# The confused flour beetle, *Tribolium confusum*

## A model for investigating *Wolbachia*-host interactions

Dissertation

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*This dissertation is dedicated to my parents,*
*Firooz & Hedieh*

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# Summary

*Wolbachia*, a group of maternally inherited intracellular endosymbiotic Alphaproteobacteria, are found in a wide range of arthropods and nematodes. It has been estimated that over 65% of insect species are infected with at least one *Wolbachia* strain. A major proportion of global biodiversity is represented by insects, and that a single symbiont can infect half of the presented species in the world shows how extraordinarily successful *Wolbachia* is. However, while *Wolbachia* is well known as a manipulative reproductive parasite specially by inducing cytoplasmic incompatibility, they are also significant regulators of host fitness by distributing in somatic tissues as well. Therefore, their role in host biology and physiology is undeniable. It has been suggested that more than 30% of *Drosophila* stocks, a ubiquitously used model system, are infected with these intracellular bacteria, *Wolbachia*, which also illuminates their fundamental role in evolutionary studies. In addition, some *Wolbachia* strains help to protect their insect hosts against viral infections, and their antiviral effects have been utilized as a biocontrol tool to inhibit the transmission of some viruses, related to human infections. Not surprisingly, *Wolbachia* also attract a great interest of biologists, since they may apply as a novel environment friendly agent to control insect pest species.

Species of the flour beetles genus *Tribolium* are small darkling beetles, two of which, *T. castaneum* and *T. confusum,* are the most notorious global pests of flour mills and stored food products, but at the same time are widely used as model organisms in fields of evolution, genetics and development. Consequently, adapting *Tribolium* beetles as a model system for *Wolbachia* research next to the widely used classical model systems such as *Drosophila* flies, *Culex* mosquitoes and nematodes, opens possibilities for a wide range of studies, taking advantage of the already established culturing methods and genetical tools. Regarding *Wolbachia*-host association, *T. confusum* is known to be naturally infected with endosymbiont bacteria, *Wolbachia*. Surprisingly, research infrastructure of *Wolbachia*-host interaction in *T. confusum* is considerably less established than other models, even though the number of such studies is recently growing. In order to contribute to the potential role of *T. confusum* beetles in host symbiosis relationship, we outlined the potential of establishing *T. confusum* as a model for *Wolbachia* research, assembled and annotated genome of the *Wolbachia* strain (*w*Tcon),

investigated the fitness effects of *w*Tcon in relation to temperature in an experimental approach and performed behavioral studies to investigate the influence on the locomotion and action selection of *Tribolium confusum* beetles. Hereby, we confirmed that the *w*Tcon genome is sharing the characteristics as the other *Wolbachia* strains and especially the ones in supergroup B. Also, we showed that *w*Tcon is temperature specific and the density of the endosymbiont reduces under heat stress, although the cytoplasmic incompatibility (CI) was intact. In addition, we observed that *Wolbachia* infection alters the locomotion of *T. confusum* beetles, so as to increase the *w*Tcon transmission in the host population.

# General introduction

Symbiosis is fundamental to ecosystem functioning, the evolution of biological complexity, and organismal health. Through mechanisms of co-evolution, host-bacterial associations have developed into prosperous relationships that affect animals' fitness and consequently influence their evolutionary dynamics (Chow et al. 2010; Douglas 2014). The majority of these bacteria occupy the host's internal and external tissues, integrating deeply into the host's biology by colonizing the intracellular niches of the host (Fisher et al. 2017). Additionally, endosymbiotic bacteria are of great interest since they play a crucial role in diversification (Mandel and Dune 2016; Sudakaran et al. 2017). Endosymbionts can be either primary or facultative. Primary endosymbionts usually transmit vertically (i.e. from mothers to offspring) and are essential for host development and reproduction, however, facultative symbionts populate a broader range of cells (intra and extra) and can transmit both vertically and horizontally, can develop a variety of essential and non-essential fitness advantages (or disadvantages) to their host (Lopez-Madrigal et al. 2019).

*Wolbachia* is one of the best-known examples of endosymbionts, estimated to infect more than 52% of terrestrial arthropod species (Weinert et al. 2015), yet the prevalence varies from one host group to another. In addition to arthropods, filarial nematodes which are responsible for some human tropical diseases carry obligate mutualistic *Wolbachia* endosymbionts (Sironi et al. 1995; Bandi et al. 1998). These findings on the specific host population of *Wolbachia* was consistent with the primary hypothesis on the origin of *Wolbachia* which stated "essentially a terrestrial phenomenon", however, not long after, screening *Wolbachia* from non-insect pancrustaceans (Cordaux et al. 2001), found for example in fully marine species such as goose barnacle *Lepas anatifera* (Cordaux et al 2012), recommends a reconsideration of the previous hypothesis (Makepeace and Gill 2016). Maternally transmitted *Wolbachia*, as one of the most successful symbionts among arthropods and filarial nematodes, has been characterized as a "pandemic" (Bordenstein et al. 2006), that can impose major evolutionary processes such as sexual selection (Jiggins et al. 2000; Charlat et al. 2003), sex determination (Rigaud et al. 1997; Bouchon et al. 2008) and speciation (O'Neill and Karr 1990; Werren 1998) to their host. The main route of transmission for *Wolbachia* is vertical, which is mainly occurs by infecting the

germplasm or colonization of somatic stem-cell niche in the germanium (Serbus and Sullivan 2007; Toomey et al. 2013). The experimental infection in *D. melanogaster* indicated the ability of *Wolbachia* to cross three barriers such as peritoneal sheath membrane, the muscle epithelium surrounding the ovariole, and the somatic tissues enclosing the germline to enter the immature egg, by constraining outside the reproductive area of the host (Frydman et al. 2006). Moreover, horizontal transfer is an alternative way that contributes to the endosymbionts spread across species, and this could be validated by the inconsistency of *Wolbachia* phylogenies with those of their hosts, e.g., as shown for diverse arthropod groups (Heath et al 1999; Gerth et al. 2014; Makepeace and Gill 2016). Similar to vertical transmission, *Wolbachia* utilizes different paths to transmit horizontally which can be mechanically by mouthparts or ovipositor of the parasitoids (Heath et al. 1999; Ahmed et al. 2015; Mascarenhas et al. 2016) or by predation, scavenging, and cannibalism among terrestrial isopods (Le Clec'h et al. 2013). For instance, the *Wolbachia* strain harbored the same allele in *Anastrepha* fruit flies and their braconid parasitoid wasps suggested the possible lateral gene transfer (Mascarenhas et al. 2016). Moreover, *Wolbachia* might transfer via plant and fungal tissues since arthropods sometimes co-feed on the same material (Sintupachee et al. 2006; Stahlhut et a. 2010; Yang et al. 2012).

Most of the previous phylogenetic analysis of *Wolbachia* strains has been based on single-gene analyses (*Wolbachia* surface protein gene- *wsp*; the 16S rRNA gene; or *ftsZ*), which separate them into phylogenetic "supergroups" (O'Neil et al. 1992; Zhou et al. 1998), however recent studies adapted on five other genes of the Multilocus Sequence Typing (MLST) system, since analyses based on the *wsp* gene evidently can be biased by recombination (Baldo et al. 2006). Thereafter, Bleidorn and Gerth (2018) concluded that the resolution of MLST might be not enough to distinguish *Wolbachia* strains and whole genome typing should be introduced as a reliable alternative. So far, based on genetic diversity and phylogenetic analysis, 17 supergroups have been proposed for *Wolbachia* (Lefoulon et al. 2020). Supergroups A and B, belong to a single monophyletic lineage, infect arthropods and adapt to a wide range of hosts, by frequently adapting to new hosts (Gerth et al. 2014). Supergroups C, D, and J are restricted to filarial nematodes that co-evolved with their hosts (Bandi et al. 1998). Interestingly, supergroup F is the only shared clade between both filarial nematodes and arthropods (Haegman et al. 2009; Gerth et al. 2014; Lefoulon et al. 2016), that is evidence for *Wolbachia*'s host shift between two major host groups, and suggested to be at least two times (Gerth et al. 2014). It has been proposed

that filarial nematodes developed a mutualistic relationship with their endosymbiont since they present growth retardation and sterilization after antibiotic treatment (Bandi et al. 1999). On the contrary, *Wolbachia* developed a parasitic relationship with most of their arthropod hosts, whereas they interfere with the reproductive process of their hosts. *Wolbachia* is able to manipulate host's reproduction with different methods to increase their chance of proliferation and transmission, including male-killing, induction of parthenogenesis, feminization, and cytoplasmic incompatibility (Werren 1997; Werren et al. 2008; Zug and Hammerstein 2012; Beckmann et al. 2017; Miyata et al. 2017; Turelli et al. 2018). Male killing occurs during embryonic development and led to unhatched male embryos, and eventually causes reduced sibling competition on food, happens in Coleoptera, Lepidoptera, Diptera, and Pseudoscorpions (Hurst et al. 2000; Fialho and Stevens 2000). *Wolbachia* can transform genetic males into functional females called feminization, especially in Malacostraca and some species of Hemiptera and Lepidoptera (Kageyama et al. 2002). In Hymenoptera, Thysanoptera, Collembola, *Wolbachia* induce parthenogenesis, where females develop from unfertilized eggs (Kremer et al. 2009). In these three methods, *Wolbachia* purposely causes impaired sex ratio to maximize their transmission. Cytoplasmic incompatibility (CI) is the most common manipulation method that *Wolbachia* among different hosts from Hexapoda, and Chelicerata, to Crustacea.

It was not until 1971, that Janice Yen and A. Ralph Barr introduce a novel reproduction manipulation strategy by *Wolbachia*, named Cytoplasmic incompatibility (CI). They discovered that the eggs of *Culex* mosquitos were sterile as a result of fertilization between *Wolbachia* infected males and aposymbiotic ($w^-$) females, therefore the cross was incompatible (Yen et al. 1971). Cytoplasmic incompatibility is the most common method that *Wolbachia* endosymbionts use to manipulate their host's reproduction, however, *Wolbachia* can employ other methods such as feminization, parthenogenesis, or male-killing (Werren 1997; Stouthammer et al. 1999). In Cytoplasmic incompatibility, sperm of infected males mating with uninfected females, modifies in a way, which leads to embryonic mortality or haplodization - in few haplodiploid species- at early stages of the offspring development (Hoffmann 1988; Bourtzis et al. 1994; Tram et al. 2003). Two types of CI have been discovered as unidirectional and bidirectional. In unidirectional CI, one strain of *Wolbachia* is involved with males and females are not infected,

whereas, in bidirectional CI, males and females are infected with different strains of *Wolbachia* (O'Neil et al. 1990; Bourtzis et al. 1996; Shropshire et al. 2020) (Fig. 1).

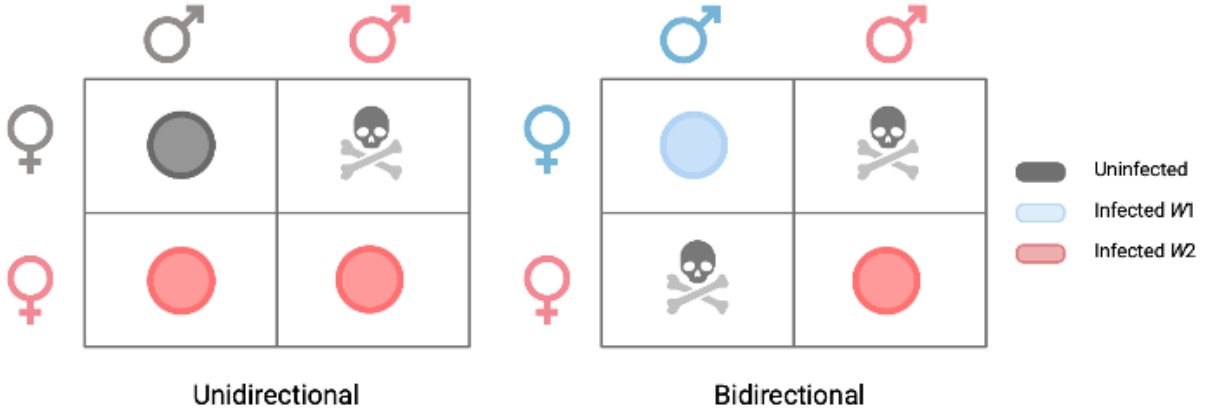


**Figure 1**. Illustration of two different types of cytoplasmic incompatibility (CI), which has been explained above. The different colors are described as grey- uninfected, blue- infected with Strain 1, and pink- infected with Strain 2 of endosymbiotic bacteria, *Wolbachia*.

Previous studies proposed two models as "mistiming" and "lock-key" to explain cytoplasmic incompatibility (Bossan et al. 2011). In the mistiming model, slow progression of sperm induced by *Wolbachia* can only be compensated with the same delayed progression modification to the ovum, so the uninfected eggs are unable to rescue the modified sperms, and this led to incompatible crosses (Charlat et al. 2001). This sperm modification is introduced as "locks" in a lock-key model, where *Wolbachia* disables the paternal chromosomes for mitosis, and this can be rescued only by installation of matching "keys" in egg cytoplasm by the same *Wolbachia* strain (Poinsot et al. 2003). Overall view of "modification-rescue" models, indicated the presence of a specific threshold level of *Wolbachia* density for production of viable eggs (Clancy and Hoffman 1998; Duron et al. 2007).

Long before, it was suggested that there must be a correlation between the presence of prophage WO within *Wolbachia* strains and CI phenotype, since neither of prophage WO and CI induction can occur in filarial nematodes and they are restricted to arthropod (Sinkins et al. 2005), and later on, this role was discovered. Recent studies illustrated the role of two prophage WO genes, $cifA_{\text{wMel}}$, and $cifB_{\text{wMel}}$, in enhancing cytoplasmic incompatibility by recapitulating. This proposed as a "two for one" model in which transgenic expression leads to the occurrence of both CI and

rescue and the dual expression of *cifA*$_{wMel}$ and *cifB*$_{wMel}$ genes can induce stronger CI in the host (LePage et al. 2017; Shropshire and Bordenstein 2019). CI mortality changes widely across different species, as an example, the level of incompatibility for four *Drosophila* species infected with CI inducing *Wolbachia* is partial and varies from 25-60%, compared to the control crosses of uninfected flies (Bourtzis et al. 1996; Mercot and Charlot 2004). In addition, CI inducing *Wolbachia* alters the fitness and especially the fecundity of their host (Engelstädter and Telschow 2009). However, primary studies mostly focused on the negative impact of CI on phenotypic traits, it may have a positive influence on its host as well. Dobson et al. (2004) reported higher fecundity in infected females of *Aedes albopictus* due to their CI inducing *Wolbachia* infection (Dobson et al. 2004). In contrast, no positive fecundity has been confirmed in *Drosophila* and *Nasonia* species regarding their *Wolbachia* infection (Bordenstein and Werren. 2000; Meany et al. 2019).

As mentioned, typically it has been believed that the relationship of *Wolbachia* with their arthropod hosts can mostly be described as parasitic, however, a considerable number of recent studies suggested otherwise. One of the first examples for a mutual dependency of *Wolbachia* within the arthropods was discovered in a parasitic wasp, *Asobara tabida,* that *Wolbachia* elimination led to *the* production of immature oocytes (Dedeine et al. 2001). Although an increased number of studies in arthropod hosts revealed more mutual dependency, it is still hard to describe it as mutualistic in most cases, since the host does not benefit from *Wolbachia*'s ability to control its mode of reproduction (Makepeace and Gill 2016). Genomic analysis has revealed that in mutualistic symbiosis, *Wolbachia* can diminish some crucial dietary resources for the host (Ponton et al. 2015). It has been confirmed that in *Aedes aegypti*, those infected mosquitos with a lower amino acid level in their diet, showed a reduction in their fertility, which can be interpreted as *Wolbachia*'s *lack* of capacity to synthesis de novo amino acids (Foster et al. 2005; Caragata et al. 2014).

Consequently, genomic data is essential to understand *Wolbachia*'s role in the symbiosis. The first assembled *Wolbachia* genome (*w*Mel) was announced for supergroup A in 2004 (Wu et al. 2004), and so far over 35 complete and 55 draft genomes (mostly from supergroup A and B) has been described (Lefoulon et al. 2020). Next generation sequencing (NGS) is the pioneer in genomic studies, by maximizing the throughput with time-efficient workflow (Fig. 2), along

with a drastic reduction of costs. Because *Wolbachia* is an intracellular bacterium and often present in relatively low abundance, sequencing the genome and the depth of the coverage of the sequencing from the host genome is extremely dependent on the density of the endosymbiont, particularly for gene content identification (Makepeace and Gill 2016).

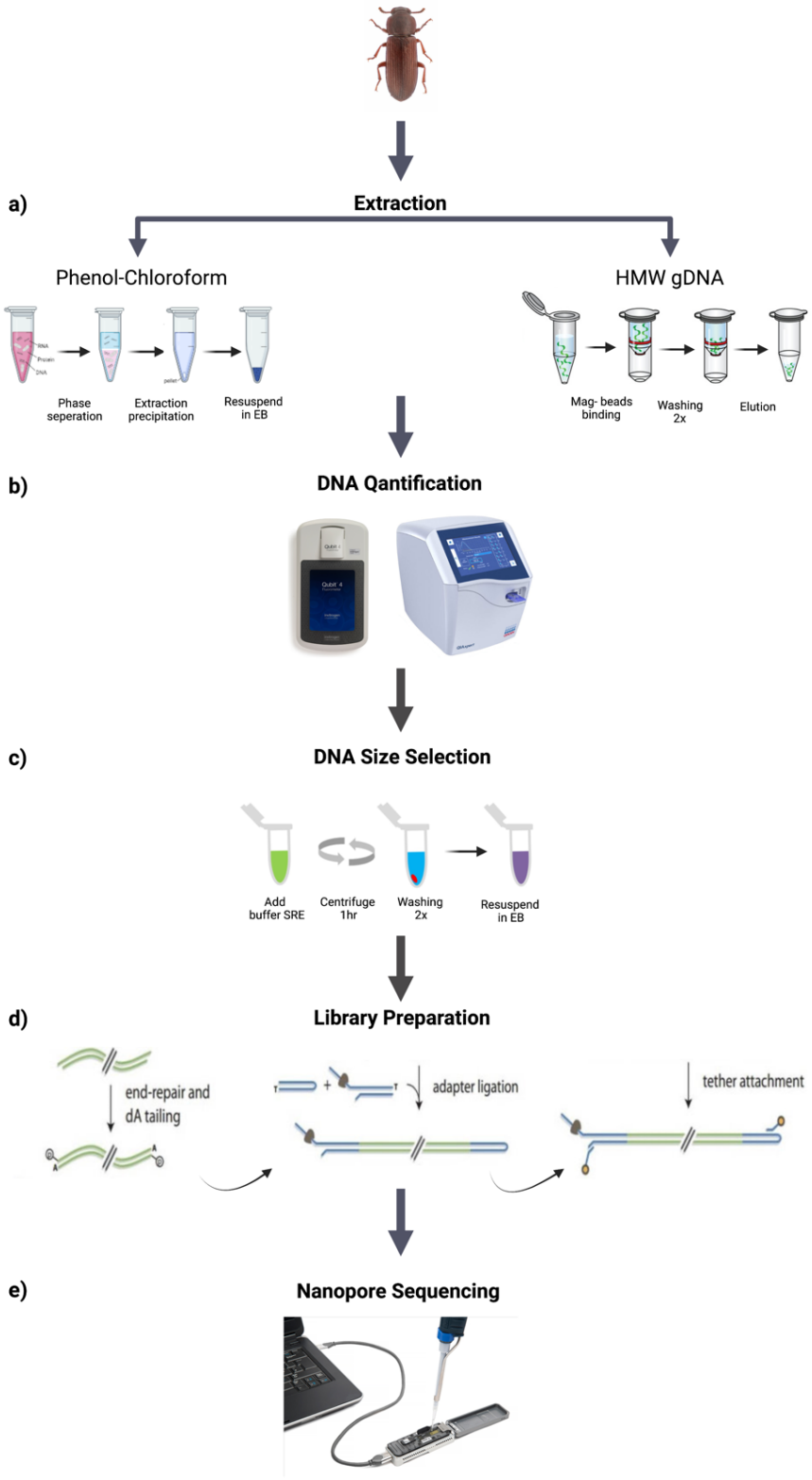


**Figure 2**. An example of a general workflow scheme for optimizing HMW DNA, quality assessment, and generating nanopore long reads data for whole-genome assembly studies. Created in BioRender.com.

The initial comparative genomic data, discovered more repeat regions, insertion sequences (IS), group II introns, and ankyrin-repeat domain proteins in *Wolbachia* strains of arthropod species and with larger genome size, in comparison with other obligate intracellular bacteria genomes and even *Wolbachia* strains in filarial nematodes (Wu et al. 2004; Foster et al. 2005; Brelsford et al. 2014).

Endosymbionts, however, must be abundant enough to fulfill their host-symbiont association, limit the cost of transmission, and ensure at least vertical transmission between generations (Campbell *et al*. 2018), and many factors such as environmental constraints and in particular temperature, which is also significant for the survival and development of the hosts (Bale et al. 2002). Accordingly, *Wolbachia*'s infection rate and the factors that might influence it, are of great interest in controlling disease vectors, considering the role of *Wolbachia* as a biocontrol using two methods as Incompatible Insect Technique (IIT), and Sterile Insect Technique (SIT), in combination or separate (Zhang et al. 2015). The incompatible Insect Technique (IIT) is a new alternative method for (SIT), where instead of releasing sterilized insects (SIT), it's based on *Wolbachia*-infected males and their incompatibility to produce viable eggs after mating with wild-type females (Pagendam et al. 2020). One of the early examples of employing *Wolbachia* as a biocontrol was for controlling *Aedes polynesiensis*, an important agent of lymphatic filariasis. These mosquitos are naturally infected with a single strain of *Wolbachia* from supergroup A, but when they microinjected with another strain from supergroup B, a bidirectional incompatibility occurred (Breslsfoard and Dobson 2009). However, controlling *A. aegypti* mosquitos, the main species responsible for infectious human viruses as Zika, dengue, yellow fever, and chikungunya, by extending *Wolbachia* transmission in the mosquito population (Edenborough et al. 2021), is substantially the most important and wide project of World Mosquito Program (WMS) (http://www.eliminatedengue.com/our-research/Wolbachia) (Fig. 3).

Following this strategy, new studies proposed a similar method to control pest species as well. Flour beetles, *Tribolium castaneum* (red flour), and *T. confusum* (confused flour), identified as one of the most cosmopolitan pests in flour mills and grain-based products, with a great economic impact on the food industry (Campbell et al. 2004). *Tribolium* beetles belong to the holometabolous group of insects, meaning that they go through different larval

stages following the pupal stage before developing as an adult (Klinger 2004). Adults are small, brown to black, and around 3-4 mm long, which originally live underneath the bark of rotten wood (Dawson 1977).

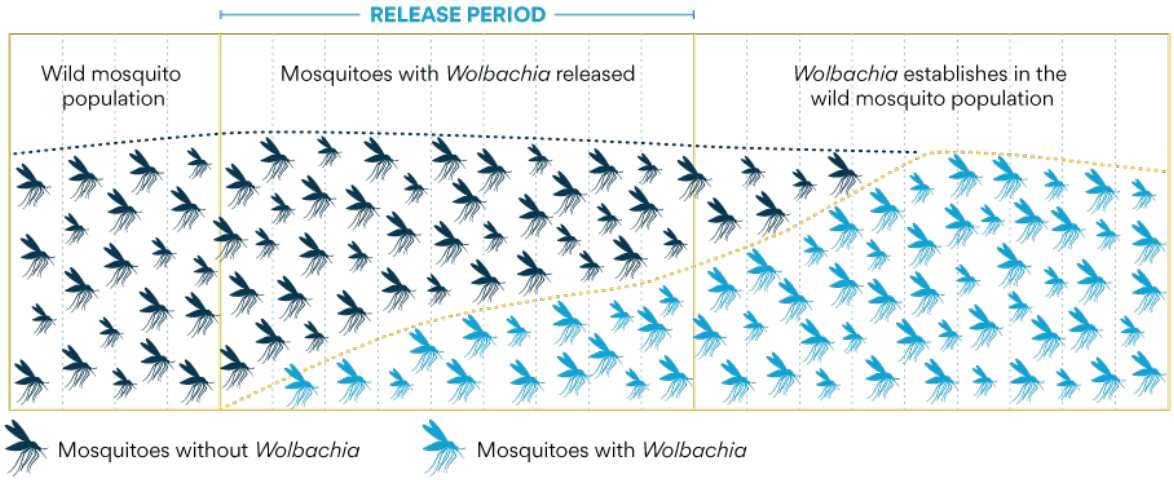


**Figure 3**. Long term monitoring of infecting *A. aegypi*, in order to control the mosquito's population against Zika and dengue. Image: www.eliminatedengue.com.

Human agriculture was the main reason for *Tribolium* worldwide dispersal, considering the discovery of dead *T. confusum* beetles in ancient Egyptian tombs dating back to 5000-7000 years ago (Anders 1931). Regarding the fact that these beetles belong to the largest animal order (Stork et al. 2015), they have been represented as one of the most convenient laboratory study systems for studying population genetics, the evolution of development (evo-devo), and ecology (Klinger 2004; El-Aziz 2011; Pointer et al. 2021). This is mainly because of their developmental biology, which is more similar to typical insects in comparison with the classical system, *Drosophila*, in addition to their relatively easy breeding process, short life culture, the capability of genetic crosses, and high fecundity (Klinger 2004; Richards et al. 2008). For a long time, functional genomic studies mostly relied on *Drosophila*, however, in the last couple of decades *Tribolium castaneum* became another emerging model system (Brown et al. 2003). Another closely related species, *Tribolium confusum,* represents an interesting but so far rather neglected model for symbiosis studies, given their natural *Wolbachia* infection. Although *T. confusum* and *T. castaneum* are genetically distinct (Ming et al. 2015), in addition to harbor distinct bacterial endosymbionts (Goodacre et al. 2015; Ming et al. 2014), the morphology is exceptionally similar, and they still consider as sibling species. *T. confusum* is infected with bacterial endosymbionts of *Rickettsia* and *Spiroplasma* and in contrast, *T. confusum* is only infected with

a single strain of the *Wolbachia* group (Goodacre et al. 2015). These findings along with further studies on the details of *T. confusum Wolbachia* strain (*w*Tcon) and their symbiosis relationship might be brought into future applications on *Wolbachia* as a biocontrol agent of notorious pests, *T. confusum,* and *T. castaneum.*

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# Thesis aim

For a long time, the potential of confused flour beetles. *Tribolium confusum* has been overlooked, since only few studies have been dedicated to *Wolbachia*-host interaction in these beetles. The principle aim of my project is to illuminate our understanding on *Wolbachia* strain of *T. confusum* (*w*Tcon), and its extensive association with its natural host. This thesis composed of four main chapters, each focused on different aspects of *w*Tcon and its impact on the host population. The first chapter is an overview on why *Tribolium confusum* is an excellent model organism regarding to *Wolbachia* infection and the knowledge on the cytoplasmic incompatibility that *w*Tcon can induce to the host, by combining already existing studies on the genomics and fitness role of *w*Tcon and new outcome from this project. In the second chapter, the focus is on assembling and annotating the genome of *w*Tcon, using two different sequencing strategies (Nanopore long reads and Illumina short reads), in order to obtain a highly continuous and covered genome. In the third chapter we tested the hypothesis that heat stress has an influence on *w*Tcon infection proliferation and replication rate, and the possible negative impact of high temperature on cytoplasmic incompatibility. In the last chapter we tested the hypothesis that *Wolbachia* infection in *T. confusum* beetles has a significant effect on behavioral traits, such as sleep duration, activity, exploratory rate, speed and thigmotaxis of the host.

# Contributions of authors

**I.** In chapter 1, the study was designed by Dr. Christoph Bleidorn and I reviewed the literature regarding *Wolbachia* infection of confused flour beetle, *Tribolium confusum*.

**II.** In chapter 2, the study was designed by Dr. Bleidorn. I carried over the laboratory work (sample extraction, library preparation, and sequencing), and the generatd date from both Illumina and Nanopore sequecing were trimmed and assembeled by Alexander Wähling and Dr. Bleidorn. I wrote the manuscript in contribution with Dr. Bleidorn. The manuscript has been published in Microbiology Resource Announcement (MRA).

**III.** In chapter 3, the study was designed by both Dr. Bleidorn and myself. As a following, I carried out the experimental (setting beetles, counting progenies under each temperature) and molecular laboratory work (real-time PCR), along with data analyses. I drafted the manuscript in contribution with Dr. Bleidorn. The manuscript is under revision for BMC Research Notes.

**IV.** In chapter 4, the study was designed by Dr. Bleidorn, Dr. Bart Geurten and myself. I perfomed the experimental work (circadian activity recording and locomotor behavior in open field) and the generated date was alanlyzed mainly by Dr. Geurten. The manuscript was written by me with contributions from Dr. Geurten. The manuscript is in preparation for Society for Applied Microbiology.

# Chapter 1

## The confused flour beetle, *Tribolium confusum,* a model for investigating *Wolbachia*-host interactions

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## Abstract

Up to 65% of insects have been infected with an endosymbiont bacteria, *Wolbachia*, which is an intracellular Alphaproteobacteria closely related to Rickettsia. *Wolbachia* is maternally inherited and induces reproductive alterations such as cytoplasmic incompatibility, male-killing, feminization, parthenogenetic development in the insect population, however, they are capable of infecting a variety of somatic tissues as well. Confused flour beetle, *Tribolium confusum* is one of the key model systems to study host-endosymbiont interactions. These beetles are naturally infected with a single strain of *Wolbachia* that induces a complete CI, which means they are a perfect case to study the genomics of *Wolbachia*. In this review, we present the current literature on different aspects of *Wolbachia* strain (*w*Tcon) involved with *T. confusum* beetles. We highlighted ongoing scientific efforts on *w*Tcon in the field of genomics, replication rate under temporal stress, and its effect on the fitness of the host. Overall, we aimed to propose confused flour beetle as an excellent model system in investigating *Wolbachia*-host interactions.

## Introduction

Novel insights into the broad range of animal–bacterial interactions have fundamentally transformed our understanding of animal biology and evolution (McFall-Ngai et al. 2013). In the case of insects, bacterial symbionts and other microbes that live inside them play critical roles in host physiology, development, immunity, stress resistance, behavior and nutrition (Chaston et al. 2014; Hague et al. 2020). The vast majority of bacterial symbiont species associated with an insect host are harboured in their guts, even though important exceptions, such as specific symbiont hosting organs ("bacteriomes") or symbionts which are found across all tissues (e.g., *Wolbachia* infections) occur (Saridaki and Bourtziz 2010; Moran et al. 2019). For interpreting the biological effects of endosymbiotic microbes on the fitness mechanism of the host, *Wolbachia* has been introduced as a model system in the last decades, since it mainly transmits vertically to the host population and influences the host's ecology (Hoffman et al. 1990; Werren 1997).

*Wolbachia,* the most abundant bacterial endosymbiont of animals, are intracellular bacteria belonging to the a-proteobacteria, which transmit to a wide range of arthropods, nematodes, and crustaceans, and are estimated to occur in 40-52% of terrestrial arthropods (Hilgenboecker et al. 2008; Werren et al. 2008; Zug and Hammerstein. 2012). *Wolbachia* is currently classified into 17 major phylogenetic clades named supergroups (A-Q), mainly based on Multilocus Sequence Typing (MLST) analysis and some supergroup demonstrate a specific type of symbiosis with their host (Baldo et al. 2006; Gerth 2016). *Wolbachia* strains from the two main supergroups (A-B) are restricted to arthropods, developed different types of symbiosis from reproductive parasitism to facultative and obligate mutualism (Zug and Hammerstein 2015; Lefoulon et al. 2016). However, supergroups C and D are major clades infecting filarial nematodes and showing an obligate mutualism, where the symbiont existence is essential for embryogenesis and larval development (Taylor et al. 2005; Comandatore et al. 2013; Makepeace and Gill 2016). Other supergroups are rather limited to one or a few host species, nevertheless, supergroup F is unique since it includes hosts from both arthropods and nematodes (Gerth et al. 2014; Lefoulon et al. 2016).

Since *Wolbachia* is an obligate intracellular bacterium for which it replicates exclusively inside the host cells, essential knowledge about its biology often has to be deduced from genome

sequencing. The first complete *Wolbachia* genome (*w*Mel) which was a CI-inducing strain was sequenced in 2004 from *Drosophila melanogaster*. Comparing the genome evolution and structure exhibited differences between *Wolbachia* and other obligate symbionts for instance *Buchnera* (Wu et al. 2004). By comparison the genomes of obligate mutualists, *Wolbachia* frequently shows signs of recombination (Baldo, Bordenstein, et al. 2006; Klasson et al. 2009), high level of gene losses and gains (Ishmael et al. 2009; Ellegard et al. 2013[a]), higher frequency of mobile elements (Wu et al. 2004; Kent and Bordenstein 2010), and contain those putative pathogenic determinants which are lost from many mutualistic endosymbionts in particular type IV secretion system (Rances et al. 2008).

Previously, Werren et al. (1995) reported the ability of different strains of *Wolbachia* to coinfect the same host (Werren et al. 1995[a]; Werren et al. 1995[b]; Kent et al. 2011), which already pointed at a broad horizontal movement of these endosymbionts between host species (Baldo et al. 2006). The pattern of horizontal movement and loss of infections is also shown in the incongruence between phylogenies of *Wolbachia* and its hosts (Baily-Bechet et al. 2017). To understand and resolve these conflicts, we would require whole-genome analysis from different host species since using other screening methods such as MLST do not reflect a precise image of the properties of the *Wolbachia* strain (Bleidorn and Gerth 2018).

Maternally transmitted *Wolbachia* can impose a variety of reproductive manipulation to their hosts such as feminization, male-killing, parthenogenesis, and cytoplasmic incompatibility (Hoffman and Turelli 1997; Stouthamer et al. 1999; Werren et al. 2008). Furthermore, *Wolbachia* infection can lead to several phenotypic changes and additionally affects host's physiology (Hedges et al. 2008; Cordaux et al. 2011). Cytoplasmic incompatibility (CI) is a phenotype, in which *Wolbachia*-infected females advance reproductively over uninfected females, so it increases the chance of persistence of *Wolbachia* in populations (Sinkins et al. 1995; Bourtzis et al. 1996). Even though *Wolbachia* transmits via the host germline, it also infects a variety of host somatic cells, such as digestive, metabolic, and nervous system cells (Dobson et al. 1999; Pietri et al. 2016). Previous studies focused excessively on the regulatory strategies that *Wolbachia* utilize to alter host reproduction, but "*Wolbachia is more than a bug in the insects' genitals*" (Saridaki and Bourtzis 2010). These endosymbiotic bacteria are capable of imposing several modifications on nutritional acquisition (Douglas et al. 2009), virus susceptibility (Hedges et al. 2008), behavioral consequences and physiology (Feldhaar et al.

2011; Hosokawa and Fukatsu 2020; Bi et al. 2020) and temperature tolerance (Brumin et al. 2011). Because of these interesting phenotypes *Wolbachia* attracted great attention due to its role in controlling vector-borne pathogens by suppressing the transmission of infection of viruses and filarial nematodes. As an example, *w*AlbB increases the West Nile virus infection in the mosquito *Culex tarsalis* (Dodson et al. 2014), however, it blocks the transmission of dengue (Mousson et al. 2012). In addition, *Wolbachia* especially the strains with the ability to induce CI can be utilized as a tool for insect pest population control by different strategies (Bourtzis 2008).

The influence of *Wolbachia* on the host is complex and difficult to predict according to "genotype-by-genotype-by environment" interactions (Thomas and Blanford 2003), meaning different environmental conditions can influence host and symbiont separately, and eventually the interactions between host and the endosymbiont affect the symbiosis association (Mouton et al. 2006). Measuring the *Wolbachia*'s infection density under different conditions would be essential in this term. Through combinations of genetic and environmental factors in the *Wolbachia*-host association, it may be possible to predict the evolution of local adaptations for regulation of infection density and host interaction (Mouton et al. 2007). Plus, the infection spread also depends on the fitness consequences of *Wolbachia* in host tissues, and initial spread from low frequencies requires positive effects from *Wolbachia* on host fitness (Barton and Turelli 2011), although their regulation on altering host fitness components is poorly studied (Ross et al. 2019). As mentioned, environmental factors and especially temperature is of primary importance in the development and survival of both the host and *Wolbachia*, by establishing direct impact on the biological and physiological processes such as host distribution and endosymbiont's virulence (Thomas and Blanford 2003; Mouton et al. 2006), which results on an effect on the regulation of infection density (Bale et al. 2002; Mouton et al. 2006).

The mechanisms of how *Wolbachia* is manipulating its hosts became better understood in recent years and much was learned from model-based research focussing on different groups of Diptera (*Drosophila* spp., *Culex* spp., *Anopheles* spp.) (Sinkins et al. 2005; Weeks et al. 2007; Glaser and Meola 2010; Baldini et al. 2014), as well as the interaction of *Wolbachia* with human-pathogenic nematodes (Filaria) (Foster et al. 2005; Slatko et al. 2014). However, outside of these models, studies become much more sporadic and punctual. In this review we want to present recent advances in studies on the influences of *Wolbachia* on the confused flour beetle, *Tribolium confusum*, a so far rather neglected model for *Wolbachia* research.

## *Tribolium* beetles

Tenebrionid beetles of the genus *Tribolium* include cosmopolitan pests of many stored products from flour to dried foods and stored products (Campbell et al. 2004; Abd El-Aziz 2011). These beetles are often feed on damaged and broken grains and wheat products, although their original habitat was expected to be under the bark of the rotten trees, thus they rather to play a role as a secondary invader (Dawson 1977). The genus *Tribolium* includes more than 36 species (Angelini and Jockusch 2008), however, the red flour beetle, *Tribolium castaneum* and the confused flour beetle *Tribolium confusum* are the most well-known species due to been utilized to a significant number of scientific contributions from ecological to evolutionary studies. Being a member of Holometabola (metamorphosing insects) and not as highly derived as *Drosophila* (the most popular model system), has underpin these beetles as versatile model systems (Brown et al. 2003). In addition to these advantages, the knowledge on their durational life cycle and their easy and affordable culture conditions is well known (Pointer et al. 2021) (Fig. 1).

**Figure 1**. The cultures of *Tribolium* beetles and the minimal equipment needed for breeding.

The life cycle of *Tribolium* beetles is relatively fast (6-12 weeks), depending the environmental conditions such as temperature (26-32°C) and humidity (±65), along with their type of nutrition (usually flour) (Smith and Whitman 1992).

These beetles are promiscuous and rapidly breed throughout the year. The eggs are white and small, following with seven instars of slender larvae, which can be differentiating by their color during their development (Ryan et al. 1970) (Fig. 2a). The pupae stage is necessary for separating the sexes, since their genitals on urogomphi is more noticeable and distinctive between male and female (Ryan et al. 1970) (Fig. 2b). Adults are between 3-6 mm and darkening in color by aging, which can be upto three years (Smith and Whitman 1992) (Fig. 2c).

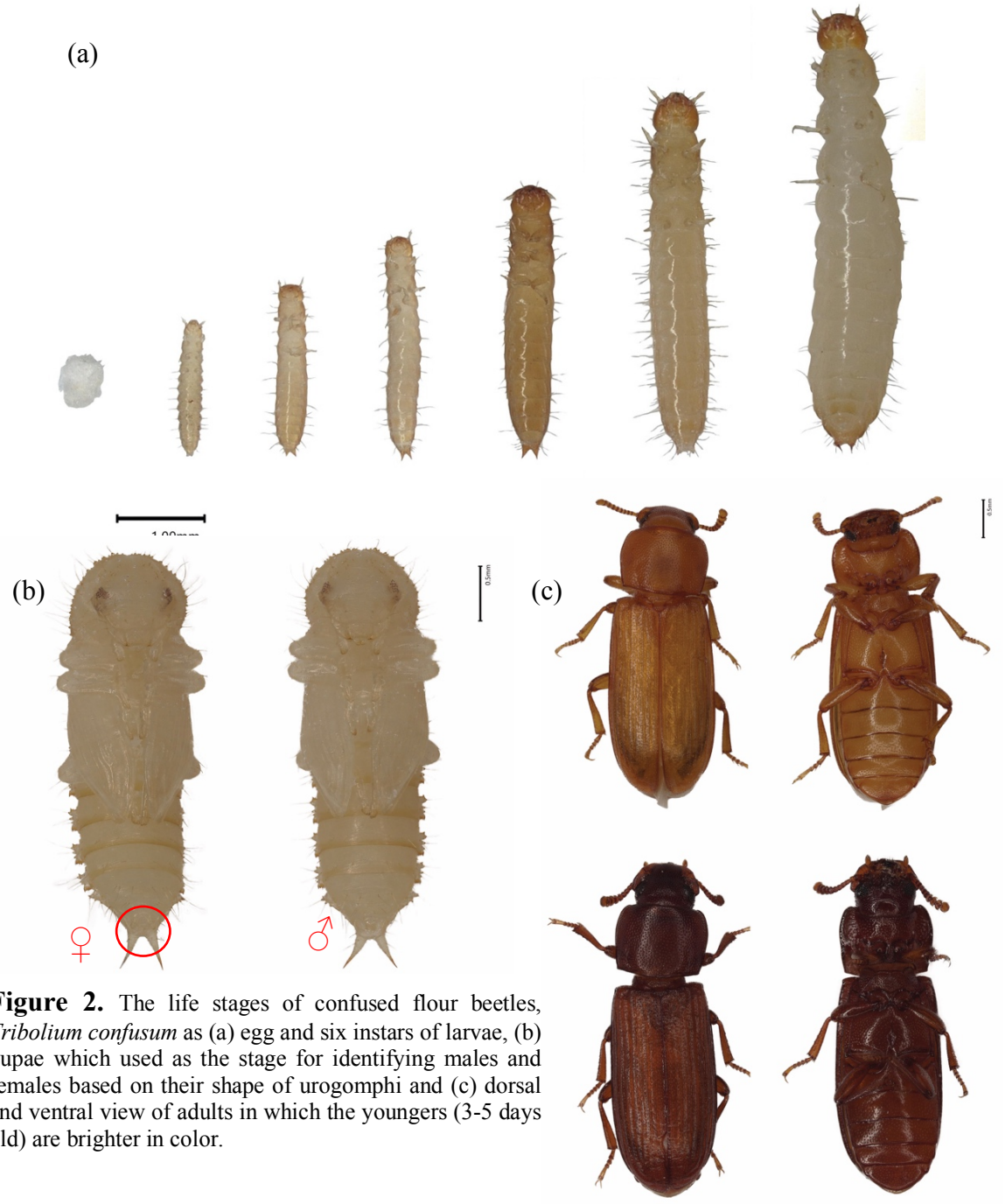


**Figure 2.** The life stages of confused flour beetles, *Tribolium confusum* as (a) egg and six instars of larvae, (b) pupae which used as the stage for identifying males and females based on their shape of urogomphi and (c) dorsal and ventral view of adults in which the youngers (3-5 days old) are brighter in color.

Due to their fast and easy to culture life-cycle, *Tribolium* beetles became a popular model system for genetic and developmental research. Consequently, many genetic techniques have been established, such as CRISPR/Cas9-mediated genome editing or RNA interfence (RNAi)-mediated gene silencing (Adrianos et al. 2018).

## *Wolbachia* in *Tribolium* beetles

Out of 10 Tenebrionidae stored product insects and also among all beetles of the genus *Tribolium*, natural *Wolbachia* infection seems mainly to be restricted to *T. confusum* (Wade and Chang 1995; Stevens et al. 1994; Lu et al. 2019). However, one recent study has isolated a single *Wolbachia* strain and a phage WO of supergroup A from *T. castaneum*, although these results need to be confirmed (Gowda et al. 2018).

Wade and Stevens (1985) first confirmed natural *Wolbachia* infection in the *Tribolium confusum* based on the incompatibility found between populations (Wade and Stevens. 1985). However, the following studies reported the probability that this might be due to the presence of two different *Wolbachia* strains, belonging to distinct phylogenic clades of arthropods, supergroup A and B (Werren et al. 1995[b]). Fialho and Stevens (1996), showed that out of eight strains of *T. confusum*, all were only infected with a single and common CI inducing strain (Fialho and Stevens. 1996; 1997; Kageyama et al. 2010; Ming et al. 2015), in which no viable offspring can result from incompatible cross of uninfected females and infected males (Yen and Barr 1971; Werren 1997). A few studies attempted to transinfected other *Tribolium* species such as *T. castaneum* with the same strain of *Wolbachia* (*w*Tcon) from *T. confusum*, yet the new host was impervious, which is probably related to either endosymbiont or host factors (Chang and Wade 1994; 1996). However, a successful microinjection was carried on from infected donor eggs of *T. confusum* to host eggs and interestingly 40% of the surviving transinfected eggs showed reproductive incompatibility. The results also indicated that microinjecting of infected cytoplasm into host eggs drastically reduces the survival of eggs to adult stage (25.1%), in comparison with injecting of uninfected cytoplasm (32.3%), or *Wolbachia* injected controls (35.9%) (Chang and wade 1996).

The localization of *Wolbachia* density in *T. confusum* from different tissues and body parts confirmed the highest *Wolbachia* infection rate in reproductive tissues and abdomen followed

by thorax and head of adult beetles (Ming et al. 2015; Lu et al. 2019). This outcome is helpful, since different levels of symbiont density may act as a factor to control the effect of *Wolbachia* in *T. confusum* (Ming et al. 2015). *Wolbachia* infection density from both males and females of *T. confusum* showed no significant difference at every age (Ming et al. 2015), yet a recent study reported higher *Wolbachia* density in females than the males especially during the pupae stage (Lu et al. 2019), pointed at this fact that the infection density differed based on the developmental stages of the host, as it was increased constantly with the host development progressed. The infection rate reached a peak in the eggs and young adults (Ming et al. 2015; Lu et al. 2019).

Environmental condition and specially temperature, as one of the primary components might influence the effect of *Wolbachia* on host reproduction by changing the density of infection (Sinkins et al. 1995; Poinsot et al. 1998), was essential to be explored. Recent study on the effect of high temperature on the density of *Wolbachia* in *T. confusum* adults showed that during two consecutive generations of *T. confusum* beetles, the transmission of *Wolbachia* decreases when the beetles reared at 34°C, which is the peak temporal degree that the host survived at, for males and females (Gharabigloozare and Bleidorn, in review). The population of *Wolbachia* reached the highest at 30°C and 31°C (favorable developmental temperatures for the host), compared to higher temperatures (Gharabigloozare and Bleidorn, in review). In addition, the difference in *Wolbachia* infection rate for males and females reared at five different temperature (30-34°C) were not significant (Gharabigloozare and Bleidorn, in review), although Lu et al. (2019) reported a gender *Wolbachia* density bias, with increased *Wolbachia* density in females rather than males (Lu et al. 2019).

Among nine different *T. confusum* strains (except for HP70), all males and females were positively infected with a single CI inducing *Wolbachia* (Wade and Stevens 1985; Fialho and Stevens 1996; Goodacre et al. 2015). Later on, detailed screening of *Wolbachia* density from the whole body of the *T. confusum* beetles revealed that young adults of both males and females retain the highest *w*Tcon density among all developmental stages (Lu et al. 2019), in verification with another study that reported high *Wolbachia* density level in eggs and adult stages of *T. confusum* (Ming et al. 2015).

## *CI* in *T. confusum*

The *Wolbachia* strain of *T. confusum* beetles *w*Tcon is of particular interest, due to their strong and complete CI (Fialho and Stevens 1997; Lu et al. 2019), since this can help to predict the induction of CI quantitively in the host population (Engelstädter and Telschow 2009). The expression of CI in *T. confusum* beetles by *w*Tcon, confirmed the positive fecundity in females regardless of male infection status (Ming et al. 2015). Since infected females increase the number of infected progenies, higher female fecundity and equality of sex ratio in the *T. confusum* beetles, increase the CI which results in higher *Wolbachia* prevalence. By all means, this suggests a mutualistic relationship between CI-inducing *Wolbachia* and *T. confusum* (Lu et al. 2019).

As mentioned before, the influence of temperature on *Wolbachia* density varies from one individual to another, so as for the expression of CI, and host's fitness related to the expression of CI. Stevens (1989) demonstrated the possibility of heat treatment (36°C for 12 days) to surpass CI in larvae of *T. confusum* beetles (Stevens 1989). Nevertheless, a recent study showed that a complete CI occurred even in higher temperatures (33-34 °C), thus heat has no significant effect on CI. Plus, there was no larval survival detected above 34°C (Gharabigloozare and Bleidorn, in review). Ming et al (2015) investigated the role of CI inducing *Wolbachia* on reproduction and egg hatch of *T. confusum* beetles. It stated that CI did not affect egg production, although a significant change in the egg hatch rate was reported in crosses of uninfected females (Ming et al. 2015). This is in line with a new study which showed that even by adding the heat factor, a significant decrease in the number of laid eggs for the beetles reared under thermal stress (34°C) and in all crosses for two consecutive generations were recorded (Gharabigloozare and Bleidorn, in review). As for the development, the same results applied to the number of hatched eggs by a drastic reduction when reared at the highest survival temperature (34°C) in all crosses. Furthermore, the egg hatch proportion for all crosses, even for the mating crosses between uninfected males and females were reported to be at a similar rate and no drastic reduction was detected, regardless of the temperature that beetles were reared at (Gharabigloozare and Bleidorn, in review). On contrary, Ming et al. detected a reduction in the number of hatched eggs in the cross between uninfected males and females of *T. confusum* beetles (Ming et al. 2015).

Additionally, heat stress appears to have no significant effect on the female ratio, as the ratio for both sets of beetles reared under optimal (30°C) and stress temperatures (34°C) were between 0.5-0.6, confirming the fact that *w*Tcon has no significant role in altering the sex ratio in confused flour beetles, *T. confusum* (Ming et al. 2015; Lu et al. 2019; Gharabigloozare and Bleidorn, in review).

## *w*Tcon genome

Genomes of several *Wolbachia* strains representing diverse supergroups have been published (31 genomes), although the acquisition of sequence data has been hindered by the difficulty of obtaining sufficient quantities and purity of *Wolbachia* gDNA due to their obligate lifestyle as an intracellular endosymbiont (Ellegard et al. 2013[b]). The annotated draft genome of the *w*Tcon strain from the confused flour beetle, *Tribolium confusum*, has been assembled based on long and short-read sequence data, taking advantage from the fact that long reads facilitate the assembly of complex repeat regions which are challenging to resolve when solely relying on short-read sequencing (Gharabigloozare et al. 2022).

The final assembly of *w*Tcon contains 12 contigs ranging in length from 29,423 to 346,899 bps (N50 value, 138,551 bp) in a total length of 1,418,452 bp, with a GC content of 34.1%. The *w*Tcon genome size is relatively similar to another CI-inducing *Wolbachia* strain from supergroup- B, wPip (*Culex pipiens*) with 1.48 mbp, whereas CI-inducing *w*Mel (supergroup-A) genome, from *Drosophila melanogaster,* is considerably smaller (Klasson et al. 2009). The annotation of the *w*Tcon genome identified 1,335 genes in total with 1,236 protein-coding genes, and 1,294 coding sequences (CDSs). The genome also encodes 34 tRNAs genes that contain cognates for all amino acids, 58 pseudogenes, 4 non-coding RNAs (ncRNAs), and 3 rRNAs (5S, 16S, and 23S), with zero number of plasmids. This genome shares general characteristics with other *Wolbachia* strains that have been sequenced before, including genome size (~0.9- 1.8 Mb), GC content (~33-35%), and an approximate number of coding sequences (CDSs) (Table. 1). Genome size and composition is in the typical range of that of other facultatively mutualistic *Wolbachia* strains, but significantly different (bigger in size, more genes) than those of obligate mutualists.

The analysis for completeness of the genome, which was assessed by measuring the proportion of expected gene content from highly conserved, single-copy orthologs (BUSCO groups), was

carried out against 364 searched BUSCO groups, and it showed that the 1,236 protein-coding genes in the *w*Tcon genome contain 353 complete and single-copy BUSCO groups, 2 complete and duplicated BUSCO groups, 11 missing and none fragmented BUSCOs, resulting in a 96.9% BUSCO completeness score (Gharabigloozare et al. 2022). In comparison with some other *Wolbachia* genomes from supergroups A and B, the completeness of assembly for *w*Tcon is considerably higher which can be interpreted as a high-quality genome assembly, thereby representing a valuable resource for future research on this particular strain.

## CI genes in *w*Tcon

It has been demonstrated that *w*Tcon can induce complete cytoplasmic incompatibility in *Tribolium cofusum*. Recent genetic studies of CI in *Drosophila* and *Culex* hosts have shown that the expression of a pair of syntenic genes, now called *cifA* and *cifB*, which are sometimes within the WO prophage genomes, are causing this phenotype (LePage et al. 2017; Bonneau et al. 2018). Among all *Wolbachia* strains, *cifA* and *cifB* happen to co-occur as a pair of neighboring genes, which based on phylogenetic analysis are distinguished in different "types", representing monophyletic, but genetically distinct entities with partly variable domain content (LePage et al. 2017; Lindsey et al. 2018). BLAST searches against available sequences of these genes (Lindsey et al. 2018) also revealed putative orthologs in the *w*Tcon genome in contig 9 neighboring each other.

## Presence of WO prophage in *w*Tcon

Prophages play an important role in *Wolbachia* biology, and in particular, it could be demonstrated that CI is fairly dependent on the prophage WO (*w*Mel) in its *Drosophila* host (Beckmann et al. 2017; LePage et al. 2017). A complete genome enables an extended search for any potential prophages in *w*Tcon. PHASTER webserver (Arndt et al. 2016), available at (http://phaster.ca), were used for the annotation of any integrated prophage sequences in the *w*Tcon genome. Various BLAST comparison was executed to identify the WO-like islands, which are the regions containing the genes and are present in WO phages and cannot be detected in genomes of phage-free *Wolbachia* (Bordenstein and Bordenstein 2016; LePage et al. 2017). The PHASTER webserver identified one intact region on contig 4 (GI725950572) and 48.4kb in size (E-Value: 7.10e-25). The GC content is 35.7% and in total 48 CDSs were detected (Fig. 3).

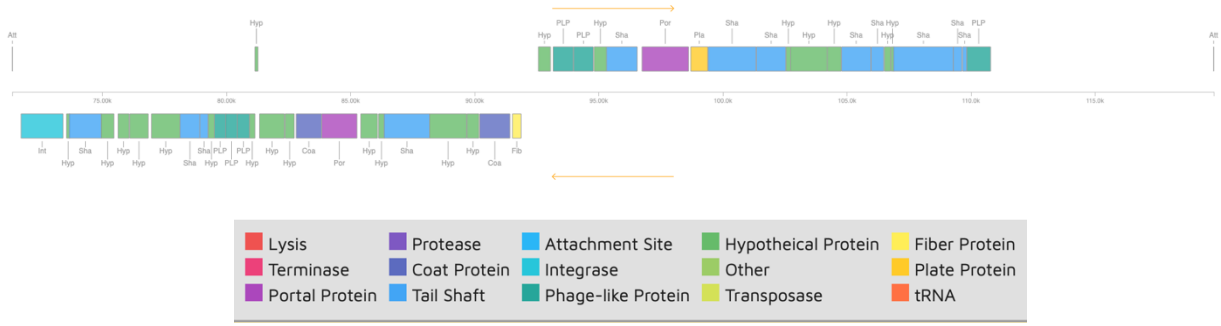


**Figure 3.** Localization of an intact prophage on contig 4 of the *wTcon* genome from *T. confusum* beetles.

## Housekeeping markers (MLST)

The MLST system has been used widely for a variety of studies such as phylogenetic inferences among strains, strain typing, identification of different strains, and recombination within genes. However, the reliability of the MLST system has been much debated (Bleidorn and Gerth 2018). Recently, a study comparing different genome sequences indicated that MLST typing is still largely compelling, especially for the detection of closely related strains and also for supergroup identification of *Wolbachia* (Wang et al. 2019).

The five housekeeping markers of the Multi Locus strain typing (MLST) system (Baldo et al. 2006; Jolley and Maiden 2010) to characterize the phylogenetic relationship of *w*Tcon with other *Wolbachia* strains. The five MLST genes in the *w*Tcon strain used for genome sequencing, showed the exact profile as available for *Tribolium confusum* (strain Tcon_B_BhAvill AK (id:20), *Wolbachia* strain type ST-30) in *the public* PubMLST database (https://pubmlst.org/).

Table 1 General assembly statistics of ten *Wolbachia* strains from supergroups A and B.

| Name | Host Organism | Super-group | Size (Mb) | GC% | rRNA | tRNA | Proteins | Other RNA | Total Genes | Pseudogenes | BUSCO Score |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***w*Au** | *Drosophila simulans* | A | 1.26 | 35.2 | 3 | 34 | 1,099 | 4 | 1,265 | 125 | 83.8 |
| ***w*Ha** | *D. simulans* | A | 1.29 | 35.3 | 3 | 35 | 1,126 | 4 | 1,263 | 95 | 83.8 |
| ***w*OneA1** | *Nasonia oneida* | A | 1.29 | 35.8 | 3 | 31 | 1,114 | 4 | 1,201 | 109 | 86.5 |
| ***w*Mel** | *D.melanogaster* | A | 1.27 | 35.2 | 3 | 34 | 1,100 | 4 | 1,270 | 129 | 82.9 |
| ***w*Ri** | *D.simulans* | A | 1.45 | 35.2 | 3 | 34 | 1,254 | 4 | 1,403 | 108 | 83.3 |
| ***w*AlbB** | *Ae. albopictus* (Aa23 cell line) | B | 1.48 | 34.4 | 3 | 34 | 1,205 | 4 | 1,434 | 188 | 81.9 |
| ***w*No** | *D.simulans* | B | 1.30 | 34.0 | 3 | 34 | 1,065 | 4 | 1,231 | 125 | 82.4 |
| ***w*Pip** | *C. quinquefasciatus* | B | 1.48 | 34.2 | 3 | 34 | 1,257 | 4 | 1,402 | 104 | 81.9 |
| ***w*Tpre** | *Trichogramma pretiosum* | B | 1.13 | 33.9 | 3 | 35 | 827 | 4 | 1,106 | 237 | 82.4 |
| ****w*Tcon** | *Tribolium confusum* | B | 1.41 | 34.1 | 3 | 34 | 1,236 | 4 | 1,335 | 58 | 96.9 |

## Outlook

*Wolbachia* is well known to host reproductive manipulators, despite their ability to distribute in various somatic tissues in the particular brain, which leads to a range of potential effects on the host's fitness, although the type and level of interaction that *Wolbachia* develops with the host is dependent on many other factors such as environmental condition and host genomic background. In this review, we introduced the potential of using *Tribolium* as a promising model for *Wolbachia* research, and this is mainly because *Tribolium* species can be kept with minimal maintenance work and access to all life-stages is given throughout the year, and by *T. confusum* harboring a single *Wolbachia* strain which induces complete CI, they represent an ideal model to investigate the effects of *Wolbachia* infections in the laboratory.

Almost all the studies mentioned in the review were performed under laboratory conditions, due to the hardship of dissecting complex behavior in the field. Further questions on the influence of *w*Tcon infection in field conditions remain pending. Therefore, molecular analysis on both *w*Tcon and *T. confusum* beetles is needed, since *Wolbachia* is capable to modify gene expression, number of proteins, and miRNAs, which emphasize the multidimensional side of *Wolbachia* to influence the host. New strategies on bioinformatics and molecular biology would assist in a more comprehensive pathway to clear the influence of *w*Tcon on confused flour beetles.

## Author contributions

**YG** wrote the manuscript with contributions from CB, and CB designed the study. All authors contributed material for the study and approved the final version.

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# Chapter 2

## Whole-genome sequence of the *Wolbachia* strain *w*Tcon, an endosymbiont of the confused flour beetle, *Tribolium confusum* (Coleoptera: Tenebrionidae)

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## Abstract

Up to 65% of insects are infected with symbiont intracellular Alphaproteobacteria of the genus *Wolbachia*, which are often able to manipulate their host reproduction. Here, we report the annotated draft genome of the *w*Tcon strain from the confused flour beetle, *Tribolium confusum*, based on long- and short-read sequence data. The assembled genome is located on 12 contigs with a total size of 1,418,452bp.

Tenebrionid beetles of the genus *Tribolium* include cosmopolitan pests of many stored products from flour to dried foods (Campbell et al. 2004). Due to minimal maintenance work and access to all life stages, two different tenebrionid beetle species, the red flour beetle *Tribolium castaneum* and the confused flour beetle *Tribolium confusum,* have been studied in the lab for decades as model systems (Pointer et al. 2021). *Tribolium confusum* is known to be naturally infected with *Wolbachia* (Fialho and Stevens 1996; Goodacre et al. 2015; Kageyama et al. 2010), an intracellular Alphaproteobacterium which has been found as a widespread endosymbiont of arthropods and nematodes (Werren et al. 2008). To support the utility of *Wolbachia* in the *Tribolium* model system, we report the *w*Tcon draft genome sequence.

DNA was extracted from adult *T. confusum* beetles of the *Wolbachia*-infected MN61 strain (originally collected in Kansas, USA), supplied by the Stored Product Insect and Engineering Research Unit of USDA-ARS. Beetles were reared on flour medium and brewer yeast (5%) at 30°C and 65% relative humidity with a 16:8-hour dark/light cycle. For ONT long reads, high molecular weight DNA from beetles (10 males and 10 females) were extracted by both MagAttract HMW DNA kit (Qiagen, Hilden, Germany) and phenol-chloroform extraction with precipitation by sodium acetate (Montero-Mendieta 2018). Two ONT libraries were prepared (SQK-LSK109) - without fragmentation or size-selection- and sequenced by MinION (FLO-MIN 106) protocols from ONT. Raw ONT reads from two separate runs were base called with Guppy v3.4.4, yielding 4,074,131 reads with N50 of 2,511 bp. Furthermore, quality assessment and trimming were performed using FastQC v0.11.9 (Andrews 2013) and NanoFilt v2.8.0 (De Coster et al. 2018), respectively. For short read sequencing, the genomic DNA was isolated by Quick DNA Miniprep Plus Kit (Zymo research, Irvine, USA). Library preparation and

sequencing was performed by Allgenetics (A Coruña, Spain), using Illumina paired-end (150-bp) libraries, sequenced by an Illumina HiSeq 2500, yielding 46,330,553 paired-end reads, which trimmed with FastQC v0.11.8 (Andrews 2013). Default parameters were used except where otherwise noted.

The initial metagenome assembly for ONT long-reads was generated by Flye v2.8.1 (Kolmogrov et al. 2020), which estimated the size of the genome around 1.4 Mbp. Using a BLAST search against the reference genome of the supergroup B *Wolbachia* strain *w*PipPel from *Culex quinquefasciatus* (NC_010981.1), we identified putative *Wolbachia* contigs in the metagenome and with mapping reads with Minimap2 v2.13-r850 (Li 2018) on these contigs, we retrieved putative *Wolbachia* ONT raw reads. For Illumina reads, we created a metagenome assembly using SPAdes v3.13.2 with a *k*-mer of 77 (Nurk et al. 2013). After mapping the trimmed raw reads on this assembly with Bowtie v2.3.4.1 (Langmead et al. 2019), we were able to extract short reads of putative *Wolbachia* origin.

The hybrid assembly of *w*Tcon using the extracted long and short reads was completed using Unicycler v0.4.9 (Wick et al. 2017), which contains 12 contigs (N50 value, 138,551 bp) in a total length of 1,418,452 bp (GC content, 34.1%). The draft genome was annotated by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al. 2016). The annotation identified 1,236 protein-coding genes, 58 pseudogenes, 34 tRNAs, 4 noncoding RNAs (ncRNAs), and 3 rRNAs (5S, 16S, and 23S). We assessed the genome completeness by using BUSCO v5.2.2 (Simão et al. 2015). Out of 364 searched BUSCO groups, 353 were complete and single copy, 2 complete and duplicated, 11 missing and none fragmented, resulting in a 96.9% BUSCO completeness score. We compared the five housekeeping markers of the Multi Locus strain typing (MLST) system (Baldo et al. 2006), namely *gatB*, *coxA*, *hcpA*, *fbpA*, and *ftsZ, with the public* PubMLST database (https://pubmlst.org/) and could confirm that our sequenced strain shows the exact profile as available for *Tribolium confusum* (strain Tcon_B_BhAvill AK (id:20), *Wolbachia* strain type ST-30).

**Data availability.** The accession number for the complete genome of *w*Con in GenBank is JAIZNT000000000. In addition, raw sequence reads for Oxford Nanopore and Illumina sequencing are deposited in the NCBI Sequence Read Archive (SRA) under the accession numbers of PRJNA767570.

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### Author contributions

**YG** and CB designed the study. All authors contributed material for the study. **YG** generated the data and AW along with CB analyzed the data. **YG** wrote the manuscript with contributions from CB. All authors approved the final version.

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# Chapter 3

## Effect of high temperature on *Wolbachia* density and impact on cytoplasmic incompatibility in the confused flour beetle, *Tribolium confusum* (Coleoptera: Tenebrionidae)

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## Abstract

### Objectives

Environmental constraints, especially temperature, have been identified as a key in understanding host-symbiont relationships, as they can directly impact the fitness of the symbiont population and the host development. Here we investigated the effect of temperature during the host development on the density of intracellular bacteria of the *Wolbachia, w*Tcon strain within the confused flour beetle, *Tribolium confusum*. The *w*Tcon can induce a complete cytoplasmic incompatibility (CI) in *T. confusum* beetles; therefore, we observed the effect of heat stress on the symbiont-mediated CI.

### Results

The density of CI inducing *Wolbachia* in the *Tribolium confusum* is temperature-specific. Our observation of the beetles reared in five different temperatures (30-34°C) measured the highest *Wolbachia* density at 30-31°C and lowest at 34°C within a single insect generation. In this species, changes in the density of *Wolbachia* related to higher temperature did not influence CI. However, the fertility of beetles reared in higher temperatures showed a substantial decrease in the number of laid and hatched eggs. Thus, we can confirm the effect of high temperature on lowering the *w*Tcon density and no impact on induction of cytoplasmic incompatibility (CI) in *T. confusum* beetles.


**Keywords:** *Wolbachia* density, fertility, cytoplasmic incompatibility (CI), heat stress

## Introduction

Environmental factors are of primary importance in the development and survival of the host-endosymbiont relationship. In particular, the temperature directly impact the ecological and evolutionary dynamics of populations and the individual's infection development and pathogen virulence (Thomas and Blanford 2003; Mouton et al. 2006). The influence of temperature in various symbiotic model systems may help to predict the evolution of local adaptations to regulate infection density (Mouton et al. 2007). However, the temperature can have a specific effect on both the symbiont and the host separately; the influence on the symbiotic interaction relies on the type of symbiosis between host and symbiont (Thomas and Blanford 2003; Mouton et al. 2006). For example, short exposure to high temperatures in pea aphids eliminates all or most of their bacterial symbiont, *Buchnera aphidicola*. This resulted in drastically lower fecundity and reduced thermal resistance of hosts due to a deficiency in the production of essential amino acids derived from the obligatory symbiont (Dunbar et al. 2007).

*Wolbachia*, arguably the most common animal endosymbiont in nature, is a maternally inherited intracellular bacteria belonging to Alphaproteobacteria, present in arthropods and nematodes (Werren et al. 1995; Werren et al. 2008; LePage and Bordenstein 2013). *Wolbachia* lineages are classified into 17 supergroups (A-H) based on their divergence in molecular phylogenetic analyses, which differ in their host range and type of symbiosis, spanning from mutualistic to parasitic (Gerth and Bleidorn 2016; LePage et al. 2017). The *Wolbachia*-host symbiosis can affect the host fitness, especially by manipulating reproduction, e.g., due to feminization, parthenogenesis, male-killing, or cytoplasmic incompatibility (CI) to eventually increase their spread in the host population (Werren et al. 1995; Saridaki and Bourtzis 2010). The effect of temperature on *Wolbachia* has always been of considerable interest, as it may influence endosymbiont density and completeness of CI (Bordenstein and Bordenstein 2011), yet this effect may vary among endosymbiont strains and hosts. Previous studies showed that extremely high and low temperatures could be lethal for the symbiont (Perrot-Minnot et al. 1996; Van Opijnen and Breeuwer 1999). In *Drosophila bifasciata*, lower *Wolbachia* density has been recorded at elevated temperature (26°C) (Hurst et al. 2000)*;* however*, D. simulans* males favor low temperature (19°C) in terms of the infection density, especially during larval development

(Clancy and Hoffmann 1998). Interestingly, *Wolbachia* is even able to manipulate the temperature preference of its hosts (Hague et al. 2020).

Here we explore the effects of high temperature by comparing *Wolbachia* density in naturally infected (MN61) and uninfected (HP70) stocks of confused flour beetle, *Tribolium confusum,* from Kansas, USA. Both *T. confusum* stocks may differ in their genetic background, and as such, this could influence the fecundity of crosses between them. Previous studies demonstrated the presence of complete CI and reproductive isolation between the beetle populations (Wade and Stevens 1985). One follow-up study reported the probability of the existence of two different *Wolbachia* strains (Werren et al. 1995); nevertheless, Fialho and Stevens (1996) showed that out of eight different stocks of *T. confusum*, all were infected with a single and common CI inducing strain (Fialho and Stevens 1996,1997; Kageyama et al. 2010; Ming et al 2015). Here, we investigated the effect of high temperature on the density of *Wolbachia* infection in *T. confusum* adults. High temperature has a significant impact on *Wolbachia* density, which might influence the host reproduction, and as such, we investigated (i) how symbiont density is affected by heat, (ii) the impact of heat on CI, and (iii) if high temperature influences sex ratio of the host regarding *Wolbachia* infection.

## Main text

### *Methods*

**Insect biology and rearing**. In this study, two stocks of *Tribolium confusum* (Coleoptera: Tenebrionidae) beetles were compared, being either infected (MN61) or uninfected (HP70) with *Wolbachia*. The beetle's stock was established from adults and transported from the Stored Product Insect and Engineering Research center of USDA in Kansas, USA. They were stored in container boxes with a feed medium containing a small proportion of brewer yeast (5%) in type 405 wheat flour and maintained at 30°C and 65±5%RH under a 16:8 (D: L) cycle. Later, beetles were sexed at the pupal stage based on their urogomphi morphology (Stanley and Grundmann 1965).

**DNA extraction and detection of *Wolbachia*.** Single adults were removed from their stock containers for DNA extraction with Roboklon tissue and bacterial DNA kit (Roboklon GmbH,

Berlin, Germany). Polymerase chain reaction (PCR) was carried out using primer pairs *wsp*F (5'-GCAGCATATATCAGCAATCCTTCAA) and *wsp*R (5'-GCATCATCCTTAGCCGCCTTAT) (Kageyama et al. 2010). PCR was performed in thermocyclers in a total volume of 25 µl (12.5 µl DreamTaq PCR master mix, 0.5 µl for each forward and reverse primer, and 10.5 µl distilled water). The PCR thermal profile used was- one cycle (the 30s 94°C) followed by 35 cycles (15s 94°C, 30s 53°C) and one cycle (30s 72°C). Gel electrophoresis demonstrated DNA bands in 1% agarose gel stained in GelRed (Zhou et al. 1998).

**Effect of heat on *Wolbachia* density** Six young infected adults (3-5 days old - 3 ♀ and 3 ♂), which were kept at the rearing temperatures of 30-34°C, were tested to measure the relative *Wolbachia* density for two consecutive generations by carrying out real-time PCR on Rotor-Gene Q (Qiagen, Hilden, Germany). Each sample was pipetted two times into a 72-well plate and run with two sets of primers and two technical replicates for each sample. As for the first set, a specific pair for *Tribolium confusum* is *Tco*261F23 (CAGGATGAACTGTTTACC) and *Tco*474R25 (GTAGGTCGTATATTAATTACTG), along with a specific TaqMan probe (FAM-ATCATCTAATATCGCTCACGGAGGAG-TAMRA) to identify *T. confusum* were used. PCR amplification in a final reaction volume of 20 µl contained 10 µl Premix ExTaq (Probe qPCR, 2X) (ThermoFisher Scientific, MA, USA), 0.4 µl specific forward primer, 0.4 µl specific reverse primer, 0.8 µl TaqMan probe, 7.4 µ l ddH2O, and 1 µl template DNA. The PCR cycler conditions were an initial denaturation at 95°C for the 30s, followed by 35 cycles of 95 °C for 5 s, 60 °C for 34 s, and a final extension at 72°C for 10 min (Zhang et al. 2016). Other sets of primers were designed to detect *Wolbachia wsp* gene as *wsp*F (5'-GCAGCATATATCAGCAATCCTTCAA) and *wsp*R (5'-GCATCATCCTTAGCCGCCTTAT) as well as specific designed TaqMan probe (5'- FAM-TGTTAGCTATGATGTAAC135TCCAGAA-TAMRA). Real-time quantitative PCR reactions with a total volume of 20 µl contained 10 µl Premix ExTaq (Probe qPCR, 2X) (ThermoFisher Scientific, MA, USA), 0.2 µl specific forward primer, 0.2 µl specific reverse primer, 0.4 µl TaqMan probe, 8.4 µ l ddH2O, and 2 µl template DNA. The temperature regime was as follows: 30s at 95°C for initial denaturation, then 40 cycles of 95°C for 5s, 60°C for 34s, and with a final extension at 72 °C for 10 min (Ming et al. 2015). PCR was carried out to attain

the crossing point (Cp) values for these markers of each beetle. Differences between the crossing point (ΔCp) of the *Wolbachia* and *Tribolium confusum* primers were calculated and then transformed by $2^n$ to reach the relative estimates of *Wolbachia* density (Lee et al 2012).

**Test for cytoplasmic incompatibility (CI).** Beetles reared at 30-34°C were used to determine the effect of *Wolbachia* density on CI expression in incompatible crosses under heat stress. Crosses were performed in four combinations, using 3-5 days old males and virgin females ($w^+$ x $w^+$, $w^+$ x $w^-$, $w^-$ x $w^+$, and $w^-$ x $w^-$) with three cross-replicates per combination. After three days, the number of eggs for each cross and assigned temperature were calculated for 30 days. Subsequently, eggs were placed in separate vials containing flour medium and were checked for hatchability. The presence of CI and the level of incompatibility were estimated from this data using temperature as the only independent variable.

**Test for reproduction and sex ratio**. The results from the last experiment, and the numbers of emerging male and female adults (in the pupal stage), were recorded for each vial and temperature every day for 30 days. This data allowed us to estimate the reproduction and survival rate plus the sex ratio (% females) of beetles in five different temperatures.

**Statistical analysis**. For statistical analysis, one-way ANOVA and Tukey post-hoc tests were conducted in JMP v16.2.0 (SAS Institute Inc., Cary, NC, USA) to assess the effect of temperature on the density of endosymbiont bacteria, *Wolbachia,* and also its impact on the fertility of *T. confusum* females. As for the sex ratio, the number of eggs for a pair of beetles was assessed for each temperature (mean± SD) by the Tukey HSD test, $p<0.05$ (Supplementory table 2).

## *Results and discussion*

**Effect of heat on *Wolbachia* density.** Females and males of *T. confusum* were reared in five different temperatures from 30°C as a favorable developmental temperature for the host to 34°C, the highest temporal degree that the host survived. The relative density of *Wolbachia* in individual beetles varies with temperature. In two consecutive generations, the density of

*Wolbachia* was not significantly different (F1: $F_{4,29}$= 5.61, p-value= 0.002, F2: $F_{9,29}$= 2.30, p-value= 0.057), however there was an obvious reduction of density for the beetles reared at 34 °C, in comparison with those reared at 30°C (F1: p-value=0.0352, F2: p-value= 0.001 ) and 31°C (F1: p-value=0.001) (Fig. 1).

Lu et al. (2019) reported *Wolbachia* density differences between sexes, and we thus analyzed the results of the two sexes separately (Lu et al. 2019). However, our results showed no significant difference in the density of males regardless of the temperature that they reared at for two consecutive generations (F1: p-value= 0.46, F2: p-value= 0.45).

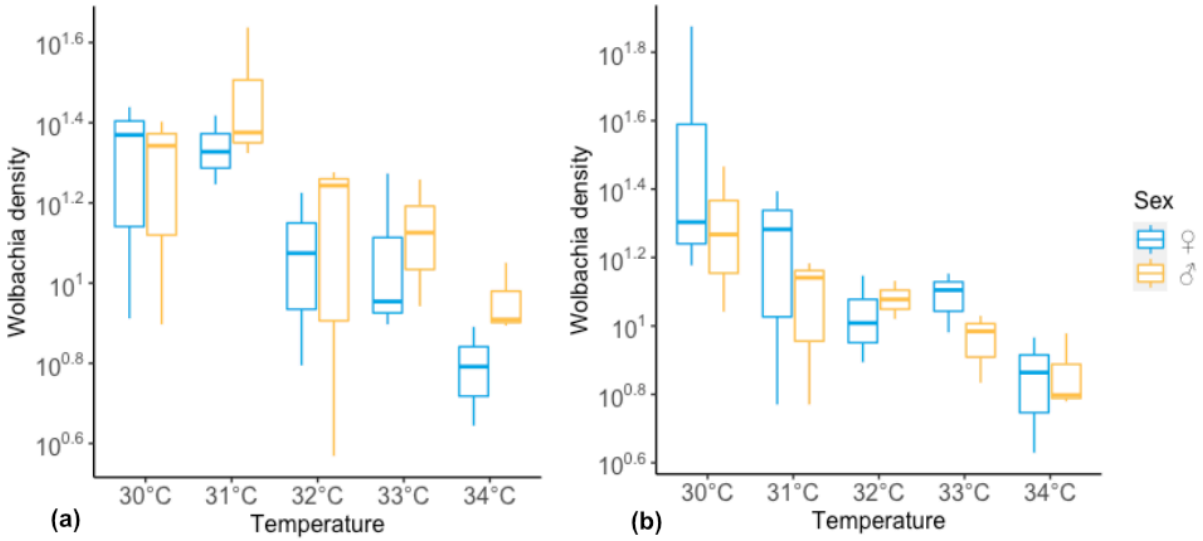


**Figure** 1. Relative density of *w*Tcon in *Tribolium confusum* reared under rearing temperature cycles of 30–34°C for F1 (a) and F2 (b).

**Impact of heat on cytoplasmic incompatibility**. Fecundity can be influenced by the temperature, so we first tested the effect of higher temperature on the fecundity of the virgin females and males in four different crosses respectively (w+ x w+, w+ x w-, w- x w+, and w- x w-). After counting the number of laid eggs for each cross in five different temperatures, no noticeable differences in the number of laid eggs in all four crosses were found (F1: $F_{4,59}$= 8.78, p-value <0.001, F2: $F_{4,59}$= 5.795, p-value =0.0006), except for an obvious decrease of egg production for the beetles reared at 34°C, in comparison with those reared at 30°C (F2: p-value= 0.001), 31°C (F1: p-value= 0.026, F2: p-value=0.001) and 33°C (F1: p-value= 0.001) (Fig. 2a-b). However, temperature had no significant impact on the number of laid eggs in crosses of the beetles which were reared at 30-33°C (F1: $F_{3,15}$ = 2.256, p-value < .05, F2: $F_{3,15}$= 3.027, p-value

< .05). In addition, the number of produced eggs for four different crosses regardless of what temperature they reared at, were not significant in both generations (F1: $F_{3,19} = 0.53$, p-value < .05, F2: $F_{3,19} = 0.874$, p-value < .05).

Afterward, we tested the effect of temperature on the completeness of CI. As for the number of the hatched eggs, the same results applied to the number of hatched eggs by a drastic reduction when reared at the highest survival temperature (34°C) in all crosses (F1: $F_{4,44} = 9.88$, p-value < 0.0001, F2: $F_{4,44} = 16.56$, p-value < 0.0001). Although a study reported a reduced number of progenies and hatched eggs from the crosses between uninfected males and females in comparison with the other two crosses (Ming et al. 2015), our results cannot validate this finding (F1: $F_{2,44} = 0.201$, p-value= 0.818, F2: $F_{2,44} = 1.02$, p-value = 0.377). As for the expression of CI under higher temperatures, we found that a complete CI occurred even in higher temperatures (33-34°C), and no hatched egg could be detected from incompatible crosses of infected males, and uninfected females reared in different temperatures; thus, heat has no significant effect on CI. To some degree, the number of hatched eggs in crosses of both infected males and females (w+ x w+) is higher than the hatched eggs for infected females and uninfected males (w+ x w-), which may result from the loss of the ability of infected females to restore compatibility with infected males (Fig. 2c-d).

Additionally, heat stress appears to have no significant effect on the sex ratio, which for both sets of beetles reared under optimal (30°C) and stress temperatures (34°C) varied between 0.5-0.6 (Supplementary table 2), confirming the suggestion that *w*Tcon has no significant role in altering the sex ratio (Kageyama et al. 2010; Ming et al. 2015).

# Conclusion

Based on our results, higher temperature alters the density of *w*Tcon in the *Tribolium confusum*. We showed that among the five different temperatures beetles were reared at, the highest *Wolbachia* density was reported at 30-31°C, which is also the most favorable temperature range for the host development, while at 34°C, the density of *w*Tcon decreased to a great extent. Furthermore, the fertility of adult females was strongly reduced at 34°C, with a drastic reduction in the number of laid and hatched eggs. However, based on our study (but with a rather low number of replicates), we found that CI was intact even in the mating crosses between the adult

beetles, reared at the highest temperature. Therefore, this might interpret as the low-density requirement of *w*Tcon in the case of inducing CI in *T. confusum*, although this demands further experiments.

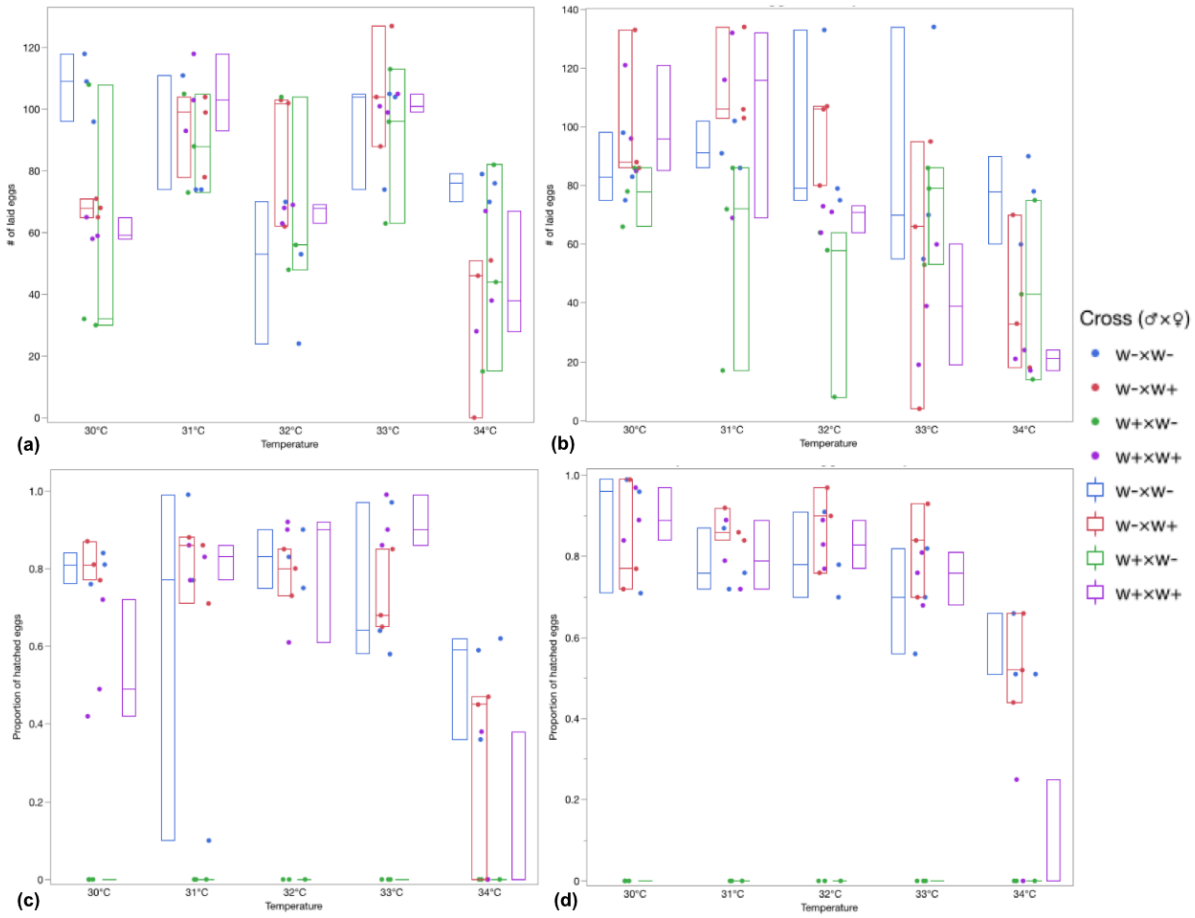


**Figure 2.** Box plots showing the effect of high temperature on the number of laid eggs for F1 (a) and F2 (b) in four cross combinations and hatch proportion in three cross combinations for F1 (c) and F2 (d) of *Tribolium confusum* beetles.

# Limitations

Changes in environmental conditions can affect the infection dynamics and the interaction of *Wolbachia* with their host and, as a result, the ability of the host to adapt in a wild population. Although our findings suggest a spatial and/or seasonal difference in *Wolbachia* densities based on our experiments, field data with wild populations would be essential to understand the effects in a natural setting. Due to time and space constraints, we limited our replicates, which should be resolved in further experiments.

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## Abbreviations

***PCR*:** Polymerase chain reaction

***qPCR*:** Quantitative polymerase chain reaction

***CI*:** Cytoplasmic incompatibility

**Ethics approval and consent to participate**

Not applicable.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Authors contributions**

YG carried out study design, the experiment, molecular laboratory work, and data analyses. CB carried out the study design. YG drafted the manuscript, and both authors read and approved the final manuscript.

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# Appendix

Supplementary table 1. Relative density of *w*Tcon for male and females of *T. confusum*, reared at different temperature based on Cq value for two consecutive generations as (a) F1 and (b) F2.

(a)

| F1 | Sex | $\bar{x}$ Cq (Tcon) | $\bar{x}$ Cq (*wsp*) | Subtract (n) | 2n |
|---|---|---|---|---|---|
| 30°C | ♀ | 21.68 | 18.65 | 3.03 | 8.16809701 |
| 30°C | ♀ | 22.28 | 17.5 | 4.78 | 27.474094 |
| 30°C | ♀ | 22.99 | 18.44 | 4.55 | 23.4253711 |
| 30°C | ♂ | 22.26 | 17.8 | 4.46 | 22.0086691 |
| 30°C | ♂ | 21.72 | 18.74 | 2.98 | 7.88986163 |
| 30°C | ♂ | 22.94 | 18.08 | 4.86 | 25.281322 |
| 31°C | ♀ | 23.67 | 19.53 | 4.14 | 17.6304819 |
| 31°C | ♀ | 23.61 | 18.9 | 4.71 | 26.1728659 |
| 31°C | ♀ | 23.61 | 19.2 | 4.41 | 21.258973 |
| 31°C | ♂ | 24.34 | 18.9 | 5.44 | 43.4113385 |
| 31°C | ♂ | 24.1 | 19.53 | 4.57 | 23.7523771 |
| 31°C | ♂ | 22.95 | 18.55 | 4.4 | 21.1121266 |
| 32°C | ♀ | 21.58 | 17.51 | 4.07 | 16.7954669 |
| 32°C | ♀ | 20.63 | 17.99 | 2.64 | 6.23331664 |
| 32°C | ♀ | 21.98 | 18.41 | 3.57 | 11.8761886 |
| 32°C | ♂ | 21.16 | 19.27 | 1.89 | 3.70635225 |
| 32°C | ♂ | 23.12 | 18.88 | 4.24 | 18.8958826 |
| 32°C | ♂ | 24.68 | 20.55 | 4.13 | 17.5086992 |
| 33°C | ♀ | 21.61 | 18.44 | 3.17 | 9.00046788 |
| 33°C | ♀ | 23.82 | 19.59 | 4.23 | 18.7653592 |
| 33°C | ♀ | 22.79 | 19.81 | 2.98 | 7.88986164 |
| 33°C | ♂ | 24.04 | 19.86 | 4.18 | 18.1261422 |
| 33°C | ♂ | 23.31 | 19.57 | 3.74 | 13.3614067 |
| 33°C | ♂ | 23.48 | 20.35 | 3.13 | 8.75434961 |
| 34°C | ♀ | 21.04 | 18.41 | 2.63 | 6.19025997 |
| 34°C | ♀ | 21 | 18.86 | 2.14 | 4.40762046 |
| 34°C | ♀ | 22.49 | 19.53 | 2.96 | 7.78123958 |
| 34°C | ♂ | 23.75 | 20.26 | 3.49 | 11.235559 |
| 34°C | ♂ | 22.29 | 19.32 | 2.97 | 7.83536238 |
| 34°C | ♂ | 21.81 | 18.79 | 3.02 | 8.11167584 |

(b)

| F2 | Sex | $\bar{x}$ Cq (Tcon) | $\bar{x}$ Cq (*wsp*) | Subtract (n) | 2n |
|---|---|---|---|---|---|
| 30°C | ♀ | 24.67 | 18.44 | 6.23 | 75.0614368 |
| 30°C | ♀ | 22.74 | 18.83 | 3.91 | 15.032364 |
| 30°C | ♀ | 23.22 | 18.89 | 4.33 | 20.112214 |
| 30°C | ♂ | 22.98 | 19.52 | 3.46 | 11.0043345 |
| 30°C | ♂ | 23.51 | 18.64 | 4.87 | 29.2426064 |
| 30°C | ♂ | 23.59 | 19.38 | 4.21 | 18.5070109 |
| 31°C | ♀ | 22.45 | 19.89 | 2.56 | 5.89707687 |
| 31°C | ♀ | 23.78 | 19.15 | 4.63 | 24.7610399 |
| 31°C | ♀ | 24.315 | 20.05 | 4.265 | 19.1596593 |
| 31°C | ♂ | 23.16 | 19.23 | 3.93 | 15.242208 |
| 31°C | ♂ | 23.32 | 19.53 | 3.79 | 13.8325957 |
| 31°C | ♂ | 22.72 | 20.16 | 2.56 | 5.89707687 |
| 32°C | ♀ | 22.65 | 18.84 | 3.81 | 14.0256915 |
| 32°C | ♀ | 22.06 | 18.71 | 3.35 | 10.196485 |
| 32°C | ♀ | 22.36 | 19.39 | 2.97 | 7.83536238 |
| 32°C | ♂ | 23.51 | 19.75 | 3.76 | 13.547925 |
| 32°C | ♂ | 23.39 | 19.81 | 3.58 | 11.958794 |
| 32°C | ♂ | 23.3 | 19.91 | 3.39 | 10.4831472 |
| 33°C | ♀ | 23.34 | 19.67 | 3.67 | 12.7285837 |
| 33°C | ♀ | 23.86 | 20.03 | 3.83 | 14.2214829 |
| 33°C | ♀ | 23.62 | 20.36 | 3.26 | 9.57982964 |
| 33°C | ♂ | 23.36 | 19.94 | 3.42 | 10.7034204 |
| 33°C | ♂ | 23.26 | 19.99 | 3.27 | 9.64646262 |
| 33°C | ♂ | 23.31 | 20.54 | 2.77 | 6.82107913 |
| 34°C | ♀ | 22.77 | 19.9 | 2.87 | 7.3106516 |
| 34°C | ♀ | 23.42 | 21.33 | 2.09 | 4.25748073 |
| 34°C | ♀ | 23.14 | 19.93 | 3.21 | 9.25350547 |
| 34°C | ♂ | 23.75 | 20.26 | 3.49 | 11.235559 |
| 34°C | ♂ | 22.29 | 19.32 | 2.97 | 7.83536238 |
| 34°C | ♂ | 21.81 | 18.79 | 3.02 | 8.11167584 |

Supplementary table 2. Results of crosses between *Wolbachia*-infected and uninfected *Tribolium confusum*, rearing continuous at 30°C (a), 31°C (b), 32°C (c), 33°C (d), 34°C (e). Statistical analysis by two-way ANOVA and Tukey/Kramer test (P=0.05) (mean ± standard error).

**(a)**

| Temperature (30°C) | crosses (♂×♀) | N | number of eggs | Eggs hatched (%) | number of F1 adults | F1 females (%) |
|---|---|---|---|---|---|---|
| **F1** | w+ ×w+ | 3 | 60.6±3.7 | 54±15.6 | 16.5±4.8 | 58±5.5 |
| | w- ×w+ | 3 | 68±3 | 81±5 | 28±5.3 | 49.4±10.4 |
| | w+ ×w- | 3 | 56.6±44.4 | 0 | 0 | 0 |
| | w - ×w- | 3 | 107.6±11 | 80±4 | 50±6.1 | 52.8±1.2 |
| **F2** | w+ ×w+ | 3 | 100.6±18.4 | 90±6.5 | 46±11.3 | 54.4±3.8 |
| | w- ×w+ | 3 | 102.3±26.57 | 82.6±14.3 | 44±19.8 | 60.1±10.8 |
| | w+ ×w- | 3 | 76.6±10 | 0 | 0 | 0 |
| | w - ×w- | 3 | 85.3±9.5 | 88.6±15.3 | 37±10.3 | 58.2±6.1 |

**(b)**

| Temperature (31°C) | crosses (♂×♀) | N | number of eggs | Eggs hatched (%) | number of adults | Females (%) |
|---|---|---|---|---|---|---|
| **F1** | w+ ×w+ | 3 | 104.6±12.5 | 82±4.5 | 59±8.8 | 56.4±2.3 |
| | w- ×w+ | 3 | 110±13.9 | 81.6±9.29 | 55±8.8 | 53.6±3.4 |
| | w+ ×w- | 3 | 88.6±8.6 | 0 | 0 | 0 |
| | w - ×w- | 3 | 86.3±16 | 62±46.3 | 39±29.1 | 56.3±8.7 |
| **F2** | w+ ×w+ | 3 | 105.6±32.7 | 80±8.5 | 46.3±12.8 | 46.6±2.9 |
| | w- ×w+ | 3 | 114.6±11.5 | 87.3±4.1 | 50±7.7 | 52.8±6.5 |
| | w+ ×w- | 3 | 92.6±36.4 | 0 | 0 | 0 |
| | w - ×w- | 3 | 93±6.6 | 78.3±7.7 | 36.5±7 | 50.3±11 |

**(c)**

| Temperature (32°C) | crosses (♂×♀) | N | number of eggs | Eggs hatched (%) | number of adults | females (%) |
|---|---|---|---|---|---|---|
| **F1** | w+ ×w+ | 3 | 66.6±3.2 | 81±17.3 | 27.6±6.5 | 53.4±4.3 |
| | w- ×w+ | 3 | 89±23.2 | 79.3±6 | 38±11 | 57.2±4.2 |
| | w+ ×w- | 3 | 69.3±3.5 | 0 | 0 | 0 |
| | w - ×w- | 3 | 49±12.2 | 82.6±7.5 | 18±7.8 | 64.1±7.2 |
| **F2** | w+ ×w+ | 3 | 69.3±3.8 | 83±6 | 28.6±4.1 | 50.8±2.7 |
| | w- ×w+ | 3 | 97.6±12.4 | 87.6±10.6 | 42.5±9.8 | 51.7±4 |
| | w+ ×w- | 3 | 43.3±22.8 | 0 | 0 | 0 |
| | w - ×w- | 3 | 95.6±26.4 | 79.6±10.5 | 37.8±13.5 | 54.3±1.3 |

**(d)**

| Temperature (33°C) | crosses (♂×♀) | N | number of eggs | Eggs hatched (%) | number of adults | Females (%) |
|---|---|---|---|---|---|---|
| **F1** | w+ ×w+ | 3 | 101.6±3 | 91.6±6.6 | 55.6±6.5 | 54.7±6 |
| | w- ×w+ | 3 | 106.3±16 | 72±6.3 | 39.6±13.2 | 54.2±5.2 |
| | w+ ×w- | 3 | 61.6±3.7 | 0 | 0 | 0 |
| | w - ×w- | 3 | 94.3±14.3 | 72.6±10.7 | 38.1±16.9 | 60.5±2.3 |
| **F2** | w+ ×w+ | 3 | 39.3±4 | 75±6.5 | 15.3±8.2 | 56.2±11.6 |
| | w- ×w+ | 3 | 65±8.6 | 82.3±11.5 | 28.1±15.2 | 58.8±17.5 |
| | w+ ×w- | 3 | 72.6±10 | 0 | 0 | 0 |
| | w - ×w- | 3 | 86.3±27.3 | 69.3±13.1 | 33.3±19.5 | 48.8±13.4 |

**(e)**

| Temperature (34°C) | crosses (♂×♀) | N | number of eggs | Eggs hatched (%) | number of adults | females (%) |
|---|---|---|---|---|---|---|
| **F1** | w+ ×w+ | 3 | 44.3±16.5 | 12.6±21.9 | 4.3±6.8 | 57.6±40.2 |
| | w- ×w+ | 2 | 32.3±28.1 | 30.6±26.5 | 7.5±6 | 57.6±4.4 |
| | w+ ×w- | 3 | 47±13 | 0 | 0 | 0 |
| | w - ×w- | 3 | 75±3.7 | 52.3±14.2 | 20.8±6.3 | 57.3±2.4 |
| **F2** | w+ ×w+ | 3 | 20.6±2.8 | 8.3±14.4 | 1±1.6 | 66.6±38.4 |
| | w- ×w+ | 3 | 40.3±21.8 | 54±11.1 | 12.8±8.9 | 60.6±8.8 |
| | w+ ×w- | 3 | 44±30.5 | 0 | 0 | 0 |
| | w - ×w- | 3 | 76±12.3 | 56±8.6 | 21.1±11.4 | 57.7±10.9 |

# Chapter 4

## *Wolbachia* (Alphaproteobacteria) modulates the locomotion of females of their host, *Tribolium confusum* to increase infection rate

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## Summary

*Wolbachia*, a maternally inherited endosymbiotic bacteria, alpha-proteobacteria, infects a wide range of host species from arthropods to nematodes. In those species, *Wolbachia* induces changes in the host reproduction to eventually promote their own transmission. In addition to its occurrence in reproductive tissues of their hosts, *Wolbachia* also infects a variety of somatic tissues, which raises the possibility of *Wolbachia*'s role as a potential factor in the behavior and fitness of the host. *Wolbachia*'s effect on the fitness of the host delineates its endosymbiont role in the whole symbiotic relationship within the host populations.

Here we characterize the effect of *Wolbachia* infection on the behavior of the confused flour beetle, *Tribolium confusum.* We found no difference in activity and activity rate between non-infected and infected beetles. However, minute but potent changes in the locomotion and exploration behavior could be observed: Relative to uninfected beetles, infected individuals show less centrophobism and wall-following behavior. More surprising, the change of behavior in the beetles treated with antibiotic to eliminate their *Wolbachia* were more severe in comparison with naturally infected and uninfected strains of the host beetles. Antibiotic-treated beetles tend to be more stationary and centrophobic, in addition, to maintaining reduced exploratory rate and speed. In female infected beetles, we could identify a sex-specific increase in locomotor activity and exploration behavior. An infected female explores 32% more area than her uninfected counterpart. This leads to more encounters with male beetles and consequently to a faster spread of the infection. We suggest that these behavioral effects, which also interact strongly with the genetic background of the host, help to explain the widespread infection of *T. confusum* beetles with *Wolbachia*. These findings might have great implications for further advances on using *Wolbachia* as environmentally friendly biocontrol agent to control pest species.

## Introduction

Microbial symbionts are common in insects, although their type of symbiosis varies from mutualistic to parasitic from one host to another. The fact that the microbial symbionts are critical in host speciation has been discussed frequently (Bourtzis et al. 2003; Hosokawa and Fukatsu 2020). The evolutionary modus operandi of symbionts in speciation remains to be characterized. One of the most widespread intracellular endosymbiotic bacteria is *Wolbachia. Wolbachia* belongs to alpha-proteobacteria and is able to infect 40-55% of organisms from insects to isopods and filarial nematodes with the estimation of infecting 65% of the insect population (Werren 1997; Hilgenboecker et al. 2008; Zug and Hammerstein 2012).

*Wolbachia's* success is attributed to its ability to increase their transmission via selective mechanisms to alter the reproduction of their host. It regulates the reproduction of the host through mechanisms like feminization, parthenogenesis, male-killing, and cytoplasmic incompatibility (CI) (Werren 1997; Bourtzis and O'Neill 1998; Charlat et al. 2003; Goodacre and Martin 2012). Cytoplasmic incompatibility is the most widespread way that *Wolbachia* manipulates the host's reproduction, in which infected males decrease the number of viable eggs when mating with uninfected females (Wade and Chang 1995; Bourtzis et al. 2003; Zheng et al. 2011).

The ability of *Wolbachia* to induce CI positions it to be an ideal model for reproduction mediation of the hosts (Zheng et al. 2011; Beckmann et al. 2017; Shropshire et al. 2018). However, recent studies identified the extensive interaction of *Wolbachia* in non-reproductive tissues as it plays a role in the physiology and ultimately in the behavior of the host (Thomas et al. 2005; Perrot-Minnot and Cézilly 2010; Bi et al. 2019). Evidences for somatic interaction can be found in many species (*Drosophila*: Dobson et al. 1999; Bennington and Hoffman 1989; Rohrscheib et al. 2015; Dobson et al. 1999; Casper-Lindley et al. 2011; Thomas et al. 2018, *Collembola*: Czarnetzki and Tebbe 2004; Xiang et al. 2019, lepidopterans: Narita et al. 2007; Narita et al. 2009) that influenced host fitness by modification of several host genes (Xi et al. 2008; Zheng et al. 2011; Caragata et al. 2017). Additionally, altering the levels of gene expression in, but not limited to, the central nervous system of the host (Thomas et al. 2005; Perrot-Minnot and Cézilly 2010; Bi et al. 2019), *Wolbachia* increased bacterial transmission by action selection (Goodacre and Martin 2012).

The most fundamental action selection, is the process of deciding to be active at all. In many higher organism active phases alternate with sleeping phases in the so-called circadian rhythm/clock [Hendricks et al. 2000; Li et al. 2018). It comes at no great surprise that *Wolbachia* has been found to influence action selection on the fundamental level of the circadian clock (Rohrscheib et al. 2015; Vale and Jardine 2015). As an example, high concentrations of three *Wolbachia* strains (*w*Mel, *w*Riv, and *w*Pop) were found in the central brain of the fruit fly *Drosophila*, where they influenced the hosts circadian clock (Albertson et al. 2013).

*Wolbachias* ability to influence host reproduction, physiology, neuronal control, and ultimately behavior gained in popularity as an environmentally friendly biocontrol factor to control insect pest populations (Zhou and Li 2016). The confused flour beetle, *Tribolium confusum* (Jacquelin du Val) (Coleoptera: Tenebrionidae), is a worldwide pest of cereal products and dried foods, that ranks among the most deleterious pests (Campbell et al. 2004). Besides being distinguished as a notorious pest insect, *Tribolium* species became a powerful model system in the area of evolution, physiology, and development due to their high representative development, due to their short life-cycle and easy to implement cultures (Klingler 2004; Pointer et al. 2021). Among 10 Tenebrionid pest species, *T. confusum* is the only naturally infected beetle with a single CI inducing *Wolbachia* strain (Wade and Stevens 1985; Fialho and Stevens 1996; 1997). The exact type of symbiosis, however, remains elusive. One specific study focused on determining the density of *Wolbachia* in different body parts and its effect on non-reproductive tissues and the behavioral pattern of *Tribolium confusum*, such as host mate choice, and mating performance (Ming et al. 2015). Yet, to further characterize the type of symbiosis between *Wolbachia* and *T. confusum*, more comprehensive studies are needed.

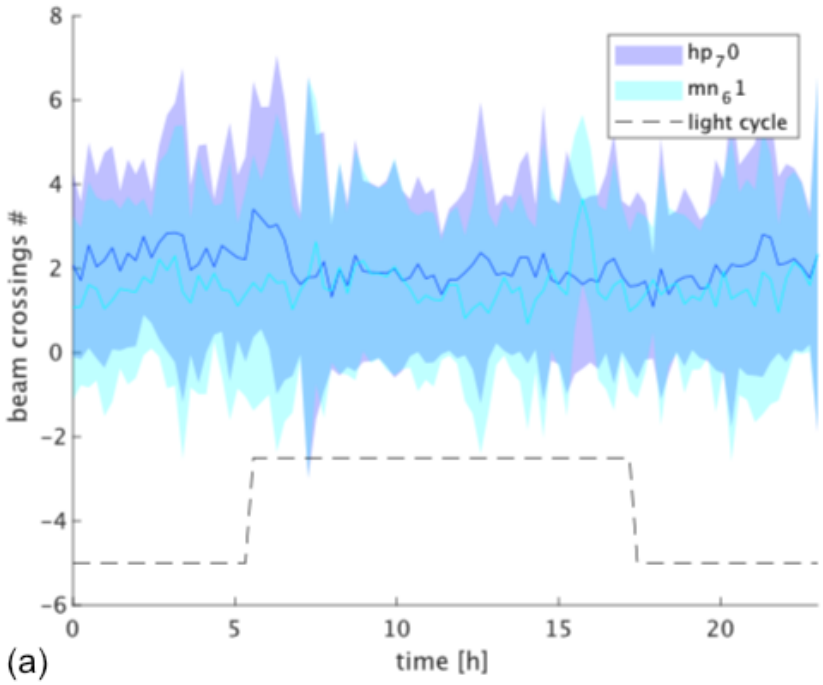
In this present study, we used simple behavioral observation to test the hypothesis that *Wolbachia* increases its own spread within the population of *Tribolium confusum* by modulating fundamental action selection of the host. We recorded the activity patterns of more than 500 beetles for over 300 days (total video recording duration) to reveal *Wolbachia's* effect on (i) circadian rhythm, (ii) sleep frequency, (iii) centrophobism (iv), and exploration rate. Based on this, we systemically explored that *Wolbachia* infected females explore more of their environment. Thereby female beetles increase their chance of copulation, revealing a further and hitherto unknown mechanism of *Wolbachia* to increase its own infection rate.

## Results

To test the hypothesis that *Wolbachia* influences the action selection and thereby the behavior of *Tribolium confusum*, we conducted a number of experiments to pin-point crucial behavioral modifications. A simple yet powerful modification would be to change the general activity level of the beetle, therefore we analyzed the circadian rhythm of uninfected and infected beetles.

### Circadian activity

We analyzed the activity pattern of *Tribolium confusum* in glass tubes of an activity monitor, which measures frequency of in-/activity based on the number of times the beetle would cross the middle of the tube via two photoelectric barriers invisible to the beetle. In the 7-10 days of observation beetles showed no obvious activity pattern regardless of their infection status (Fig. 1a, p-value = 0.109), however infected *T. confusum* beetles (MN 61) had longer phases of inactivity (Fig. 1b, p-value < 0.001) and later sleep onset (Fig. 1c, p-value = 0.004). Therefore, it is likely that *Wolbachia* infection changes the activity rate in a fashion that is not detectable with this very simple setup, as activity that does not cross the midline of the glass vial might be undetected. Hence, we decided to trace movements of the beetles for 24-hours via video recording.



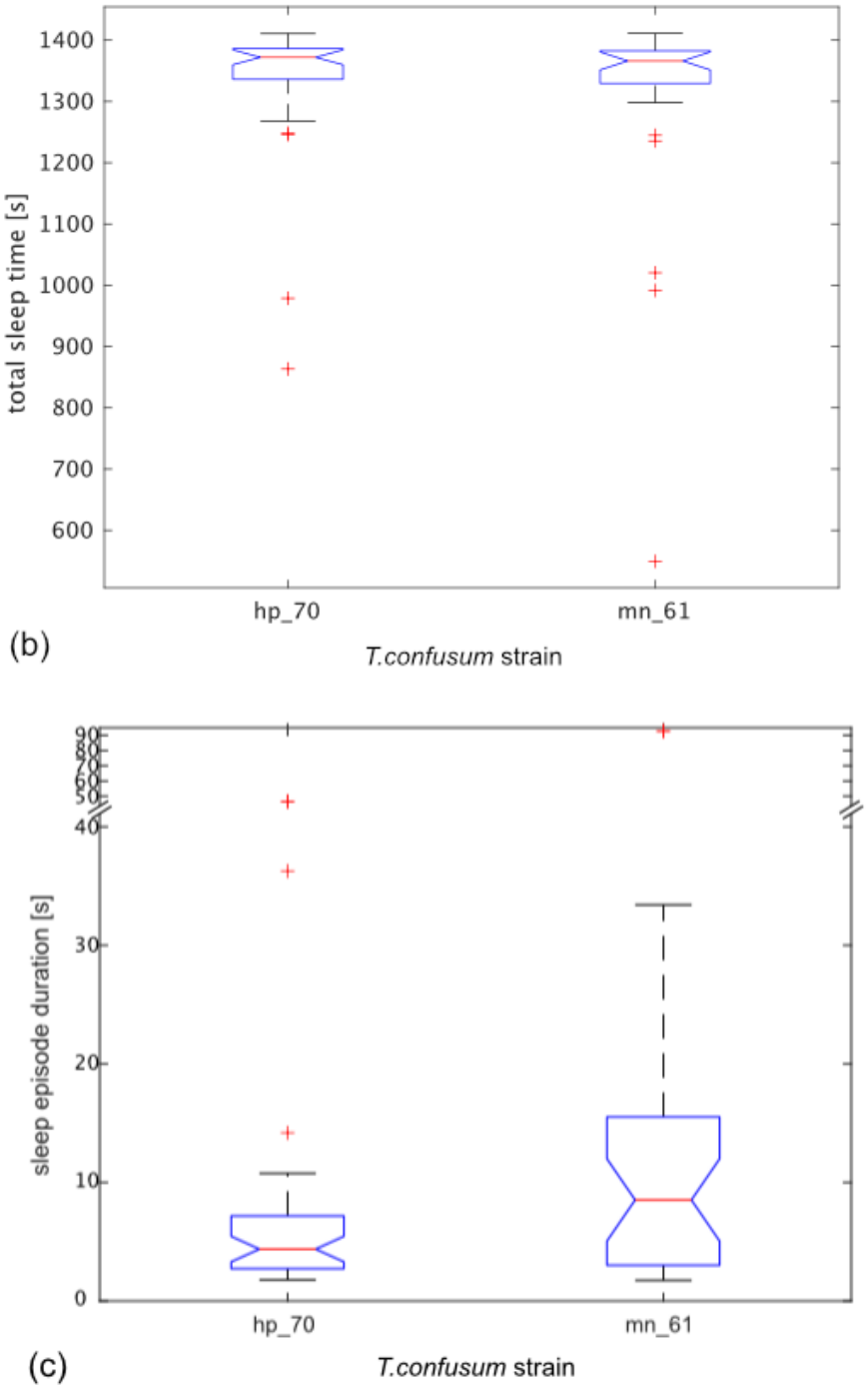



**Figure 1.** *Wolbachia* infection on the circadian activity of infected (MN61, 48 ♂and 48 ♀) and uninfected (HP70, 48 ♂and 48 ♀) confused flour beetle, *Tribolium confusum*. (a) Beam crossing and activity pattern (b) phases of inactivity/ sleep time, and (c) sleep onset/sleep episode duration during 7-10 days. Sleep episode was defined as any quiescence for at least 5 minutes.

## Activity rate and pattern

We observed three groups of *T. confusum* beetles (50 ♂and 50 ♀ per group): infected, uninfected (healthy status), and infected animals treated with antibiotics. We calculated the locomotion velocity as the Euclidean distance between the position of the beetle in two successive video frames and regarded velocities exceeding 2 mm per second as activity. Generally, all beetles showed peak activity on 12-14 hours after being transferred into the observation arena, with the exception of antibiotic treated females, which exhibited the peak activity 2-3 hours later (Fig. 2a). This effect does not show in our circadian data as the first 24h are excluded due to an acclimatization period, with antibiotics showed less and slower locomotion activity. In addition, the rate of activity of infected and uninfected beetles showed no considerable difference for both males and females (p-value > 0.05). However, the antibiotic-treated beetles were less active during the observation time of 24h, for both males (p-value < 0.05) and females (p-value < 0.05) (Fig. 2b). Age itself typically plays a notable role in the beetle activity, yet in this experiment, all the individual beetles were in a similar age range (5-10 days old). In addition, all three strains of beetles maintained an interesting activity pattern.

## Centrophobism

Our results showed that the *T. confusum* beetles, regardless of their *Wolbachia* infection, preferentially stay at the outlet of the arena during a 24 h period. In general, both infected females and males are slightly less centrophobic, in comparison with uninfected and antibiotic treated individuals, and between these two strains, uninfected females tend to be less centrophobic (Fig. 3).

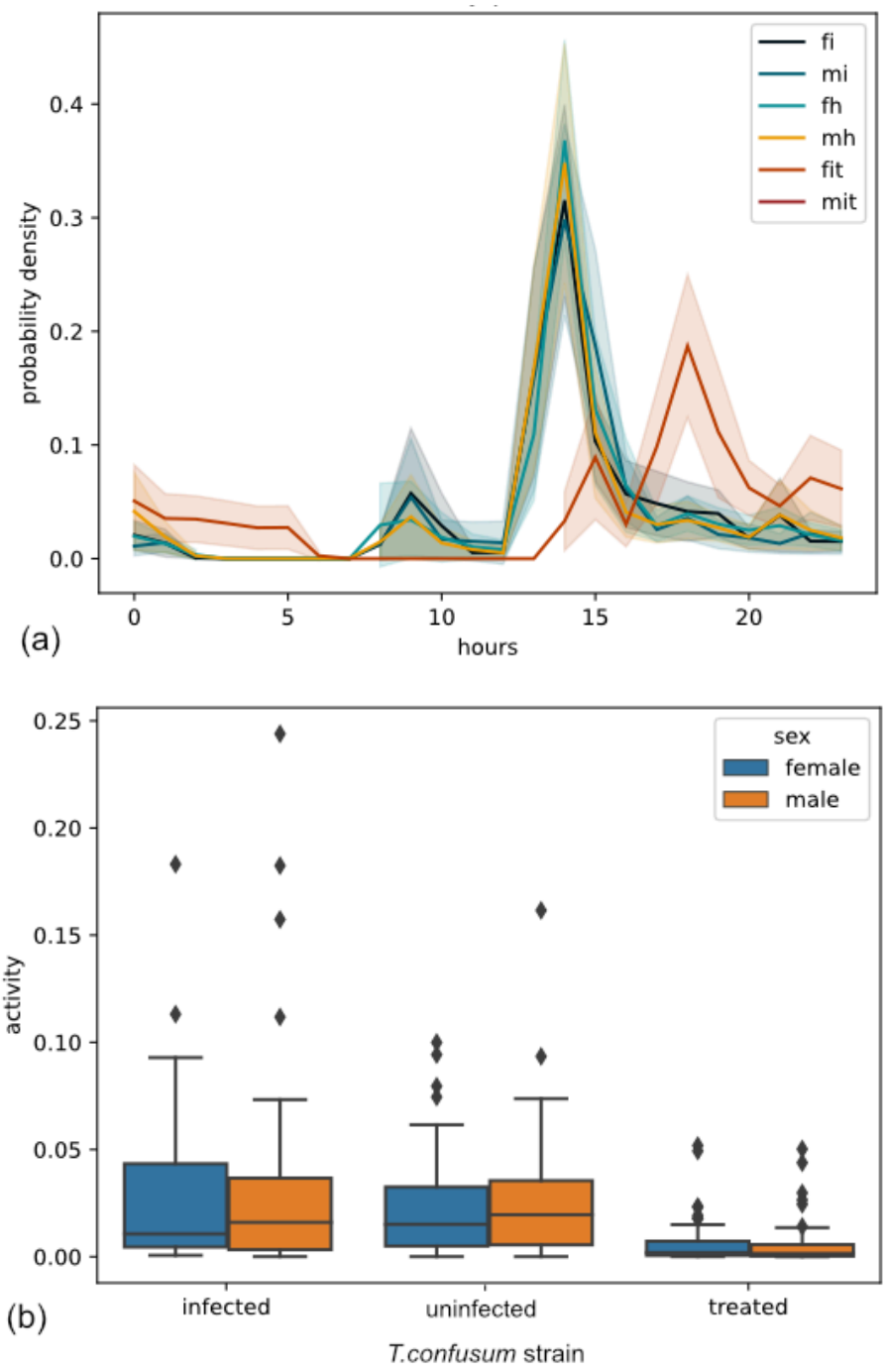



**Figure 2.** Effect of *Wolbachia* infection on (a) the activity pattern and (b) activity rate of *T. confusum* beetles. Three strains of beetles were tested as infected (50 ♂and 50 ♀), uninfected (50 ♂and 50 ♀), and treated (50 ♂and 50 ♀). The lines in the activity pattern histogram are explained as, fi (female infected), mi (male infected), fh (female healthy/uninfected), mh (male healthy/uninfected), fit (female treated), and mit (male treated).

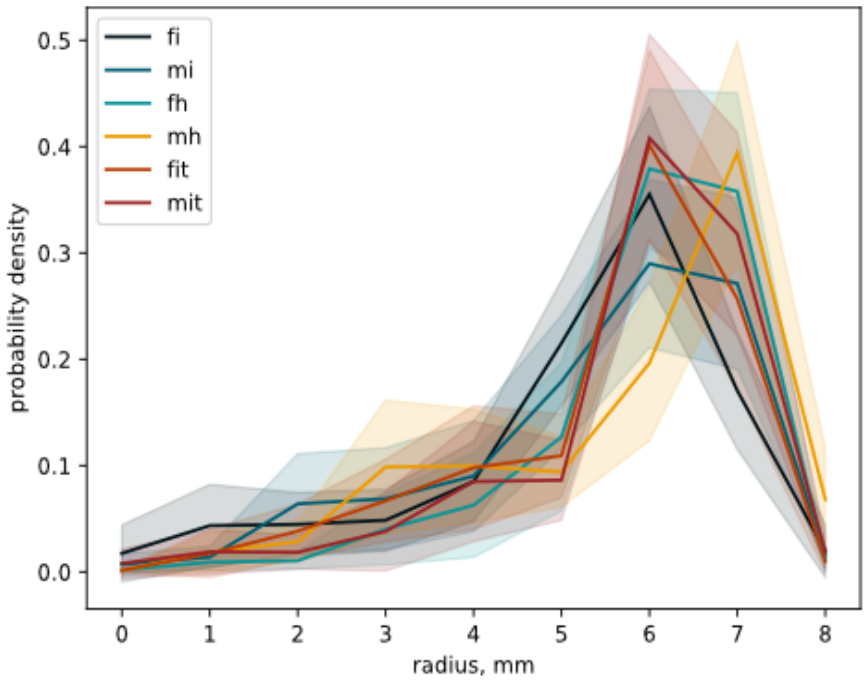



**Figure 3.** Effect of *Wolbachia* infection on centrophobism rate of *Tribolium confusum* beetles. Each line indicates as fi (female infected), mi (male infected), fh (female healthy/uninfected), mh (male healthy/uninfected), fit (female treated), and mit (male treated).

## Exploratory walking behavior

As previous data indicated that *Wolbachia* infected beetles are less centrophobic in comparison with uninfected and antibiotic-treated beetle strains, it was essential to carry out further tests on the exploratory walking behavior and the speed of the beetles to clarify the possible effect of *Wolbachia* on the host. Overall, *Wolbachia* infection altered the median walking speed between infected and uninfected females (p-value < 0.05), yet no obvious change in the walking speed of infected and uninfected males could be detected (p-value > 0.05). Moreover, the speed rate between males and females of the similar strain of infected and uninfected was not significant (p-value > 0.05). Once more, the median walking speed for both males and females of treated beetles is considerably lower, in comparison with males and females of infected and uninfected beetles (p-value < 0.05), even so within treated beetles, females walking slightly faster than males (p-value < 0.05) (Fig. 4a). Interestingly, the exploration rate index revealed no difference, between the uninfected and infected males (p-value > 0.05) and females (p-value > 0.05), and even between the males and females of a similar strains (p-value > 0.05). Continually with previous observations, the exploratory rate for both males and females of treated beetles is considerably lower, in comparison with males (p-value < 0.05) and females (p-value < 0.05) of

infected and uninfected beetles (Fig. 4b). These males and females of treated strain also maintain the relative exploratory rate (p-value < 0.05).

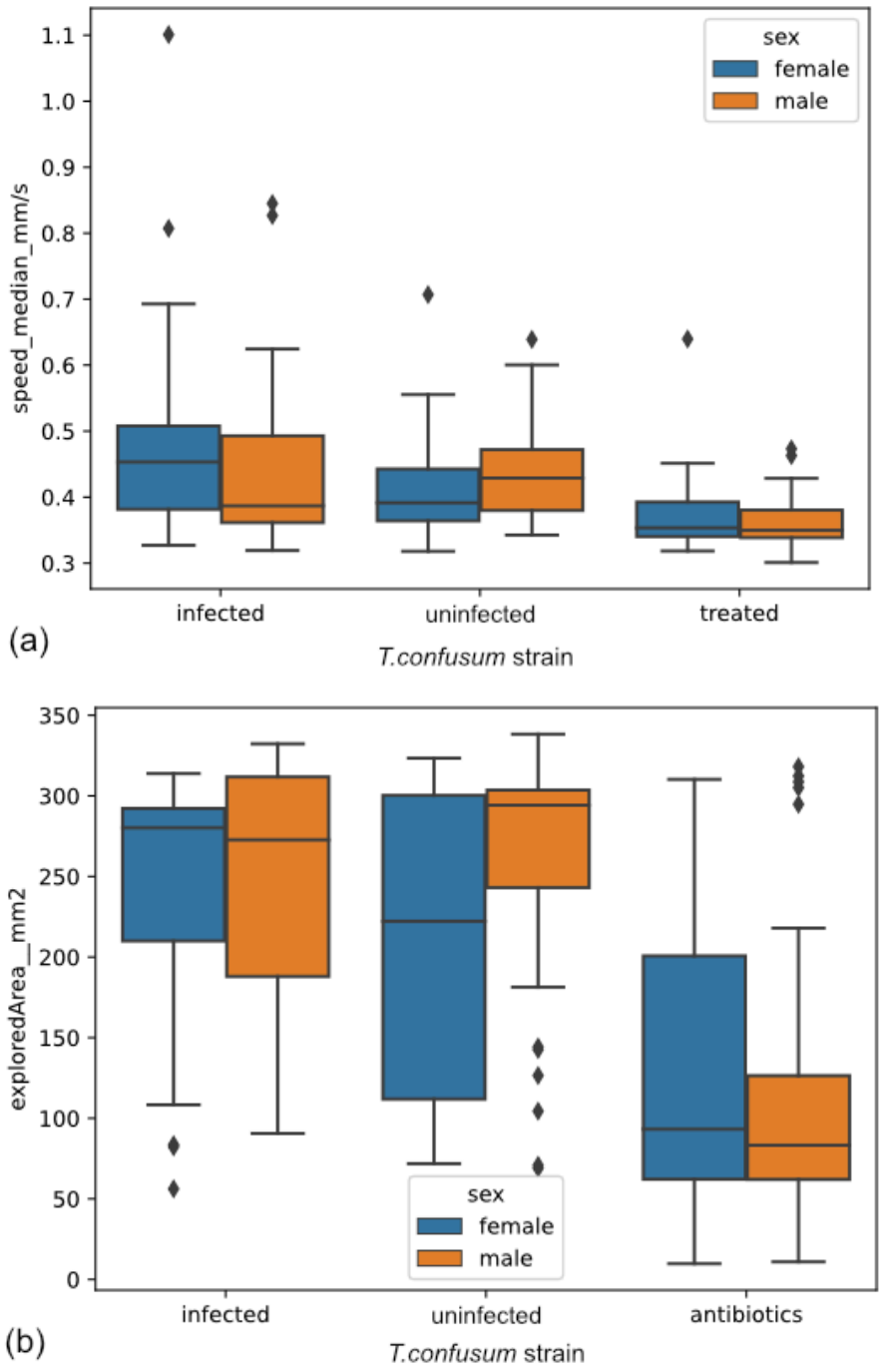



**Figure 4.** Effect of *Wolbachia* infection on (a) the walking speed and (b) the exploration rate of infected (50 ♂and 50 ♀), uninfected (50 ♂and 50 ♀), and treated (50 ♂and 50 ♀) of confused flour beetle, *Tribolium confusum*.

## Discussion

Our primary assumption was that *w*Tcon would operate as regulator on certain fundamental action selection of the beetle, in order to increase its own transmission within the beetle population. The effect of *Wolbachia* on the behavior and activity of many hosts specially for *Drosophila* fruit flies has been described in detail, however, only a single study on the role of *w*Tcon on the male mating preference and performance of *T. confusum* beetles has been documented (Ming et al. 2015). Accordingly, *Wolbachia* infection did not affect the male mating behavior in confused flour beetles (Ming et al. 2015), as well as in two-spotted spider mite *Tetanychus urticae* (Zhao et al. 2013) and butterfly *Acraer encedon* (Jigjins et al. 2002). However multiple studies suggested that *Wolbachia* has the ability to cause alterations in mating behavior in *Drosophila* (Liu et al. 2014; He et al. 2018), and even on mating preference, mating time, mating frequency of some other hosts (de Crespigny et al. 2006; Panteleevet al. 2007; Goodacre and Martin 2012).

Our primary observations revealed that *w*Tcon infection alters the exploration behavior of female *T. confusum* adults. Overall, beetles had no noticeable difference in their activity regards their *Wolbachia* infection, however, infected beetles showed longer pauses during their locomotion, along with later sleep onset. This is consistent with findings on *Drosophila* fruit flies, in which *w*Mel infection would increase sleep time through dopamine pathways (Albertson et al. 2013, Vale and Jardine 2015; Bi et al. 2018), although infected individuals demonstrated an increase in nocturnal activities (Morioka et al. 2018) and sleep latency (Bi et al. 2018), relative to uninfected flies. Based on our observations from 24h walking arena recordings of *T. confusum* beetles, regardless of their infection, were active in a similar rate, yet antibiotic treated beetles showed the opposite pattern by an obvious decrease in their activity rate and pattern.

Moreover, we can confirm a positive influence of *w*Tcon on the performance of exploratory walking and the beetle's exploration rate. Both males and females of infected beetles tend to walk at higher speed, in addition, to be more exploratory. This observation suggests a role of the endosymbiont in favor of its proliferation, as it could be interpreted that when *Wolbachia*-infected females manage to explore more space, they are more accessible for mating with both infected and uninfected males. Generally meaning that infected females might outperform and outbreed healthy females. Previously, it was reported that *Wolbachia* affected the locomotion in

*Drosophila*, though the alterations yielded different impacts based on the environmental conditions and the background of the hosts (Peng et al. 2008; Peng and Wang 2009; Caragata et al. 2011). This effect has been demonstrated in *Aedes aegypti* mosquitos, as the study showed an increase in locomotor activity and metabolism when mosquitos are infected with *Wolbachia* endosymbiont (Evans et al. 2009).

The direct negative effect of antibiotic treatment (Tetracycline hydrochloride) on the insect can be observed during the locomotor activity experiment. The antibiotic-treated beetles showed a notable decrease in their activity and exploratory walking rate (speed), and furthermore, an increase in their centrophobism. Behavioral changes would augment the findings of other studies in which the usage of tetracycline was shown to be detrimental to mitochondria (Ballard and Melvin, 2007).

To minimize any possible effects on physiological phenotypes, the beetles in this experiment were collected 3-4 generations post-treatment prior to the experiment. Nonetheless, using antibiotics has the additional consequence of removing microbial gut flora, in addition to *Wolbachia* elimination. Alternatively, the fact that we see few differences between naturally infected and uninfected beetles, but significant differences in treated beetles, could be the outcome co-evolution between the beetles and its *Wolbachia* hosts, and treatment leads to less well-adapted beetles' strain. Further experiments would be essential to clarify if treated beetles behave differently due to detrimental effects of the antibiotics or the loss of a needed symbiosis partner - *Wolbachia*.

As a conclusion, our study confirms that based on changes in the behavioral pattern mostly in favor of *Wolbachia* infected females, as in locomotor activity and exploratory rate, and along with effect of infection on the fecundity of the host, *w*Tcon have developed a mutualistic relationship with *T. confusum*. Understanding the mechanism underlying *Wolbachia*-induced changes in *T. confusum* is of great interest for the advancement of further areas to facilitate biocontrol strategy for these pests. Determining whether these differences in activity and metabolism that prompted by *Wolbachia,* can also change beetles feeding frequency, aggression, or any other traits, which eventually lend to a better understanding their biological function and symbiont-host interactions. Hence, we believe there is an enormous potential for adjusting specifically designed setups to advantage in further studies of symbiont host interaction of pests and specially *Tribolium* beetles.

## Experimental procedures

### Insect biology and rearing

In this study, two strains of *Tribolium confusum* (Coleoptera: Tenebrionidae) beetles were used, as infected (MN61) and uninfected (HP70) with *Wolbachia*. The beetle's stock was established from adults, transported from the Stored Product Insect and Engineering Research center of USDA in Kansas, USA. They were stored in container boxes with a feed medium containing a small proportion of brewer yeast (5%) in type 405 wheat flour and maintained at 30°C and relative humidity of 65±5, under 16:8 dark-light (DL) cycle. Later, beetles were sexed at the pupal stage based on their urogomphi morphology.

### Antibiotic treatment of *Wolbachia*-infected adults

Both males and female adults were reared on wheat flour (405) containing brewer yeast (5%), along with 0.1% and 0.3% w/w Tetracycline hydrochloride (Carl Roth GmbH, Karlsruhe, Germany). After 2 weeks of antibiotic treatment, DNA from those adults that were fed on two different concentrations of Tetracycline hydrochloride was examined to trace the infection and *Wolbachia* elimination, by general *wsp* primers (Zhou et al. 1998) in PCR amplification. Tetracycline treatment was repeated for two generations, and then the third generation was tested for the locomotor activity experiments.

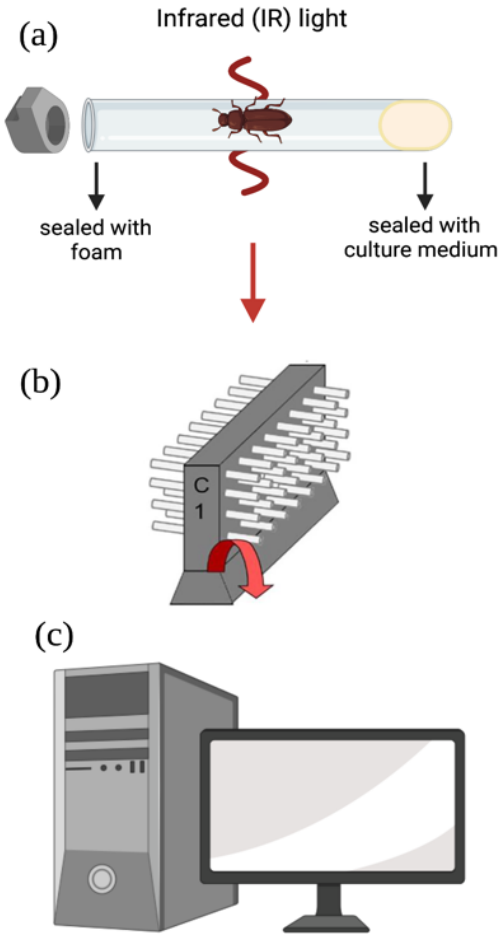



**Figure 5**. Measuring locomotor activity in *Tribolium confusum*. Experimental setup placed inside an incubator (25°C) to create a control environmental condition. The setup showing (a) individual beetle, infected and non-infected with endosymbiont, *Wolbachia* placed inside the activity tubes and then (b) every tube loaded into Trikinetics monitors (DAM system), so infrared beams ran through the tubes to (c) measure any activity levels.

## Circadian activity recording and analysis

This experiment was recorded in *Drosophila* Activity Monitors (DAM; Trinkinetics system, Waltham, MA, United States) at 25°C under LD 12:12 cycles. Trinkinetics system can record the activity of the insect individuals simultaneously and each DAM contains 32 activity monitors, an interface device, and the software to visualize the collection of data (Schlichting and Förster 2015). We set three runs for each strain of adult beetles (males and females) as infected (MN61, N ♀= 48, N ♂= 48) and un-infected (HP70, N ♀= 48, N ♂= 48), and every set was recorded for 7-10 days. Individual beetles were placed in glass tubes (4mm x 65 mm) that contain medium with a porous plug on one end and foam on the other end. An infrared (IR) light covers the tube and is detected by the photodetector. The number of beam crosses is saved in a specific period for each beetle, created by DAM system software. The experiment is illustrated in Figure 5.

## Locomotor behavior of *T. confusum* in an open field

The setup for obtaining the trajectory and position of the beetles in the fairly open walking arena is illustrated in Figure 6. An individual beetle of males and females from each strain (infected and uninfected) along with newly antibiotic-treated beetles placed in a 20 individual observation area with 20 mm radius and 3 mm depth, in which milled intro a slap of polyoxymethylene (POM). The surface area of 314 $mm^2$ was specifically designed for *Tribolium* beetles where the walls of each observational area are at 45° angle to avoid wall following and thigmotaxis. During the recording, the arena was covered with a transparent thin plastic ceiling, and infrared LEDs (940 nm: Pollin GmbH, Pförring, Germany) were used to illustrate the areas from below. For this experiment, 320 beetles (50 males and 50 females from each strain) were recorded in 15 movies with 24h duration, resulting in 432,000 frames for each beetle. The behavior of the beetles was videotaped on Ximea MQ2300 camera (Ximea GmbH, Münster, Germany) for 18-24 hours at 5 fps (frame/second). The sampling rate captured the trajectory adequately and also allowed for fine-grained analysis. Following the accession to recorded data, the position of an individual beetle was tracked in each frame. Eventually, we collected 276,480,000 detections for all animals and arenas, which can be converted to 320 days of recording.

### *Statistical analysis and software tools*

We trained artificial intelligence (AI), to minimize the tracking of each beetle and each arena in every frame, by using Inception v2 (Ioffe and Szegedy 2015), we could retrain an image recognition AI based on the Faster R-CNN (Ren et al. 2015). AI was previously originated on the Oxford-III Pets dataset (Visual Geometry Group - University of Oxford, https://www.robots.ox.ac.uk/~vgg/data/pets/, accessed 1 Mar 2022), and the latest version can be found in tensor-flow model collection (https://github.com/tensorflow/models/blob/master/research/object_detection/samples/configs/faster_rcnn_inception_v2_pets.config). Subsequently, each arena and beetle were labeled in 180 images resulting in 3600 labels for each, then the dataset was expanded by using Imgaug (Jung et al. 2020). Using an AI, all beetles could be directly detected in all frames end-to-end. The procedure for the AI image recognition was generated in Python 3.5 (Van Rossum and Drake 2009), with a focus on three main libraries as NumPy (Harris et al. 2020), Pandas (McKinney 2011), and tensor flow (Abdi et al. 2016) along with others.

For statistical analysis, Fisher's permutation test (Fisher 1936; Collingridge 2013) (implementation: https://github.com/cmohl2013/permutation_test) was executed for the statistical analysis of the behavioral data. This test enables us to assess the significance of the medians of the respective measured variables. The Benjamini-Hochberg false detection rate procedure (Benjamini and Hochberg 1995) was implemented to correct the p-values resulting from the multivariable analysis (Seabold and Perktold 2010).

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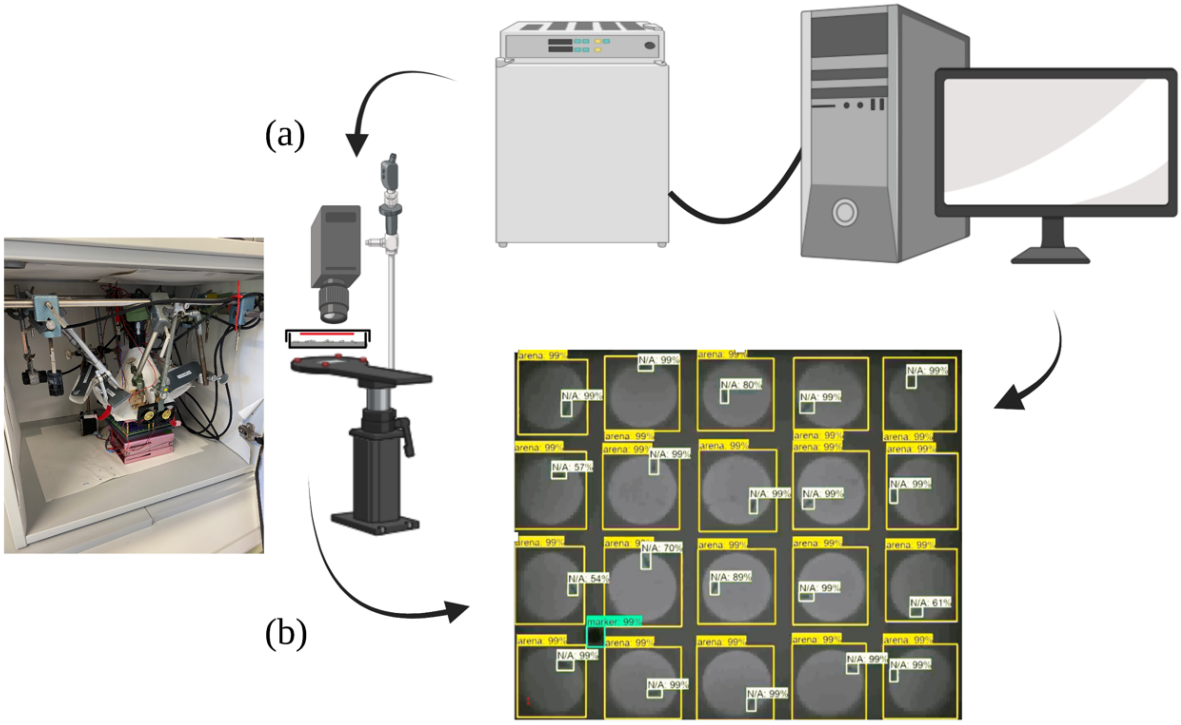


**Figure 6**. Trajectory setup for characterization of locomotor behavior of *Tribolium confusum* different strains based on their microbial infection. (a) the beetles placed in the arena were videotaped for 18-24hours under a visible light source. (b) Data acquisition for locomotion, centrophobism, and positional data.

## Author contributions

**YG** carried out study design and experimental work. BG was involved in study design and analyzed the data. CB carried out study design. **YG** drafted the manuscript. All authors contributed material for the study and approved the final version.

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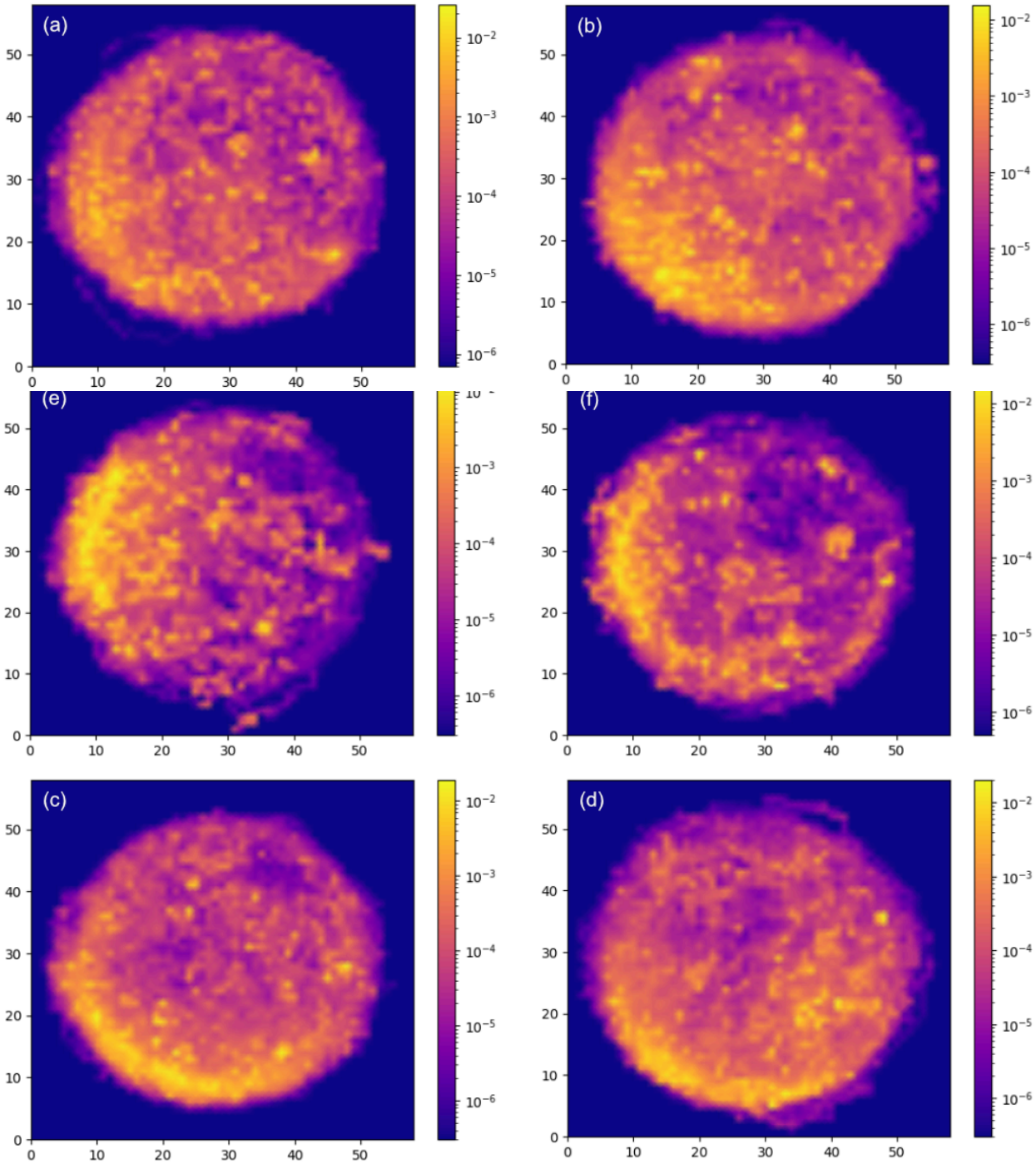
# Appendix




Supplementary figure 1. Transition plots which indicates the relative frequency of the beetle passage at each position is plotted (orange denotes a high frequency, and darker spots means beetles were rarely present. (a). infected female, (b) infected males, (c) healthy/uninfected females (d) healthy/uninfected males, (e) antibiotic treated females, and (f) antibiotic treated mal

# General discussion

## Advantages of the *Tribolium* model system

As for understanding complicated ecological and evolutionary theories, scientists forced to choose between focusing on of a few feasible and easy to culture model organisms (Sommer 2009), or utilizing a mixture of diverse models in order to expand their knowledge (Bolker 2012). It has been stablished that research model systems are essential tools to explain complex diversity research and concepts by adapting a more simplified system for experimenting and investigating. However, "true" model organism should present important criteria such as being informative and adaptive enough with more complex organisms and qualifying us for elevated experimental controls and replications (Pointer et al. 2021). In general, the number of prominent "animal models" are limited, it has been proposed that few numbers of systems might restrict science only to those questions that can be answered by traditional models (Bolker et al. 2012).

In the last decades, *Tribolium* beetles has been investigated as a modern model system in a variety of evolutionary developmental study, since they represent the most species rich Eukaryotes group of animals, the Coleoptera (Daly et al. 1978), and their elevated experimental tractability in different areas from evolutionary ecology to population genetics (Pointer et al. 2021). Additionaly, *Tribolium* beetles, two of which, *T. confusum* and *T. castaneum* are considered as major pests of food industry (Abd El-Aziz 2011). Recent studies illuminate the advantages of *Tribolim* beetles over classical model organism especially *Drosophila* fruit flies and *Culex* mosquitos, as these beetles are considered to maintain many plesiomorphic features of holometabolous insects (Brown et al. 2003; Grimaldi and Engel 2005), alongside their simple and inexpensive breeding condition, relatively short developmental time, high fecundity and longer adulthood interval (Klingler 2004; Wang et al. 2007). These characteristics of *Tribolium* beetles motivated us to investigate host symbiosis relationship between *Wolbachia*, one of the most versatile endosymbiotic bacteria, in association with these beetles.

*Wolbachia*-host interaction has been documented comprehensively for the traditional dipteran model systems, *Drosophila*, *Culex* and *Anopheles* regarding the effect of endosymbiont bacteria on the behavior and fitness of the host (Bi et al. 2020) to their role as a host manipulator (Sinkins

et al. 2005; Weeks et al. 2007; Baldini et al. 2014) and *Wolbachia* genomics (Wu et al. 2004; Klasson et al. 2009). This great focus on these dipteran species is especially due to the potential of *Wolbachia* to impose protection against viruses (Hedges et al. 2008; Glaser and Meola 2010), which lead to their role as a bio-control agent for vector-borne diseases (Mousson et al. 2012; Dobson et al. 2014). As mentioned, *Tribolium* beetles are one of the most cosmopolitan pests, and it has been proposed that *Wolbachia* might act as a possible factor to control pests as well (Goodacre et al. 2015; Ming et al. 2015), still the number of studies on the symbiosis relationship of *Wolbachia* and *Tribolium* beetles are limited.

*Tribolium confusum* (Coleoptera: Tenebrionidae) is the only Tenebrionid beetle that has been naturally infected with a single CI inducing *Wolbachia* (*w*Tcon) (Werren et al. 1995; Fialho and Stevens 1996, 1997). Based on the final assembly of the *w*Tcon genome, the total length of this endosymbiont is 1.41 MP, contains in 12 contigs (Gharabigloozare et al. 2022), which is at the same range of *w*Pip (*Culex pipiens*), another well-known CI-inducing *Wolbachia* strain from the same supergroup B (Klasson et al. 2009). The general characteristics of *w*Tcon also confirms the similarity between this strain and other sequenced genomes of *Wolbachia*, in particular the genome size (~0.9- 1.8 Mb), GC content (~33-35%), and coding sequences (CDSs) (~800-1250). These numbers are numbers are typical for *Wolbachia* strains which show a facultative symbiosis with their hosts, as obligate mutualists have usually a reduced genomes size and reduced gene number. Previous studies on *T. confusum* were mainly on the effect of *Wolbachia* distribution and fecundity of the host (Ming et al. 2015; Li et al. 2016; Lu et al. 2019). Thus, we specifically expanded upon previous studies on *Wolbachia*-host systems by executing further experiments to gain better understanding on this specific symbiosis relationship and subsequently introduce *T. confusum* as an outstanding system in regards to host-symbiosis interactions.

## Effect of temperature on *Wolbachia* density and CI

The sensitivity of *Wolbachia*'s infection rate to temperature in different host species has always been debated. Observations on the reaction of *Wolbachia* density on extremely high and low temperatures indicate that symbiotic populations are negatively impacted (Perrot-Minot et al. 1996; Van Opijnen and Breeuwer. 1999). In many hosts, the higher temperature reduces the transmission efficiency of *Wolbachia*, and for that reason, heat treatment is proposed as a method

to eliminate *Wolbachia,* especially in hosts with parasitic relationships, nonetheless, this curative temperature varies between host populations (Hurst et al. 2001). For instance, van Opijnen and Breeuwer (1999) reported that *Wolbachia* prevalence in red spider mite, *Tetranychus urticae* reduced over four generations for those reared at 32°C (fully recovered after six generations), and only 29% of mites, were still infected with *Wolbachia* after two generations (Van Opijnen and Breeuwer. 1999). In *T. confusum*, the effect of high temperature on the reduction of *Wolbachia* density has been confirmed. Those beetles reared under heat stress (34°C), showed significant decrease in their *Wolbachia* replication rate, especially in comparison with those beetles reared at host's favorable temperature (30-31°C) (*Gharabigloozare and Bleidorn 2022, in review).

As mentioned before, the influence of temperature on *Wolbachia* density varies from one individual to another, so as for the expression of CI, and host's fitness related to the expression of CI. As an example, CI expression can be eliminated for *Aedes polynesiensis* larvae, when they are exposed to 32-33 °C for 5-7 days (Wright and Wang 1980). However, in *Drosophila simulans*, CI is only decreased when the larvae are exposed to a shock heat, and still with no impact on fecundity or survival resulting from CI (Feder et al. 1999). Stevens (1989) demonstrated the possibility of heat treatment (36°C for 12 days) to surpass the expression of CI in larvae of *T. confusum* beetles (Stevens 1989). Nevertheless, a number of studies showed that a complete CI occurred even in higher temperatures, thus heat has no significant effect on CI (Li et al. 2016; Gharabigloozare and Bleidorn, in review). Ming et al (2015) investigated the role of CI inducing *Wolbachia* on the fecundity of the host according to egg production and hatch of *T. confusum* beetles. It stated that CI did not affect egg production, although a significant change in the egg hatch rate was reported in crosses of uninfected females (Ming et al. 2015). This is congruent with our findings, in which, even by adding the heat factor, a significant decrease in the number of laid eggs for the beetles reared under thermal stress (34°C) and in all crosses for two consecutive generations were recorded (Gharabigloozare and Bleidorn, in review). As for the development, the same results applied to the number of hatched eggs by a drastic reduction when reared at the highest survival temperature (34°C) in all crosses. Furthermore, the egg hatch proportion for all crosses, even for the mating crosses between uninfected males and females were reported to be at a similar rate and no drastic reduction was detected, regardless of the temperature that beetles were reared at (Gharabigloozare and Bleidorn, in review). On contrary,

Ming et al. detected a reduction in the number of hatched eggs in the cross between uninfected males and females of *T. confusum* beetles (Ming et al. 2015). Additionally, heat stress appears to have no significant effect on the female ratio, as the ratio for both sets of beetles reared under optimal (30°C) and stress temperatures (34°C) were between 0.5-0.6, confirming the fact that *w*Tcon has no significant role in altering the sex ratio in confused flour beetles, *T. confusum* (Ming et al. 2015; Lu et al. 2019; Gharabigloozare and Bleidorn, in review).

## Effect of *Wolbachia* on the sleep behavior of *T. confusum*

Sleep behavior of some insects such as *Drosophila* with prolonged reversible inactivity and increased arousal thresholds exhibited similarities with mammalian sleep (Hendricks et al. 2000; Shaw et al. 2000). In invertebrates, sleep-like states are conditioned on the behavioral analysis of inactivity, increased arousal threshold, and rest after prolonged waking (Tobler and Neuner-Jehle 1992). The circadian rhythm which occurs approximately 24h, is a system to measure the timing of sleep. Nowadays, sleep in insects, especially for flies can be measured by using *Drosophila* Activity Monitoring (DAM) System, in which locomotor activity was collected every minute and the times with zero activity counts (at least 5 continues minutes) defined as sleep (Huber et al. 2004; Schlichting and Helfrich-Föster 2015).

A significant correlation between the presence of microbial symbionts, in this case, *Wolbachia*, and the sleep time of some host species such as *Drosophila* and *Aedes*, has been described (Albertson et al. 2013). However, the number of studies on sleep behavior and inactivity of confused flour beetle, *Tribolium confusum*, regarding their *Wolbachia* infection, has been accomplished. *Wolbachia* infection in *T. confusum* had no obvious difference in their activity, yet the infected beetles showed longer inactivity phases along with later sleep onset (Chapter 4) (Fig. 1). This suggests that in comparison to *Wolbachia* infected beetles, uninfected individuals are disturbed more frequently even by a minor distraction during their sleep episode, and by all means, infected beetles showed a reduced arousal threshold. This observation is similar to *Drosophila*, where *Wolbachia* infection led to a growth of the number of nighttime sleep bouts (Bi et al. 2018).

## Effect of *Wolbachia* on activity rate

The process of movement and activity of insects can alter by many aspects such as host background, environmental condition, food and shelter distribution (Turchin 1998; Campbell and Hagstrum 2002). However, studies addressing how symbiosis might modify the locomotion in host species are few. Peng et al. (2008) revealed the ability of *Wolbachia* infection to modify the locomotion in flies, although this alteration is dependent on the environmental condition and the background of the host (Peng et al. 2008; Peng and Wang 2009). *Wolbachia-derived* modifications on *Drosophila* and *Aedes* species uncovered the inconstancy of *Wolbachia*'s role in the activity level of host species. As an example, transinfected *A. aegypti* mosquitos with *w*MelPop, rated increased levels of locomotor activity (Evans et al. 2009), while *w*MelPop and *w*Mel infection of their native host (*D. melanogaster*), are not able to cause any change in activity levels (Peng et al. 2008). The results from *D. melanogaster* are consistent with the observation of *Wolbachia* infection in males and females of *T. confusum* since no alteration in the activity rate and pattern could be detected when two different strains of beetles were compared (Chapter 4). Surprisingly, this statement does not apply to antibiotic-treated *T. confusum* beetles. The results showed a significant decrease in the activity rate of antibiotic-treated beetles, and additionally their activity pattern was also slower in comparison with the other two relative strains (Chapter 4) (Fig. 1). These findings can illuminate our further understanding of utilizing *Wolbachia* as a biocontrol agent in controlling the *T. confusum* population.

## Exploratory walking behavior

Along with other behavioral traits, the effect of *Wolbachia* on the foraging behavior (walking activity and speed), might be influenced by other factors such as temperature. As an example, the walking activity of *Trichoderma Atopovirilia* female wasps is strongly dependent on temperature, however, even in different temperatures, no significant difference was found between *Wolbachia* infected and uninfected wasps (Almeida et at.2010). In contrast, *Wolbachia* influences the exploratory walking and crawling rate of *D. nigrospara* adults and larvae respectively, since the infected flies manage to walk and crawl especially in comparison with antibiotic-treated individuals (Detcharoen et al. 2020). This statement congregates with the documented results of *Wolbachia* effect on the exploratory walking behavior of *T. confusum*

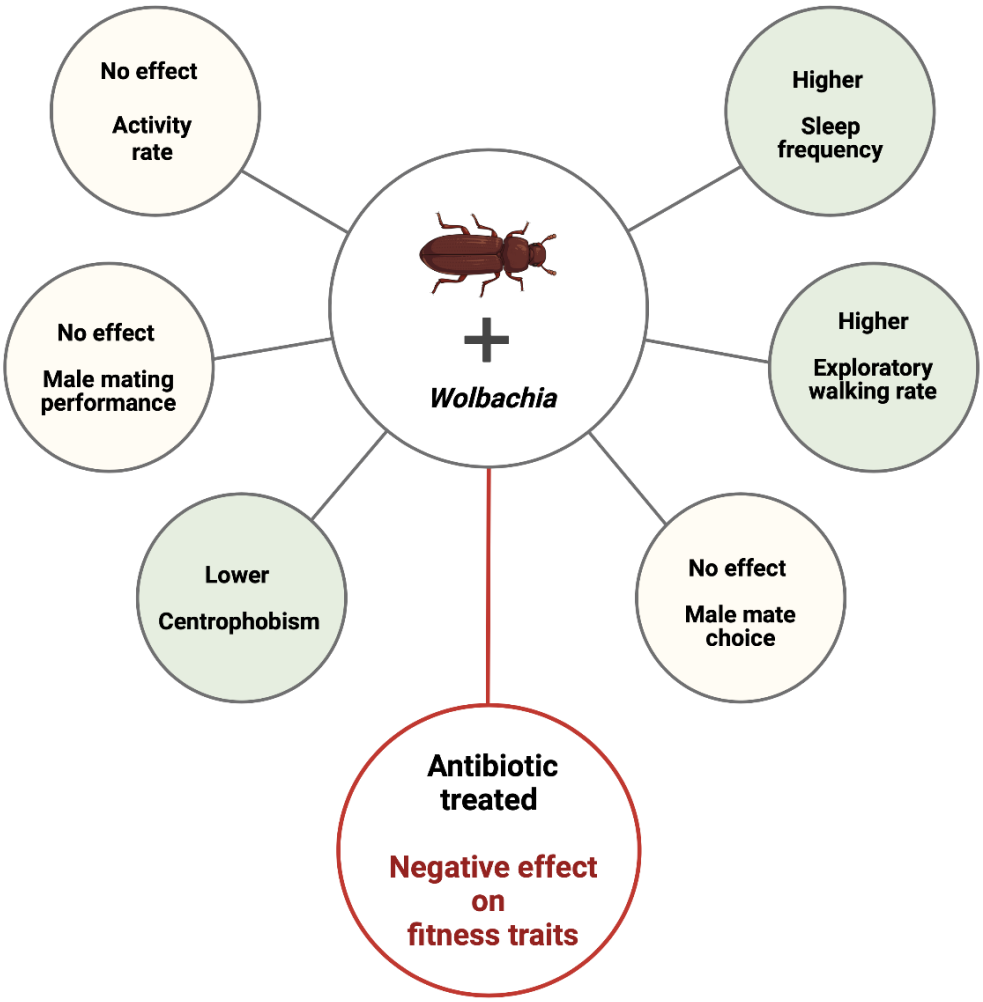



**Figure 1.** A simple model of the effects of *Wolbachia* infection on the behavior of confused flour beetle, *Tribolium confusum*. Detail description has been mentioned.

beetles of both males and females. During a 24 h time period. The exploratory walking rate for infected and uninfected beetles were similar, except for infected females, in which they interestingly explore more of the area with higher speed rate (Chapter 4). Similar to *D. nigrospara*, the antibiotic-treated beetles demonstrated a significant decrease in their walking speed and exploration rate, in comparison with both infected and uninfected beetles (Chapter 4). These results confirm the ability of *Wolbachia* to increase the chance of transmission since *Wolbachia-infected* females tend to maintain the highest exploration rate, meaning that infected females are more accessible in case of mating with either infected or uninfected females (Chapter 4). In addition to exploratory behavior, *Wolbachia* infection is a significant factor in the

thigmotaxis of *T. confusum* beetles. Among three strains of *T. confusum* male and female beetles, infected individuals were more present in the center in comparison with the other two strains, and antibiotic-treated beetles avoided walking in the center, and generally, they were more centrophobic and stationary (Chapter 4) (Fig. 1).

## Conclusion

This study along with previous ones, demonstrated the evolution of *Wolbachia* as a facultative mutualist in confused flour beetle, *Tribolium confusum*, according to their genomic data (genome size and composition), and the benefits on the behavior traits of the host. In addition, the observations on *w*Tcon infection density regarding to different temperatures will help for further questions on the biological function and the symbiosis interaction. However, more detailed knowledge on *w*Tcon is needed, for instance the effect of low temperature on the density of *Wolbachia* and induction of CI, replication rate of *w*Tcon in various host tissues under the heat stress. Also, studies exploring the differential expression of genes between *Wolbachia*-infected and uninfected strains are desirable. In addition, questions on impact of *Wolbachia* on other fitness traits including host aggressiveness and learning memory would also be compelling in order to construct a stronger foundation for symbiosis interaction of *Wolbachia* and *T. confusum* beetles. Due to commensal lifestyle of *Tribolium confusum* beetles with humans and their contrasting behavior in the field, an extensive molecular analysis along would also allow us to escalate *Wolbachia*'s role as a biocontrol agent for controlling pest insects.

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# List of publications

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**Gharabigloozare, Y**., & Bleidorn, C. (2022). Effect of high temperature on *Wolbachia* density and impact on cytoplasmic incompatibility in confused flour beetle, *Tribolium confusum* (Coleoptera: Tenebrionidae). **In review** for *BMC research notes*.

**Articles in preparation**

**Gharabigloozare, Y**., Bleidorn, C., Geurten, B. (2022). The effect of the endosymbiont, *Wolbachia* on the behavior of confused flour beetle, *Tribolium confusum* (Coleoptera: Tenebrionidae). **In preparation** for *Society for Applied Microbiology*.