327.1
			Wolbachia endosymbiont of *Anastrepha striata* outer surface protein precursor (*wsp*) gene, partial cds	929	929	98%	0.0	99.42%	EU116319.1
			Wolbachia endosymbiont of *Ostrinia furnacalis* strain *w*Fur7 outer surface protein precursor (wsp) gene, partial cds	928	928	98%	0.0	99.41%	GU166594.1
			Wolbachia endosymbiont of *Echinothrips americanus* strain Morgan outer surface protein precursor (wsp) gene, partial cds	928	928	98%	0.0	99.41%	JN315668.1
			Wolbachia endosymbiont of *Drosophila prosaltans* isolate SG2 outer surface protein (*wsp*) gene, partial cds	928	928	98%	0.0	99.41%	DQ118779.1
			Wolbachia endosymbiont of *Drosophila septentriosaltans* strain *w*Spt BCI2 outer surface protein (*wsp*) gene, partial cds	928	928	98%	0.0	99.41%	AY620210.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
wsp	81F	-female-	*Wolbachia* endosymbiont of *Drosophila septentriosaltans* strain *w*Spt BCI1 outer surface protein (*wsp*) gene, partial cds	928	928	98%	0.0	99.41%	AY620209.1
	691R	-female-	*Wolbachia* endosymbiont of *Drosophila* sp. isolate D3L surface protein (*wsp*) gene, partial cds	931	931	99%	0.0	99.42%	MN900912.1
			Wolbachia pipientis strain *w*Mel_ZH26 chromosome, complete genome	929	929	97%	0.0	99.80%	CP042445.1
			Wolbachia pipientis strain *w*Mel_I23 chromosome, complete genome	929	929	97%	0.0	99.80%	CP042444.1
			Wolbachia pipientis strain *w*Mel_N25 chromosome, complete genome	929	929	97%	0.0	99.80%	CP042446.1
			Wolbachia endosymbiont of *Rhyzopertha dominica* isolate *w*RhdA outer surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	KY781898.1
			Wolbachia endosymbiont of *Drosophila melanogaster* isolate *w*MelPop outer surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	KX650073.1
			Wolbachia endosymbiont of *Drosophila melanogaster* isolate *w*Mel outer surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	KX650072.1
			Wolbachia endosymbiont of *Hypothenemus hampei* isolate A_control_2 outer surface protein (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	KX436089.1
			Wolbachia endosymbiont of *Hypothenemus hampei* isolate A_control_1 outer surface protein (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	KX436088.1
			Wolbachia endosymbiont of *Hypothenemus hampei* isolate H_control_1 outer surface protein (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	KX436087.1
			Wolbachia endosymbiont of *Drosophila melanogaster* isolate Beijing outer surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	KU870673.1
			Wolbachia endosymbiont of *Drosophila incompta* strain *w*Inc_Cu genome	929	929	97%	0.0	99.80%	CP011148.1
			Wolbachia endosymbiont of *Anastrepha fraterculus* clone 46.1 outer surface protein (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	KC589027.1
			Wolbachia endosymbiont of *Rhyzopertha dominica* strain WrdoA surface protein (w*sp*) gene, partial cds	929	929	97%	0.0	99.80%	JN603592.1
			Wolbachia endosymbiont of *Drosophila* sp. isolate D3G surface protein (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	MN900914.1
			Wolbachia endosymbiont of *Drosophila* sp. isolate D3E surface protein (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	MN900913.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
wsp	691R	-female-	*Wolbachia pipientis* strain *w*Mel isolate Varva outer surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	HM775086.1
			Wolbachia endosymbiont of *Ostrinia furnacalis* strain *w*Fur7 outer surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	GU166594.1
			Wolbachia endosymbiont of *Echinothrips americanus* strain Morgan outer surface protein precursor (wsp) gene, partial cds	929	929	97%	0.0	99.80%	JN315668.1
			Wolbachia sp. *w*Mel isolate Yunnan outer surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	FJ403332.1
			Wolbachia sp. *w*Mel isolate Wuhan outer surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	FJ403330.1
			Wolbachia endosymbiont of *Bactrocera dorsalis* strain *w*DorHN1 surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	DQ834379.1
			Wolbachia pipientis strain *w*MelPop outer surface protein precursor, gene, complete cds	929	929	97%	0.0	99.80%	AF338346.1
			Wolbachia sp. Wmel outer surface protein precursor (*wsp*) gene, partial cds	929	929	97%	0.0	99.80%	AF020065.1
			Wolbachia endosymbiont of *Drosophila septentriosaltans* strain *w*Spt BCI1 outer surface protein *(wsp*) gene, partial cds	929	929	97%	0.0	99.80%	AY620209.1
			Wolbachia endosymbiont of *Drosophila melanogaster*, complete genome	929	929	97%	0.0	99.80%	AE017196.1
wsp	81F	-male-	*Wolbachia* endosymbiont of *Drosophila incompta* strain *w*Inc_Cu genome	1072	1072	99%	0.0	99.49%	CP011148.1
			Wolbachia endosymbiont of *Ostrinia furnacalis* strain *w*Fur7 outer surface protein precursor (*wsp*) gene, partial cds	1072	1072	99%	0.0	99.49%	GU166594.1
			Wolbachia endosymbiont of *Echinothrips americanus* strain Morgan outer surface protein precursor (wsp) gene, partial cds	1072	1072	99%	0.0	99.49%	JN315668.1
			Wolbachia endosymbiont of *Drosophila septentriosaltans* strain *w*Spt BCI1 outer surface protein (*wsp*) gene, partial cds	1072	1072	99%	0.0	99.49%	AY620209.1
wsp	691R	-male-	*Wolbachia pipientis* strain *w*Mel_ZH26 chromosome, complete genome	689	689	53%	0.0	96.84%	CP042445.1
			Wolbachia pipientis strain *w*Mel_I23 chromosome, complete genome	689	689	53%	0.0	96.84%	CP042444.1
			Wolbachia pipientis strain *w*Mel_N25 chromosome, complete genome	689	689	53%	0.0	96.84%	CP042446.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
wsp	691R	-male-	*Wolbachia* endosymbiont of *Rhyzopertha dominica* isolate *w*RhdA outer surface protein precursor (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	KY781898.1
			Wolbachia endosymbiont of *Drosophila melanogaster* isolate *w*MelPop outer surface protein precursor (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	KX650073.1
			Wolbachia endosymbiont of *Drosophila melanogaster* isolate *w*Mel outer surface protein precursor (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	KX650072.1
			Wolbachia endosymbiont of *Hypothenemus hampei* isolate A_control_2 outer surface protein (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	KX436089.1
			Wolbachia endosymbiont of *Hypothenemus hampei* isolate A_control_1 outer surface protein (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	KX436088.1
			Wolbachia endosymbiont of *Hypothenemus hampei* isolate H_control_1 outer surface protein (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	KX436087.1
			Wolbachia endosymbiont of *Drosophila melanogaster* isolate Beijing outer surface protein precursor (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	KU870673.1
			Wolbachia endosymbiont of *Drosophila incompta* strain *w*Inc_Cu genome	689	689	53%	0.0	96.84%	CP011148.1
			Wolbachia endosymbiont of *Rhyzopertha dominica* strain WrdoA surface protein (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	JN603592.1
			Wolbachia endosymbiont of *Drosophila* sp. isolate D3G surface protein (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	MN900914.1
			Wolbachia endosymbiont of *Drosophila* sp. isolate D3E surface protein (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	MN900913.1
			Wolbachia endosymbiont of *Ostrinia furnacalis* strain *w*Fur7 outer surface protein precursor (wsp) gene, partial cds	689	689	53%	0.0	96.84%	GU166594.1
			Wolbachia endosymbiont of *Echinothrips americanus* strain Morgan outer surface protein precursor (wsp) gene, partial cds	689	689	53%	0.0	96.84%	JN315668.1
			Wolbachia sp. *w*Mel isolate Yunnan outer surface protein precursor (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	FJ403332.1
			Wolbachia sp. *w*Mel isolate Wuhan outer surface protein precursor (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	FJ403330.1
			Wolbachia endosymbiont of *Bactrocera dorsalis* strain *w*DorHN1 surface protein precursor (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	DQ834379.1
			Wolbachia pipientis strain *w*MelPop outer surface protein precursor, gene, complete cds	689	689	53%	0.0	96.84%	AF338346.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
wsp	691R	-male-	*Wolbachia* endosymbiont of *Drosophila septentriosaltans* strain *w*Spt BCI1 outer surface protein (*wsp*) gene, partial cds	689	689	53%	0.0	96.84%	AY620209.1
			Wolbachia endosymbiont of *Drosophila melanogaster*, complete genome	689	689	53%	0.0	96.84%	AE017196.1
ftsZ	494F	-female-	*Wolbachia* endosymbiont of *Drosophila ananassae* strain W2.1 chromosome, complete genome	1279	1279	98%	0.0	100.00%	CP042904.1
			Wolbachia endosymbiont of *Carposina sasakii* isolate *w*CauA chromosome, complete genome	1279	1279	98%	0.0	100.00%	CP041215.1
			Wolbachia endosymbiont of *Drosophila simulans w*Au genome assembly, chromosome: 1	1279	1279	99%	0.0	99.86%	LK055284.1
			Wolbachia endosymbiont of *Drosophila simulans w*Ha, complete genome	1279	1279	99%	0.0	99.86%	CP003884.1
			Wolbachia sp. *w*Ri, complete genome	1279	1279	98%	0.0	100.00%	CP001391.1
	494F	-male-	*Wolbachia* endosymbiont of *Drosophila ananassae* strain W2.1 chromosome, complete genome	1265	1436	99%	0.0	100.00%	CP042904.1
			Wolbachia endosymbiont of *Carposina sasakii* isolate *w*CauA chromosome, complete genome	1265	1432	99%	0.0	100.00%	CP041215.1
			Wolbachia endosymbiont of *Drosophila simulans w*Au genome assembly, chromosome: 1	1265	1432	99%	0.0	100.00%	LK055284.1
			Wolbachia endosymbiont of *Drosophila simulans w*Ha, complete genome	1265	1432	99%	0.0	100.00%	CP003884.1
			Wolbachia sp. *w*Ri, complete genome	1265	1436	99%	0.0	100.00%	CP001391.1
	1262R	-female-	*Wolbachia* endosymbiont of *Drosophila ananassae* strain W2.1 chromosome, complete genome	1288	1288	99%	0.0	99.86%	CP042904.1
			Wolbachia endosymbiont of *Carposina sasakii* isolate *w*CauA chromosome, complete genome	1288	1288	99%	0.0	99.86%	CP041215.1
			Wolbachia endosymbiont of *Drosophila ananassae* isolate *w*RiDL1 cell division protein *ftsZ* gene, partial cds	1288	1288	99%	0.0	99.86%	MK955789.1
			Wolbachia endosymbiont of *Drosophila ananassae* isolate *w*RiOR1 cell division protein *ftsZ* gene, partial cds	1288	1288	99%	0.0	99.86%	MK955788.1
			Wolbachia endosymbiont of *Drosophila ananassae* isolate *w*RiRmp1 cell division protein *ftsZ* gene, partial cds	1288	1288	99%	0.0	99.86%	MK955787.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
ftsZ	1262R	-female-	*Wolbachia* endosymbiont of *Drosophila simulans* isolate *w*RiPant1 cell division protein *ftsZ* gene, partial cds	1288	1288	99%	0.0	99.86%	MK955786.1
			Wolbachia endosymbiont of *Drosophila simulans w*Au genome assembly, chromosome: 1	1288	1288	99%	0.0	100.00%	LK055284.1
			Wolbachia endosymbiont of *Drosophila simulans w*Ha, complete genome	1288	1288	99%	0.0	100.00%	CP003884.1
			Wolbachia sp. *w*Ri, complete genome	1288	1288	99%	0.0	99.86%	CP001391.1
	1262R	-male-	*Wolbachia* endosymbiont of *Drosophila ananassae* strain W2.1 chromosome, complete genome	1216	1421	100%	0.0	100.00%	CP042904.1
			Wolbachia endosymbiont of *Carposina sasakii* isolate *w*CauA chromosome, complete genome	1216	1384	100%	0.0	100.00%	CP041215.1
			Wolbachia endosymbiont of *Drosophila ananassae* isolate *w*RiDL1 cell division protein *ftsZ* gene, partial cds	1216	1216	100%	0.0	100.00%	MK955789.1
			Wolbachia endosymbiont of *Drosophila ananassae* isolate *w*RiOR1 cell division protein *ftsZ* gene, partial cds	1216	1216	100%	0.0	100.00%	MK955788.1
			Wolbachia endosymbiont of *Drosophila ananassae* isolate *w*RiRmp1 cell division protein *ftsZ* gene, partial cds	1216	1216	100%	0.0	100.00%	MK955787.1
			Wolbachia endosymbiont of *Drosophila simulans* isolate *w*RiPant1 cell division protein *ftsZ* gene, partial cds	1216	1216	100%	0.0	100.00%	MK955786.1
			Wolbachia endosymbiont of *Drosophila simulans w*Au genome assembly, chromosome: 1	1216	1384	100%	0.0	100.00%	LK055284.1
			Wolbachia endosymbiont of *Scleroderma guani* cell division protein (*ftsZ*) gene, partial cds	1216	1216	100%	0.0	100.00%	KC823117.1
			Wolbachia endosymbiont of *Drosophila simulans w*Ha, complete genome	1216	1384	100%	0.0	100.00%	CP003884.1
			Wolbachia sp. *w*Ri, complete genome	1216	1421	100%	0.0	100.00%	CP001391.1
			Wolbachia endosymbiont of *Drosophila simulans* strain *w*Ha cell division protein (*ftsZ*) gene, partial cds	1216	1216	100%	0.0	100.00%	AY508998.1
			Wolbachia endosymbiont of *Spalangia cameroni* FtsZ (*ftsZ*) gene, partial cds	1216	1216	100%	0.0	100.00%	AF289701.1
			Wolbachia endosymbiont of *Spalangia cameroni* FtsZ (*ftsZ*) gene, partial cds	1216	1216	100%	0.0	100.00%	AF289697.1
			Wolbachia endosymbiont of *Spalangia cameroni* FtsZ (*ftsZ*) gene, partial cds	1216	1216	100%	0.0	100.00%	AF289696.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
16S rRNA	CLO-f2	-female-	*Cardinium* endosymbiont of *Aphytis* sp. 16S ribosomal RNA gene, partial sequence	248	248	44%	6e-66	77.10%	AY327473.1
	rP2	-female-	*Cardinium* endosymbiont of *Bemisia tabaci* partial 16S rRNA gene, clone 29_2_3	549	549	92%	1e-156	77.57%	LN829689.2
			Cardinium sp. Jabalpur2014 gene for 16S ribosomal RNA, partial sequence, isolate: Jabalpur2014	545	545	91%	2e-155	77.57%	LC159289.1
			Cardinium endosymbiont of *Bemisia tabaci* partial 16S rRNA gene, clone 20_3_2	545	545	91%	2e-155	77.57%	LN829677.2
			Cardinium endosymbiont of *Bemisia tabaci* partial 16S rRNA gene, clone 23_1_8	545	545	91%	2e-155	77.57%	LN829684.1
			Cardinium endosymbiont of *Bemisia tabaci* partial 16S rRNA gene, clone 32_3_3	545	545	91%	2e-155	77.57%	LN829682.1
			Cardinium endosymbiont of *Bemisia tabaci* partial 16S rRNA gene, clone 37_2_5	545	545	91%	2e-155	77.57%	LN829679.1
			Cardinium endosymbiont of *Bemisia tabaci* strain Bhatinda 16S ribosomal RNA gene, partial sequence	545	545	91%	2e-155	77.57%	JN204479.1
			Candidatus *Cardinium* sp. clone S5 16S ribosomal RNA gene, partial sequence	545	545	91%	2e-155	77.57%	MN187322.1
			Cardinium endosymbiont of *Pezothrips kellyanus* from Algeria 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640330.1
			Cardinium endosymbiont of *Pezothrips kellyanus* from Spain 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640329.1
			Cardinium endosymbiont of *Pezothrips kellyanus* from Turkey 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640328.1
			Cardinium endosymbiont of *Pezothrips kellyanus* from Cyprus 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640327.1
			Cardinium endosymbiont of *Pezothrips kellyanus* from New Zealand 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640326.1
			Cardinium endosymbiont of *Pezothrips kellyanus* from Australia 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640325.1
			Cardinium endosymbiont of *Pezothrips kellyanus* from Australia 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640324.1

S4 Tab. 5 – Continued

Sample information			NCBI BLAST® results						
Gene	**Primer**	***E. americanus***	**Description**	**Max score**	**Total score**	**Query Cover**	**E value**	**Per. Ident.**	**Accession number**
16S rRNA	rP2	-female-	*Cardinium* endosymbiont of *Pezothrips kellyanus* from Australia 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640323.1
			Cardinium endosymbiont of *Pezothrips kellyanus* from Australia 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640322.1
			Cardinium endosymbiont of *Pezothrips kellyanus* from Australia 16S ribosomal RNA gene, partial sequence	521	521	89%	3e-148	77.32%	KP640321.1

S4 Supplementary reference

Jilge, M. 2016. Entwicklung und Einsatz künstlicher Diäten zur gezielten quantitativen Applikation spezifischer Wirksubstanzen bei phytosugen Terebrantia-Arten (*Frankliniella occidentalis*, *Echinothrips americanus*). M.S. thesis, Martin-Luther-University Halle-Wittenberg, Halle.

CHAPTER S5

Distribution patterns of *Wolbachia* and *Cardinium* during female progenesis and embryonic development of *Echinothrips americanus* and *Hercinothrips femoralis*

S5 Suppl. method: Giemsa staining

For the Giemsa staining, thrips females of the laboratory culture were fixed, dehydrated and embedding after following conditions:

Tab.1. Preparation procedure of specimens for analysis by light microscopy

Procedure	Chemicals	Time conditions
Fixation	Carnoy solution (composed of 60 ml 100% ethanol, 30 ml chloroform, 10 ml glacial acetic acid)	1 h
Dehydration	70% ethanol	1 h
	80% ethanol	1 h
	90% ethanol	1 h
	100% ethanol	1 h
Transition solvent	Isopropyl alcohol I	2 h
	Isopropyl alcohol II (fresh)	2 h
	Isopropyl alcohol III (fresh)	2 h
Infiltration	50 : 50, isopropyl alcohol : paraplast	24 h
	Paraplast I	24 h
	Paraplast II (fresh)	24 h
	Paraplast III (fresh)	24 h
Embedding	Pure paraplast in petri dishes	Room temperature

The embedding specimens were sliced into histological sections (6 µm) on the microtome (SM 2010 R, Leica). Subsequently, the sections were transferred in a drop of distilled water on a microscope slide (coated with albumin-glycerol) and dried at 37 °C on a hot plate. For staining the dried sections were deparaffinised (Tab. 2) and coloured after the modified Giemsa staining method in Romeis Mikroskopische Technik 2010.

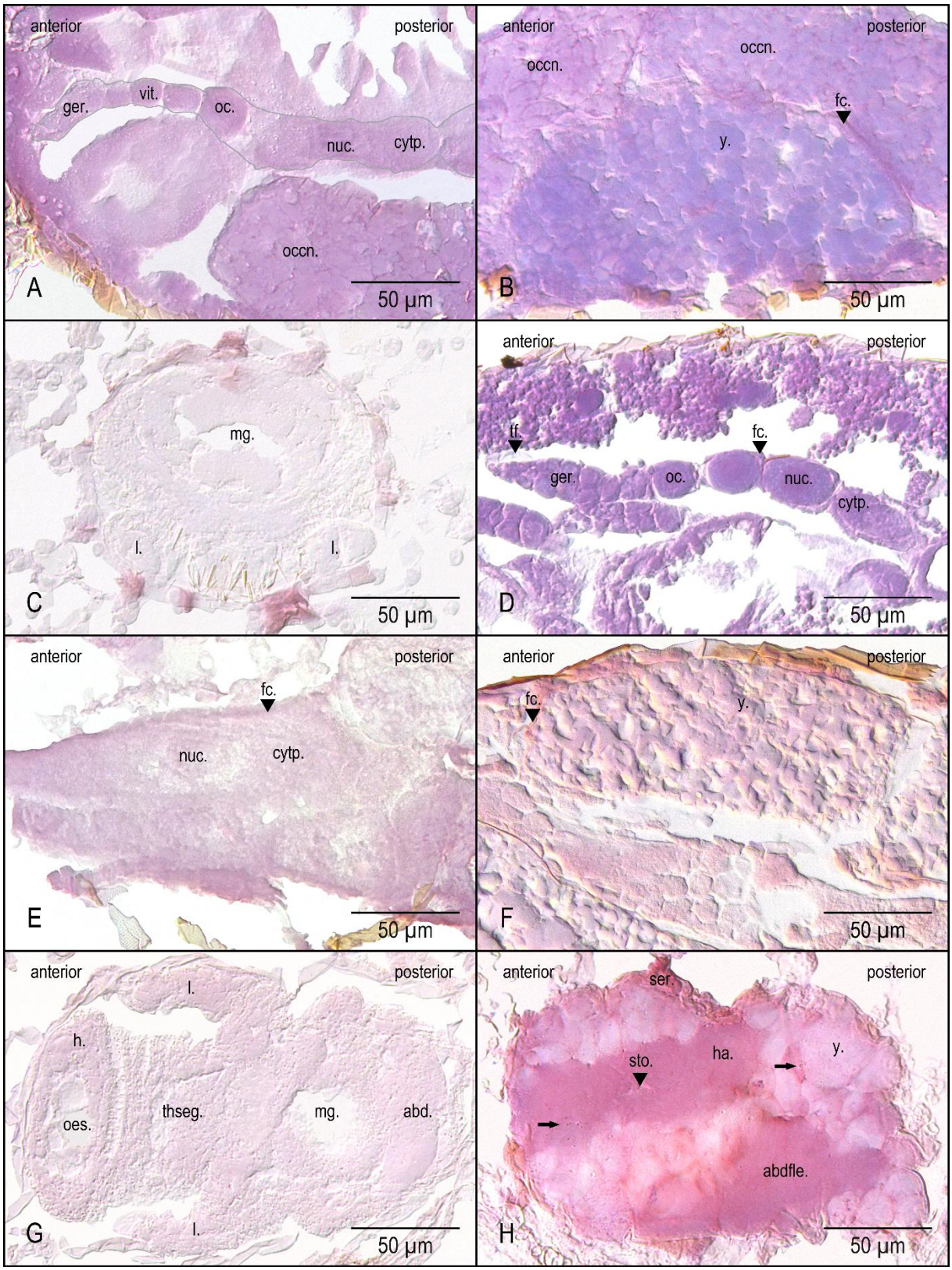
Tab. 2. Deparaffinization of histological sections.

Chemicals	Time conditions
Xylene	5 min
Isopropyl alcohol	3 min
96% ethanol	3 min
80% ethanol	3 min
60% ethanol	3 min
Distilled water	3 min

Tab. 3. Dehydration of histological sections.

Chemicals	Time conditions
96% ethanol	1 min
Isopropyl alcohol I	1 min
Isopropyl alcohol II (fresh)	1 min
Xylene	1 min

The specimens were dyed for 30 s in the Giemsa working solution (composed of 10 ml Giemsa stock solution (Merck KGaA, Germany), 190 ml distilled water, 4 drops glacial acetic acid) and afterwards washed two times for 5 min under constant distilled water. For the microscope investigations, the sections were dehydrated (Tab. 3) and mounted in Entellan® (Merck KGaA, Germany). The visualisation of the histological sections was realized with the light microscope (Leitz DMRBE, Leica, Germany). Pictures were taken with a Leica DFC450 C digital microscope camera and processed with Photoshop CS6 (Adobe, Ireland).


anterior
posterior
ger.
vit.
oc.
nuc.
cytp.
occn.
A
50 µm
anterior
posterior
occn.
occn.
fc.
y.
B
50 µm
mg.
l.
l.
C
50 µm
anterior
posterior
tf.
fc.
ger.
oc.
nuc.
cytp.
D
50 µm
anterior
posterior
fc.
nuc.
cytp.
E
50 µm
anterior
posterior
fc.
y.
F
50 µm
anterior
posterior
l.
h.
oes.
thseg.
mg.
abd.
l.
G
50 µm
anterior
ser.
posterior
sto.
ha.
y.
abdfle.
H
50 µm

S5 Fig. 1. *In situ* hybridization investigations during oogenesis and embryonic development of *F. occidentalis* and *H. femoralis* (red-brown signals, arrows). Fig. 1A–C *F. occidentalis*, control for *Cardinium* detection: (A) ovariole and oocytes, (B) vitellogenic egg for oviposition, (C) egg 4 d ± 12 h with embryo (transversal section), Fig 1D–G *F. occidentalis*, control for *Wolbachia* detection: (D) ovariole, (E) oocyte, (F) vitellogenic oocyte for oviposition, (G) egg 4 d ± 12 h with embryo, (H) *H. femoralis*, detection of *Wolbachia* in the 4 d ± 12 h egg with germ ligament. (abd.– abdomen, abdfle.–abdominal flexure, cytp.– cytoplasm, fc.– follicular epithelial cells, ger.– germarium, h.– head, ha.– head anlage, l.– leg/extremity, mg.– midgut, nuc.– nucleus, oes.– oesophagus, oc.– oocyte, occn.– oocyte with cleavage nucleus, ser.– serosa, sto.– stomodaeum, tf.– terminal filament, thseg.– thoracic segment, vit.– vitellarium, y.– yolk).

S5 Tab. 1. Preparation of the used chemicals.

Chemicals	**Preparation of chemicals**
2.5% glutaraldehyde	▪ 25% glutaraldehyde diluted in Sorensen's phosphate buffer (pH 7.4)
Lead citrate, pH 12 after Venable and Coggeshall (1965)	▪ 0.035 g lead citrate and 0.1 ml 10N NaOH, dissolved in 10 ml ddH_2O (boiled at least 5 min) ▪ Lead citrate dissolved with constant shaking ▪ Centrifugate before usage ▪ Always prepare fresh
2% osmium tetroxide	▪ 0.5 g osmium tetroxide dissolved in 25 ml Sorensen's phosphate buffer (pH 7.2)
2% paraformaldehyde	▪ 2 g PFA dissolved in 20 ml ddH_2O ▪ Warm up solution to 65 °C while stirring ▪ Add NaOH dropwise until the solution is clear, afterwards filter ▪ 10 ml PFA added to 40 ml 0.1 M Sorensen's phosphate buffer (pH 7.4)
4% paraformaldehyde	▪ 2 g PFA dissolved in 10 ml ddH_2O ▪ Warm up solution to 65 °C while stirring ▪ Add NaOH dropwise until the solution is clear, afterwards filter ▪ 10 ml PFA added to 40 ml 0.1 M Sorensen's phosphate buffer (pH 7.2)
Proteinase K	▪ 10 mg dissolved in 1 ml ddH_2O
Sorensen's phosphate buffer, pH 7.2	▪ 36 ml of 0.2 M Na_2HPO_4 buffer ▪ 14 ml of 0.2 M NaH_2PO_4 buffer ▪ Added to 100 ml ddH_2O
Sorensen's phosphate buffer, pH 7.4	▪ 40.5 ml of 0.2 M Na_2HPO_4 buffer ▪ 9.0 ml of 0.2 M NaH_2PO_4 buffer ▪ Added to 100 ml ddH_2O
2% uranyl acetate (aqueous)	▪ 0.5 g uranyl acetate dissolved in 25 ml ddH_2O ▪ Stored at 4 °C

S5 Tab. 2. Preparation procedure of specimens for diagnosis by electron microscopy.

Procedure	Chemicals	Time conditions
Primary fixation	Mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer	2 h (room temperature)
Wash	Sorensen's phosphate buffer, pH 7.4	4 x 20 min
Wash	Sorensen's phosphate buffer, pH 7.4	Overnight at 4 °C
Secondary fixation	2% osmium tetroxide	1 h (room temperature)
Wash	Distilled/deionized water (ddH_2O)	4 x 20 min
Dehydration	30% ethanol	2 x 10 min
	50% ethanol	2 x 10 min
	70% ethanol	2 x 10 min
	80% ethanol	2 x 10 min
	95% ethanol	2 x 10 min
	100% ethanol	2 x 10 min
Transition solvent	Propylene oxide	2 x 20 min on rotator
Infiltration	50 : 50, propylene oxide : araldite	2 x overnight on rotator
Embedding	Pure resin in embedding silicone rubber molds	48 h at 65 °C

S5 Tab. 3. Measured values for the large and small observed bacterial microorganisms in the cytoplasm and in the follicle epithelium of the oocytes during vitellarium in *E*. *americanus* and *H*. *femoralis* (TEM investigations). For each morphological type, 20 bacteria were measured. (bm. – bacterial microorganism).

	Measured values of large bm.		Measured values of small bm.	
Cell number	Length [nm]	Width [nm]	Length [nm]	Width [nm]
1	264	169	412	198
2	403	231	290	206
3	298	203	174	152
4	349	203	105	49
5	356	234	103	58
6	466	225	84	43
7	281	269	146	62
8	679	321	85	33
9	388	230	85	30
10	291	146	120	44
11	366	183	165	68
12	353	271	177	73
13	380	214	145	56
14	356	207	177	68
15	407	315	145	80
16	298	180	221	80
17	464	136	229	68
18	434	159	177	76
19	508	247	185	72
20	393	220	225	64
Mean	386.70	218.15	172.50	79.00
Standard deviation	94.98	50.23	78.55	48.94

S5 Supplementary reference

Mulisch, M., and U. Welsch. 2010. Romeis Mikroskopische Technik, vol. 18. Spektrum Akademischer Verlag, Heidelberg.

CHAPTER S6

Toxic impact of the entomopathogenic bacterium *Photorhabdus luminescens* on the survival rate of different Thripidae

S6 Suppl. method 1: Determination of the bacteria number per µl

For the determination of the bacteria number per µl liquid culture a Thoma chamber (Fein-Optik, Bad Blankenburg, Germany) was used. This chamber possesses 16 large central squares, each of them is divided in 16 mini squares with a area of 0.0025 mm^2, and a chamber depth of 0.1 mm. To ascertain the number of bacteria per µl, 10 µl of *P. luminescens* liquid culture (bacteria strain: BPl (e-nema, Schwentinental, Germany)) were added to 100 ml growth medium (15 g/l peptone, 3 g/l yeast extract, 6 g/l sodium chloride and 1 g/l glucose (pH 7.5)) and incubated for 72 h. After the incubation time, the optical density of the bacteria liquid culture was determined at 600 nm. The *P. luminescens* liquid culture possessed a mean optical density of 0.627. For the bacteria counting, 10 µl of an 1 : 10 diluted solution (dilute in 0.9% sodium chlorid solution) *P. luminescens* liquid culture was given in the Thoma chamber. To ascertain the mean bacteria number 5 large central squares were counted through microscope (Leitz DMRBE, Leica, Germany; phase contrast, optical magnification 40 x). Altogether four samples were counted to determine the mean bacteria number.

Tab. 1. Counted number of bacteria per large central square in four samples. The total mean of the bacteria number is calculated through the mean per sample.

Large central squares	Sample 1	Sample 2	Sample 3	Sample 4
1	135	144	138	137
2	129	149	134	148
3	108	143	114	156
4	133	166	120	146
5	129	140	131	150
Mean per sample:	634	742	637	737
Total mean:	687.5 bacteria per 5 large central squares			

The *P*. *luminescens* number per µl liquid culture was calculate after following formula:

Formula:

$$\text{Bacteria per µl volume} = \frac{\text{Counted bacteria}}{\text{Counted surface (mm}^2\text{)} \bullet \text{Chamber depth (mm)} \bullet \text{Dilution}}$$

Calculation:

Counted bacteria: 687.5 bacteria

Counted surface: 5 group squares, equivalent to 0.2 mm^2

Chamber depth: 0.1 mm

Dilution: 1 : 10

$$\text{Bacteria per µl volume} = \frac{687.5 \bullet 10}{0.2 \bullet 0.1 \bullet 1}$$

$$\text{Bacteria per µl volume} = \underline{\underline{343750}}$$

The calculation ascertained 343750 *P*. *luminescens* per µl volume liquid culture by a mean optical density at 0.627 (absorbance at 600 nm). For the calibration line the bacteria number by an optical density at 600 nm from 0.01 to 0.85 were calculated and graphical visualized in figure 1.

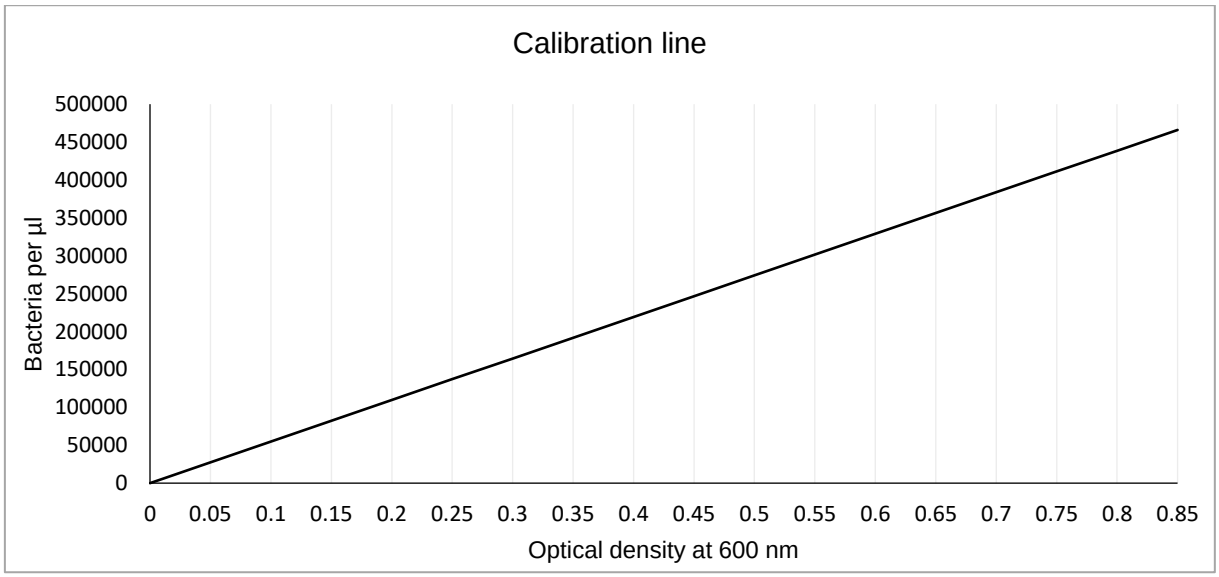


Fig. 1. Calibration line for the determination of the bacteria number per µl liquid culture by an optical density at 600 nm.

S6 Suppl. method 2: DNA extraction of *P. luminescens*

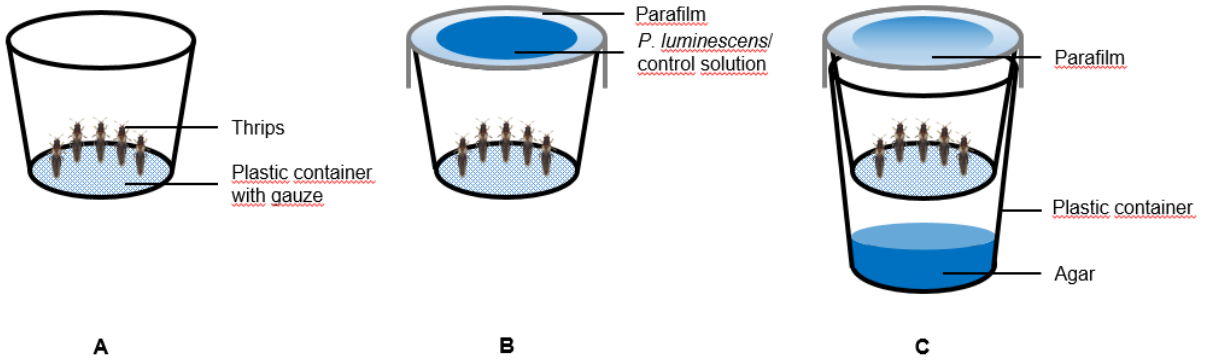
For the DNA extraction of *P. luminescens* 1 ml of an overnight culture (bacteria strain: BPl (e-nema, Schwentinental, Germany)) was centrifuged at 13000 rpm. The supernatant was discarded. To the sediment was added 0.5 ml of 0.5 M sodium chloride and 20 µl Proteinase K (1 mg/ml, Carl Roth GmbH + Co. KG, Germany). The sediment was washed for 5 min at 10000 rpm and the supernatant was discarded again. Subsequently, the sediment was resuspended in 0.5 ml 1x TE buffer (pH 7.5) and 20 µl Proteinase K, mixed and then incubated for 2 h at 37 °C. After the incubation time, the solution was mixed, 0.5 ml phenol: chloroform (proportional 1 : 1) (substances were obtained from: Carl Roth GmbH + Co. KG, Germany) were added and the solution was centrifuged for 5 min at 13000 rpm. The supernatant (aqueous phase) was transferred into a new reaction tube, mixed with 1 ml chloroform and centrifuged for 5 min at 13000 rpm. This step was repeated, before the supernatant was transferred again in a new reaction tube and mixed with 1 vol. isopropyl alcohol and 1/10 vol. 3 M sodium acetate (substances were obtained from: Carl Roth GmbH + Co. KG, Germany). The solution was incubated for 2 h at -20 °C and afterwards centrifuged for 20 min at 13000 rpm. Then, the liquid supernatant was discarded and DNA supernatant was dried for 10 min at 45 °C. Finally, the DNA was resuspended in 50 µl 1x TE buffer (pH 7.5). Afterwards, the isolated *P. luminescens* DNA was used for the amplification of the 16S rRNA (see Chapter 6).

10 x TBE	900 mM TRIS (Carl Roth GmbH + Co. KG, Germany)
pH 8.2, autoclaved solution	900 mM boric acid (Carl Roth GmbH + Co. KG, Germany)
	25 mM EDTA (Serva Electrophoresis GmbH, Germany)
	Aqua dest.

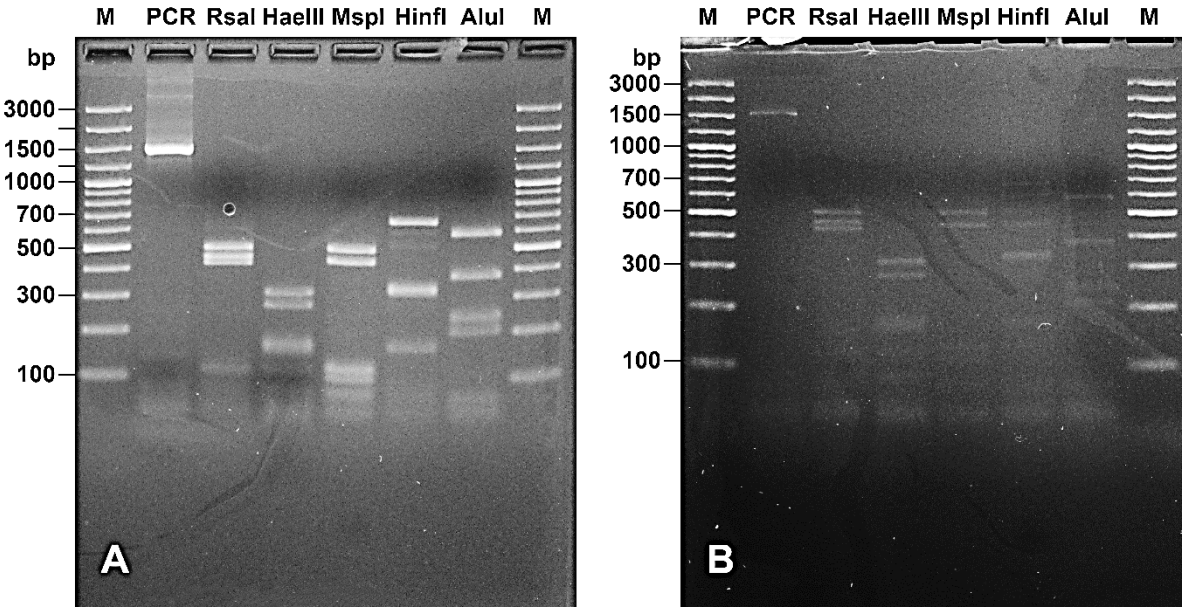
S6 Suppl. method 3: Restriction fragment length polymorphism (RFLP)

The restriction digestion analysis was used to verify the PCR results (Chapter 6). For that method the PCR products (detection of 16S rRNA, fD1 (Weisburg et al. 1991)/1492r (Lane 1991) of the *P*. *luminescens* DNA (S6 Suppl. method 2) and the thrips samples were used. Each attempt consisted of five reaction tubes for the enzymes RsaI, HaeIII, MspI, HinfI and AluI (Promega, Madison WI USA). In each reaction tube a restriction mix of: 7 µl PCR product, 1 µl reaction buffer (Promega, Madison WI USA), 0.1 µl bovine serum albumin (BSA) (Promega, Madison WI USA), 0.5 µl of the respective enzyme (RsaI, HaeIII, MspI, HinfI, AluI), and 1.5 µl ddH_2O was given. The restriction attempt was mixed and after that incubated for 2 h at 37 °C (restriction digestion). Subsequently, the reaction was stopped with 2 µl loading buffer. The restriction digestion products were verified through a 2% agarose gel (fractionation clamping: 4 h at 70 V) and visualized with the gel documentation system DIAS-III from SERVA as well as documented with GelScan V6.0.

10 x TBE pH 8.2, autoclaved solution	900 mM TRIS (Carl Roth GmbH + Co. KG, Germany) 900 mM boric acid (Carl Roth GmbH + Co. KG, Germany) 25 mM EDTA (Serva Electrophoresis GmbH, Germany) Aqua dest.
6x Loading buffer	30% glycerol (Carl Roth GmbH + Co. KG, Germany) 0.25% bromphenol blue (Carl Roth GmbH + Co. KG, Germany) 0.25% xylencyanol (Carl Roth GmbH + Co. KG, Germany) Aqua dest.
2% agarose gel	2% agarose (Carl Roth GmbH + Co. KG, Germany) 1 x TE buffer 10 µl ethidium bromide stock solution
Ethidium bromide (stock solution)	5 µg/ml ethidium bromide (Carl Roth GmbH + Co. KG, Germany) 1 ml ddH_2O



S6 Fig. 1. System for application of liquids with bacteria (*P. luminescens* solution) or without (control solution) on thrips. (A) Plastic container with gauze on one side and thrips inside. (B) On the closed plastic container (closed with parafilm) the application or control solution is applied. (C) The complete system is closed with a second layer of parafilm and placed in a plastic container with a layer on agar (1.4%) on the bottom.



S6 Fig. 2. RFLP of the PCR products (for the 16S rRNA, primers: fD1/1492r) of the used *P. luminescens* strain and *F. occidentalis* sample (exemplary for all tested thrips samples). The restriction digestion of each PCR product was realized with the enzymes RsaI, HaeIII, MspI, HinfI and AluI. S6 Fig. 2A: PCR product of *P. luminescens* strain (not digested sample) and restriction digestion products of the enzymes. S6 Fig. 2B: PCR product of *F. occidentalis* (not digested sample) and restriction digestion products of the enzymes. (M – Marker, Gene Ruler™ 100 bp (Thermo Scientific, Waltham)).

S6 Tab. 1. Number of used thrips females per *P*. *luminescens* and control group and the bacteria concentration per µl at the beginning of application.

	Number of used thrips females		Number of bacteria per µl	
Thrips species	***P. luminescens* group**	**Control group**	**Bacteria liquid culture**	**Application solution**
E. *americanus*	48	30	~ 315000 bacteria	~ 118125 bacteria
F. *occidentalis*	56	24	~ 330000 bacteria	~ 123750 bacteria
H. *femoralis*	48	24	~ 348000 bacteria	~ 130500 bacteria
T. *tabaci*	49	27	~ 365000 bacteria	~ 136875 bacteria

S6 Supplementary references

Lane, D.J. 1991. 16s/23s RNA sequencing, pp. 115–176. In E. Stackebrandt, and M. Goodfellow (eds.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd, Baffins Lane, Chichester, West Sussex PO19 1UD, England.

Weisburg, W.G., S.M. Barns, D.A. Pelletier, and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697–703.