FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

WOLBACHIA INFECTION DYNAMICS AND REPLICATION RATES WITHIN NATURALLY INFECTED *DROSOPHILA* HOST

An Undergraduate Honors Thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science

in

BIOLOGICAL SCIENCES

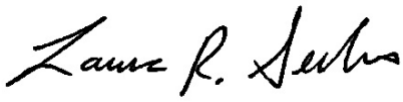
WITH HONORS

By

Hani M. Samarah

2020

This Undergraduate Honors Thesis in Biological Sciences, written by *Hani M. Samarah* entitled, "Wolbachia Infection Dynamics and Replication Rates Within Naturally Infected *Drosophila* Host," is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor(s) have read this thesis. We recommend that it be approved.



Dr. Laura R. Serbus

Honors Research Supervisor

Dr. Walter M. Goldberg

Chairperson, Undergraduate Honors Committee

Dr. Steven Oberbauer

Chairperson, Department of Biological Sciences

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Abstract of the thesis

Wolbachia Infection Dynamics and Replication Rates

Within Naturally Infected Drosophila Host

by

Hani Samarah

Florida International University, 2020

Miami, Florida

Professor Laura R. Serbus, Major Professor

Wolbachia pipientis is one of the most abundant endosymbionts carried by approximately half of all insect species as well as some filarial nematodes, mites, and crustaceans. Because *Wolbachia* are pervasive in nature, studies that gain insight into the molecular and cellular basis for *Wolbachia* infection will provide significant insight into endosymbiosis generally. Furthermore, because *Wolbachia* infection has been demonstrated to suppress transmission of viral pathogens like Dengue and Zika by *Aedes* mosquitoes, there are practical implications for understanding mechanisms that regulate *Wolbachia* infection. The absolute number of *Wolbachia* carried by host germline cells relies upon vertical inheritance from stem cells, horizontal invasion of *Wolbachia* into cysts, and binary fission within the germ cells. However, little is currently known about how *Wolbachia* is controlled within host somatic tissue, nor how much binary fission contributes to the ultimate number of *Wolbachia* per insect. The aim of this research is to establish the replication rate of *Wolbachia* within naturally infected *Drosophila* models. Here I present qPCR data measuring *Wolbachia* dynamics through the host life cycle. I

expect this research to be among the first to examine *Wolbachia* infection dynamics within both the male and female host throughout the entire life cycle, providing greater insight into our collective understanding of *Wolbachia*-host interactions.

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INTRODUCTION

Professor Heinrich Anton de Bary first applied symbiosis in a biological context, where he defined the term as, "a phenomenon in which dissimilar organisms live together" (Bary 1879; Paracer et al. 2000; Oulhen et al. 2016). Symbiosis is still used to describe any close, long-term biological interaction between two organisms (Moran 2006). For instance, symbiotic relationships between a symbiont and another organism (host) can be mutualistic, commensalistic, or parasitic. Additionally, microbes can live within the tissues or cells of an organism, a phenomenon referred to as endosymbiosis, as opposed to ectosymbiotic organisms that live on a host (Paracer 2000; Moran 2006).

Wolbachia is a genus of gram-negative endosymbiotic bacteria estimated to infect about half of insect species, as well as mites, crustaceans, and filarial nematodes (Serbus et al. 2008; Weinert et al. 2015; Werren et al. 2008; Zug & Hammerstein, 2012). *Wolbachia*-host interactions are host- and environment-dependent, and encompass the entire symbiotic spectrum (Bordenstein et al. 2008). For instance, the operational sex ratio in the butterfly, *H. bolina*, is altered at such extreme degrees that *Wolbachia* are considered parasitic (Dyson et al. 2004). By contrast, *Wolbachia* are largely beneficial to filarial nematodes, providing essential nutrients to *B. malayi* and providing benefits to nematode fertility (Foster et al. 2005; Taylor et al. 2005). *Wolbachia* are carried by a broad range of host-taxa, thus serving as an important research model for the molecular and cellular basis for endosymbiotic interactions. Additionally, *Wolbachia* colonize somatic and germline tissues in *Drosophila spp.*, spanning the entire spectrum of symbiosis. For instance, *Wolbachia* and *D. paulistorum* are obligate mutualists, while *D.*

innubila, *D. mauritiana*, *D. melanogaster*, and *D. simulans* typically share facultative mutualistic relationships with *Wolbachia* (Fry et al. 2004; Weeks et al. 2007; Miller et al. 2010; Fast et al. 2011; Unckless et al. 2012). Conversely, *W. popcornia* infection within D. *melanogaster* is particularly virulent, causing tissue degeneration and death in the adult host (Min et al. 1997). The symbiotic plasticity shared between *Drosophila* and *Wolbachia* provides an opportunity to study the diverse relationship between a symbiont and a natural, well-developed model system (Frydman et al. 2006; Serbus et al. 2007; Newton & Sheehan 2015; Sheehan et al. 2016; Yamaguchi et al. 2018).

The success of *Wolbachia* is credited in part to their ability to efficiently transmit through the maternal germ-line, a process known as vertical transmission (Stouthamer et al. 1999; Serbus et al. 2008). *Wolbachia* reliably transmit through the host germ-line by maintaining a high abundance (titer) through oogenesis (Hadfield et al. 1999; Veneti et al. 2004; Serbus & Sullivan 2007, Serbus et al. 2008). *Wolbachia* pass from a differentiating germline stem cell to a daughter cystoblast or cystocyte. The differentiated cystocyte, will mitotically divide four times, partially executing cytokinesis to form an interconnected cyst of sixteen germline cells, referred to as an egg chamber or follicle (Gilbert 2000). This structure will maturate into an embryo in which *Wolbachia* are homogenously distributed, while some *Wolbachia* localize at the posterior pole to be included into the reproductive-tissues of the adult insect (Boyle et al. 1993; Hadfield 1999; Clark et al. 2002; Veneti 2004; Serbus & Sullivan 2007, Serbus et al. 2008).

After oogenesis it is not clear how *Wolbachia* density is regulated, yet its understanding is of practical importance. For instance, Martinez et al. (2014) found *Wolbachia*-infected *Drosophila* are resistant to Flock House Virus (FHV) and *Drosophila*

C Virus (DCV). It was later found *Wolbachia* also suppress Zika, dengue, and chikungunya infections in *Aedes* mosquitoes, an important disease-causing vector (Hedges et al. 2008; Blagrove et al. 2013; Dutra et al. 2016; Xue et al. 2018). Notably, high *Wolbachia* titer correlates with higher efficacy of viral suppression (Lu et al. 2012; Van den Hurk et al. 2012; Dutra 2016; Schultz et al. 2017). Moreover, some *Wolbachia* strains induce cytoplasmic incompatibility in *Aedes*, a lethal phenotype in which crosses between infected males and uninfected females produce unviable embryos (Werren et al. 1997). These findings have generated interest into the cellular processes that regulate *Wolbachia* titer. Understanding these mechanisms can expand the arsenal of tools that prevent the spread of infectious diseases.

Most analyses of *Wolbachia* titer regulation have focused only on mitotic inheritance in the maternal germline. For instance, the Gurken (*grk*) gene in fruit flies is an important gene that encodes a ligand for Epidermal growth factor receptor (Schupbach 1987), was found to promote *Wolbachia* growth and division (Serbus et al. 2011). Additionally, host cytoskeletal and motor proteins are linked to the regulation of *Wolbachia* localization patterning and titer during oogenesis (Ferree et al. 2005; Serbus & Sullivan 2007; Newton Savytskyy et al. 2015). Recently, *Wolbachia* titer within the germline has also been shown to be sensitive to host diet. *Drosophila* who are exposed to yeast-enriched food, show a significant reduction in *Wolbachia* loads within their stage 10 egg chambers (Serbus et al. 2015). Investigations into germline titer dynamics suggest that 3000-18,000 endosymbionts will be carried within each embryo (Boyle 1993; Clark 2002).

It is not clear if mechanisms implicated in *Wolbachia* titer during development are generalized to the adult host. Recent studies into the effect of yeast-enriched diet on body-wide *Wolbachia* titer provide some clues. Unlike what is measured in ovarian tissues, optimized absolute qPCR indicates that *Wolbachia* titer body-wide is not sensitive to a yeast enriched diet (Christensen et al. 2019). In other words, *Wolbachia* abundance within the whole body remains stable despite a decrease in titer within the ovaries. One of the possible explanations for this data is that the yeast-triggered insulin pathway suppresses *Wolbachia* replication within the germline during oogenesis while promoting binary fission in somatic tissues. This interpretation is limited by what is known about *Wolbachia* infection dynamics and replication rates through host development. There is evidence supporting an exponential growth curve by *Wolbachia popcornia*, whereas *Wolbachia melanogaster/Canton-S* exhibit a stable population through the adult male *Drosophila* life cycle from ages 0 to 50-days (Chrostek et al. 2013). However, this study quantified a virulent strain of *Wolbachia* and relied on relative qPCR quantification; consequently, *Wolbachia* titer analysis through the host life cycle is still desired. Recent optimization for measuring local and body-wide *Wolbachia* titers enable greater resolution for this endosymbiont across the host life cycle (Christensen 2019). The central aim of this work is to evaluate *Wolbachia* infection dynamics within different *Drosophila-Wolbachia* systems. Ultimately, the overall objective is to determine how much *Wolbachia* replication contributes to the ultimate number of bacteria carried by each insect, by measuring the rate *Wolbachia* divide during the *Drosophila* host life cycle.

MATERIALS AND METHODS

Drosophila Stocks

The following fly strains obtained from Indiana University Bloomington Stock center (Bloomington, IN), were used in this project: *D. melanogaster* with the genotype w; Sp/Cyo; Sb/TM6B carrying endogenous *w*Mel *Wolbachia* (DB *w*Mel); Oregon-Red strain of *D. melanogaster*, carrying an introduction of the same *w*Mel strain as above (Ore-R *w*Mel); *D*. *simulans* infected with the endogenous strain, *Wolbachia* Riverside (*D. sim w*Ri), and D. *simulans* transinfected with *w*Mel (*D*. *sim w*Mel). *Wolbachia* infections were confirmed with PCR (Figure 1).

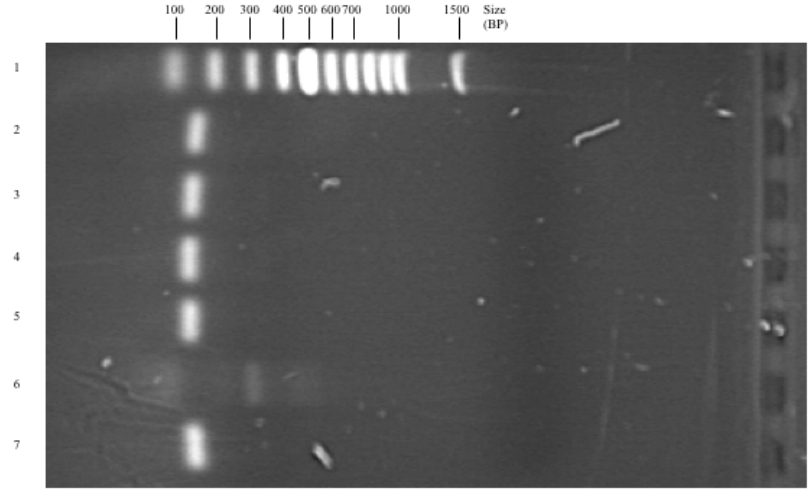

Figure 1. Agarose gel electrophoresis (1.5%) of PCR amplified products for Wolbachia surface protein (wsp) gene. Each lane corresponds to the following DNA extraction: Lane 1: 100 bp Ladder, Lane 2: Ore-R (wMel), Lane 3: D. sim (wRi), Lane 4: D. sim (wMel), Lane 5: DB (wMel), Lane 6: DB (uninfected) negative control, and Lane 7: DB (wMel) positive Control. Bands between 100-200 base pairs represent positive test for wsp gene.

Food Preparation and Fly Rearing

Drosophila lines were raised in square bottom fly bottles at 25℃ in an incubator with 24-hour ambient light cycle on a modified cornmeal recipe from the Bloomington Drosophila Stock Center. The media contained 20 L of water, 96 g of agar, 1325 g of corn meal, 190 g of soy flour, 337 g of dry active yeast, 1.5 L of light corn syrup, and 94 mL of propionic acid. Fly bottles were seeded with 30 female flies and 10 male flies to control for density-related variations in *Wolbachia* titer. Recently eclosed flies were collected daily during to be used for age dependent screening assays.

Age-Dependent Assay

Wolbachia titer across adult host age for each strain was assessed using vials containing 2 mL of standard cornmeal fly media. Eight male and female 0-day old flies were transferred into vials with 2 mL of media labeled 0-day-old, 1 day old to, 10-day old (Figure 2). I conducted the experiment twice, where two biological replicates for each group were measured in parallel. For the next ten days the flies were transferred into new vials containing fresh media. DNA extractions were conducted on 5 female flies and 5 male flies from each biological replicate every day for ten days. *Wolbachia* titers were evaluated via qPCR of the *wsp* gene (Christensen 2019).

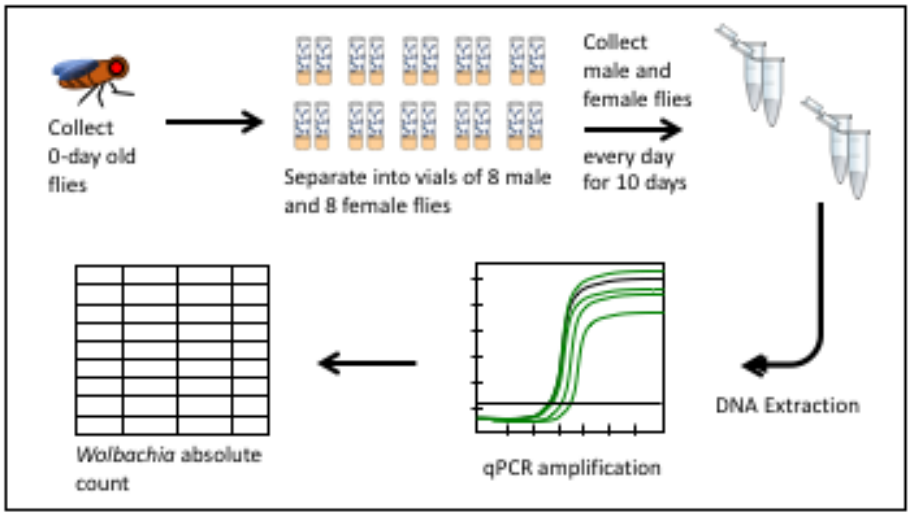

Figure 2. Approach used to measure age-dependent-*Wolbachia* titer in all fly strains used.

DNA Extraction and qPCR for *Wolbachia* Titer

Wolbachia measurements were obtained from each sample by homogenizing tissue in 2 µL of 20 mg/mL of proteinase K, and 200 µL of buffer containing 10 mM Tris HCl (pH 8.0), 1 mM EDTA and 25 mM NaCl, and 1% SDS. The samples were then incubated at 56° C for one hour. The proteinase K was deactivated by heating the samples to 95° C for three minutes. The samples were then centrifuged at 17,970 x g for 15 minutes at 4°C. A 100 µL sample of the supernatant was removed from the sample and added to a new tube along with 10 µL of 3M Sodium Acetate and 250 µL of absolute ethanol. The ethanolic mixtures were gently mixed and kept at -20° C for one hour after which they were centrifuged at 17,970 x g for 15 minutes at 4° C. The resulting supernatant was decanted without disturbing the DNA pellet and was further washed with 500 µL of 70% ethanol and centrifuged at 17,970 x g for 15 minutes at 4° C, after which it was air dried. The centrifugate was then resuspended in 100 µL of TE buffer (pH 8.0) and used directly for qPCR.

Quantitative PCR was carried out with a CFX96 real-time PCR detection system (Hercules, CA). Established primers specific for *wsp* gene were used to amplify the DNA (Chrostek 2013; Christensen et al. 2016). The reactions were performed at 11 µL final volume containing 5 µL of Maxima SYBR green-fluorescein qPCR master-mix (Thermo Scientific, Waltham, MA), 3.5 µL of DNAse/RNAse free ddH_2O, 0.25 µL of 5 mM forward and reverse primer, and 2µL of each DNA sample. The thermal cycling protocol used a two-minute incubation at 50° C, and 10-minute denaturation at 95° C. Following that, forty cycles of denaturing at 95°C for 30 s, annealing at 57° C for 1-minute, and extension at 72° C for 30 s. The data were then analyzed using the default threshold settings on Bio-Rad CFX manager 3.1 (Bio-Rad, Hercules, California).

Critical threshold values (C_q) for each group were collected and directly analyzed. The C_q, also known as the cycle quantification value, represents the cycle number where the reaction transects the threshold line (Pabinger et al. 2014). The threshold line is point in which fluorescent intensity can be detected beyond background fluorescence levels. It is important to note that lower C_q values represent higher *Wolbachia* measurements because it took less cycles for the *wsp* gene to be detected at the threshold point.

Data Analysis

Data collected in this study was tested for normality and homoscedasticity using Shapiro-Wilk test and Levene's test, respectively (Shapiro et al. 1965; Lim et al. 1996; Mohd Razali et al. 2011). I used the F-test to compare the variances between groups. Outcomes of these tests dictated the statistical method I used to analyze the data. I determined if *Wolbachia* titer was associated with host age using a linear regression (Galton 1886). Comparisons between groups within normal-homoscedastic data sets were tested with

one-factor ANOVA, followed by Tukey's post hoc test or students t-test between groups. Kruskal-Wallis test followed by Dunn post hoc analysis and Mann-Whitney test were used on non-parametric-homoscedastic data sets (Tukey 1949; Kruskal et al. 1952). I used Welch's ANOVA followed by Games-Howell post hoc analysis to compare groups within non-parametric-heteroscedastic data sets (Dunn 1964).

RESULTS

W. riverside measurements do not change with age in male *D. simulans*

To assess if *Wolbachia* levels change in female and male adult *Drosophila* over time, I measured the *Wolbachia* titers within endogenously infected *D. simulans* flies (*D. sim w*Ri) every day from newly eclosed flies to ten-day old flies (Figure 2). Regression analysis of the data collected from experiment replicate one (Figure 3) implies a correlation between age of the female host and *w*Ri measurements ($R^2 = 0.4889$, $p < 0.000001$, n = 66) but not the male host ($R^2 = 0.03088$, $p = 0.158162$, n = 66). Additionally, an analysis of variance (ANOVA) is consistent with an increase in titer within adult females (Kruskal-Wallis ANOVA $p = 0.0005$, n = 66). A post hoc Dunn's test indicates that zero-day old female flies differed significantly from ten-day old flies at $p = 0.0121$. Conversely, a significant difference between zero-day old and ten-day old age groups was not detected at this time (Dunn's multiple comparisons test $p = 0.3061$, n = 66).

In contrast, the second experiment suggests a decrease in titer within females with age ($R^2 = 0.2696$, $p = 0.000008$). Further, an ANOVA followed by Dunn's post hoc test indicates a significant decrease in *Wolbachia* titer between zero-day old and ten-day old

flies (Dunn's multiple comparisons test $p = 0.0012$, n = 66). Consistent with the first experiment, male titers do not exhibit a change in titer with age ($R^2 = 0.2696$, $p = 0.255758$), and do not exhibit differences between each age group (Kruskal-Wallis ANOVA $p = 0.1154$, n = 66). Taken together, it is not possible to conclude how *w*Ri loads change within the female host because of disparate results between experimental replicates (Figure 3). However, these results do not indicate that whole-body wRi levels change in males with age.

Table 1. Results of regression analysis and ANOVA comparing changes in *w*Ri measurements within D. *sim* host between age groups 0 and 5, as well as 0 and 10

			ANOVA	
	Sex	Regression	0 vs 5	0 VS 10
REPLICATE 1	**Male**	$y = -0.07355x + 15.35$, $R^2 = 0.4889$ **$p < 0.05$**	N.S.	**$p < 0.05$**
	Female	$y = 0.05061x + 14.91$, $R^2 = 0.2696$ **$p < 0.05$**	N.S.	**$p < 0.05$**
REPLICATE 2	**Male**	$y = -0.1847x + 17.44$, $R^2 = 0.03088$ $p = 0.158162$	N.S.	N.S.
	Female	$y = -0.02591x + 16.40$, $R^2 = 0.0005676$	N.S.	N.S.

Statistical analysis employed a Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons tests. Significance levels are shown in bold ($\alpha = 0.05$; n = 66)

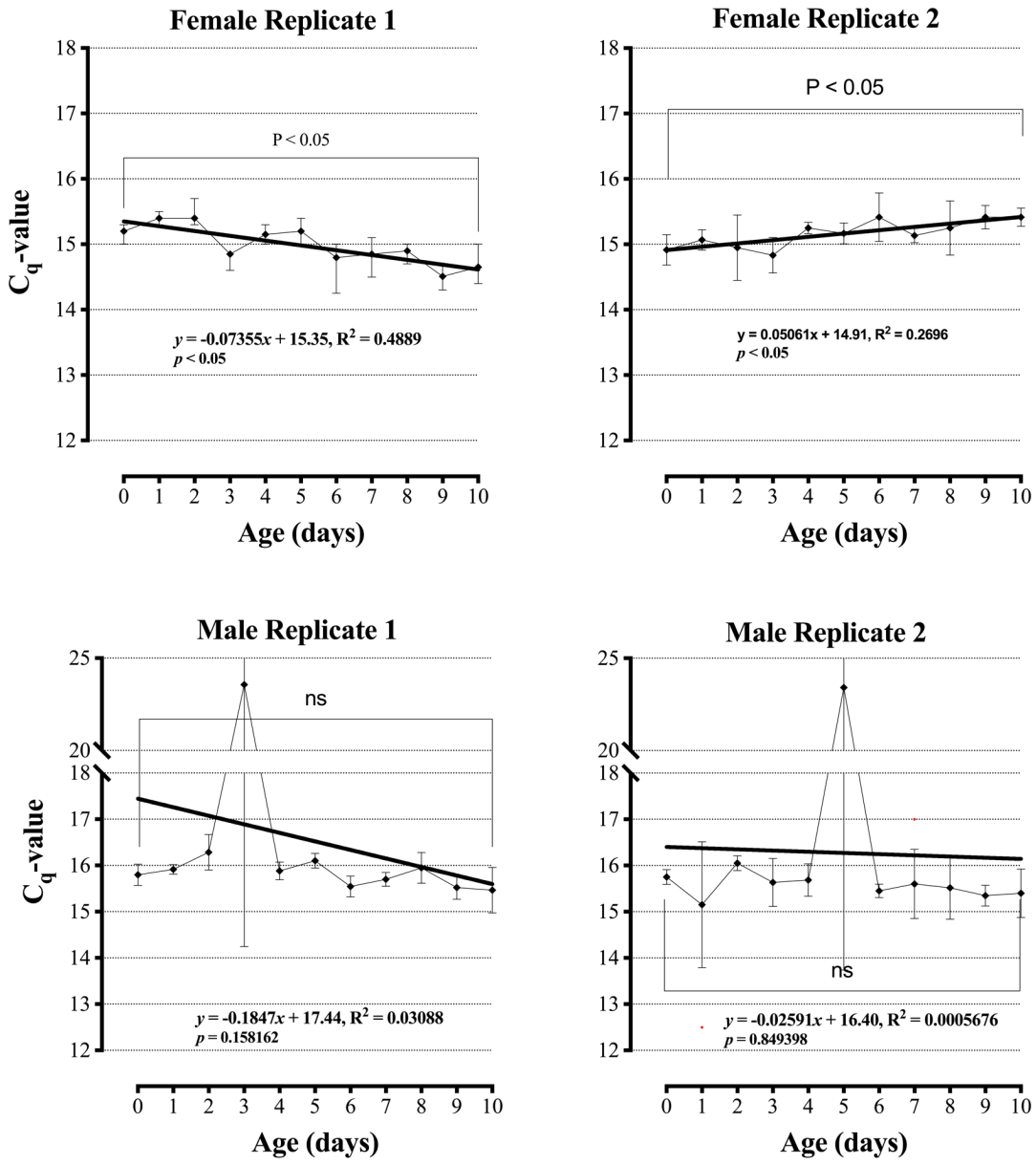

Figure 3. ***w*Ri titers within male and female adult *D. simulans* graphed by age.** A lower C_q-value is associated with a higher *Wolbachia* measurement ($\tilde{x}$ ± SD, n = 6)

***W. melanogaster* titer linearly increases with age within female and male D. simulans**

To determine if other *Wolbachia* strains exhibit similar titer stability with age in *D. simulans*, I measured transinfected *Wolbachia mela*nogaster in *D. simulans* (*D. sim w*Mel) every day from newly-eclosed to ten-day old flies (Figure 2). In contrast to bacterial measurements of endogenously infected flies, graphic appraisal of *w*Mel measurements in *D. sim* imply an increase in *w*Mel titer with age in both sexes (Figure 4). Regression analysis and ANOVA of each data set is consistent with this interpretation.

Table 2. Results of regression analysis and ANOVA comparing changes in *w*Mel measurements within D. *sim* host between age groups 0 and 5, as well as 0 and 10

			ANOVA	
	Sex	Regression	0 vs 5	0 VS 10
REPLICATE 1	**Male**	$y = -0.05412x + 15.46$, $R^2 = 0.1914$ **$p < 0.05$**	N.S.	N.S.
	Female	$y = -0.09056x + 15.16$, $R^2 = 0.3994$ **$p < 0.05$**	N.S.	**$p < 0.05$**
REPLICATE 2	**Male**	$y = -0.03985x + 16.18$, $R^2 = 0.1288$ **$p < 0.05$**	N.S.	**$p < 0.05$**
	Female	$y = -0.02652x + 16.02$, $R^2 = 0.09676$ **$p < 0.01$**	**$p < 0.05$**	N.S.

Statistical analysis employed a Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons tests. Significance levels are shown in bold ($\alpha = 0.05$; $n = 66$)

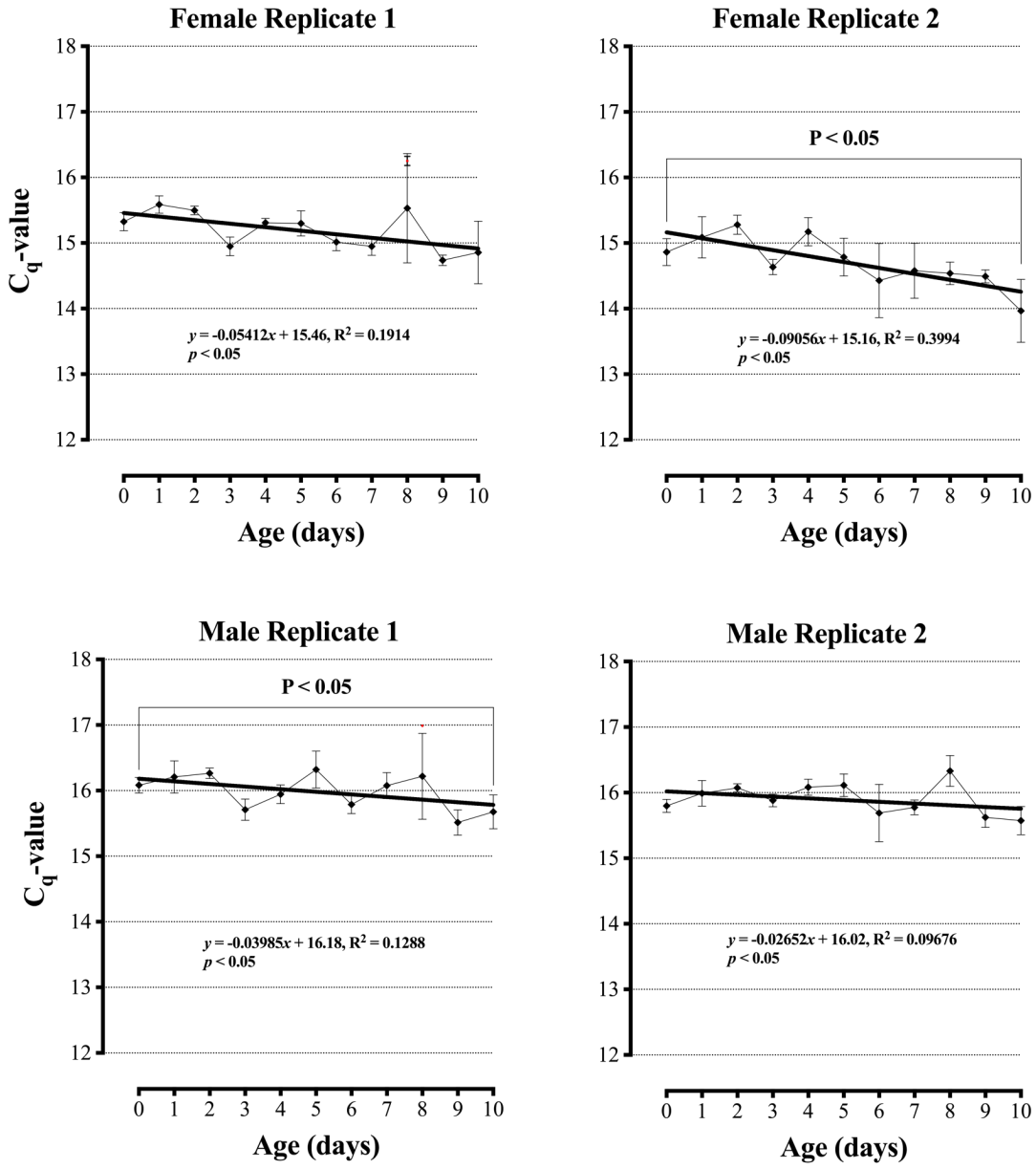

Figure 4. *w*Mel titers within male and female adult *D. simulans* graphed by age. A lower C_q-value is associated with a higher *Wolbachia* measurement (*n* = 6 ± SD).

Increase in *Wolbachia* concentration with age is associated with *w*Mel strain

I assessed the increases in *D. sim w*Mel titers to determine whether they are associated with the *w*Mel *Wolbachia* strain itself. Daily measurement of *Wolbachia* in Ore-R *w*Mel of newly-eclosed- to ten-day old flies (Figure 2) shown in Figure 5 implies a consistency

with results from the *D. sim w*Mel model system. Regression analysis and ANOVA of each experimental replicate supports the graphical interpretation of the data collected (Table 4).

Table 3. Results of regression analysis and ANOVA comparing changes in *w*Mel measurements within Ore-R host between age groups 0 and 5, as well as 0 and 10

			ANOVA	
	Sex	Regression	0 vs 5	0 VS 10
REPLICATE 1	**Male**	$y = -0.1085x + 15.26$, $R^2 = 0.4365$ **$p < 0.05$**	**$p < 0.05$**	**$p < 0.05$**
	Female	$y = -0.07803x + 16.57$, $R^2 = 0.3336$ **$p < 0.05$**	N.S.	**$p < 0.05$**
REPLICATE 2	**Male**	$y = -0.08818x + 16.12$, $R^2 = 0.3265$ **$p < 0.05$**	N.S.	**$p < 0.05$**
	Female	$y = -0.06394x + 17.49$, $R^2 = 0.1235$ **$p < 0.05$**	N.S.	**$p < 0.05$**

Statistical analysis employed a Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons tests. Significance levels are shown in bold ($\alpha = 0.05$; $n = 66$)

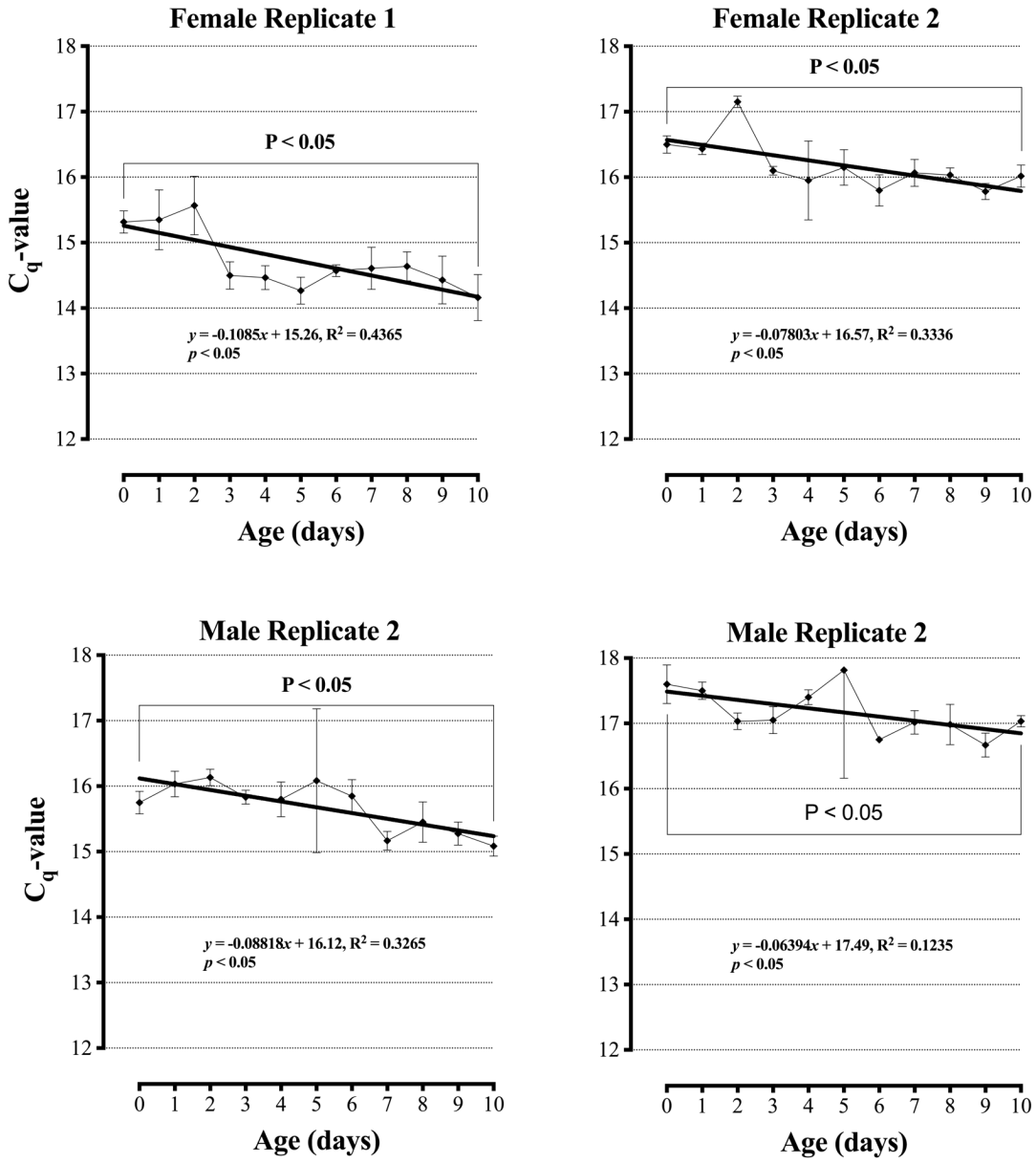

Figure 5. *w*Mel titers within male and female adult Oregon-Red strain of *D. melanogaster* graphed by age. A lower C_q-value is associated with a higher *Wolbachia* measurement ($\tilde{x} \pm SD$, n = 6)

Host genetic background may influence sex specific wMel titer increases with age

Using the same experimental protocol for Ore-R *w*Mel (Figure 2), I assessed if *w*Mel increases with age are generalized across *D. melanogaster* genetic backgrounds. For

consistency across analysis, I measured *Wolbachia* titers within *D. melanogaster* with the genotype w; Sp/Cyo; Sb/TM6B (DB *w*Mel) carrying the exact same *Wolbachia* strain that was used in the Ore-R *w*Mel experiment above. Graphically, the data is consistent with an increase in *w*Mel titer in female flies with age in both experimental replicates (Figure 6). However, unlike what was observed in the other *Drosophila* species-*w*Mel model system, contrasting results were observed within the male host between experimental repeats. In experimental replicate one, a regression analysis of *w*Mel measurements with age within the male host was negative ($R^2 = 0.009896$, $p = 0.437921$, n = 66). Whereas, in experimental replicate two, a positive regression test indicated an increase in *w*Mel levels with age in male flies ($R^2 = 0.1351$, $p = 0.003864$, n = 66). Further an ANOVA followed by Dunn's post hoc test on data collected from experiment one indicates no difference in *w*Mel measurements between zero-day old and 5-day old flies ($p = 0.7686$, n = 66) and between zero-day old and ten-day old flies ($p = 0.0504$, n = 66). While the same analysis generated significant measurements between zero-day old and 5-day old flies in experimental replicate two ($p = 0.0046$, n = 66). As shown in Figure 6 and table 4, statistical analysis of both experimental replicates, indicates that *w*Mel measurements increase with age in the female host. As a group, this preliminary data suggests that *w*Mel titer increases in females, implying that the genetic background of *D. melanogaster* may influence which sex will exhibit an increase in *w*Mel titer with age.

Table 4. Results of regression analysis and ANOVA comparing changes in *w*Mel measurements within DB host between age groups 0 and 5, as well as 0 and 10

			ANOVA	
	Sex	Regression	0 vs 5	0 VS 10
REPLICATE 1	**Male**	$y = -0.01205x + 17.81$, $R^2 = 0.00999$ $p = 0.438$	***p* < 0.05**	***p* < 0.05**
	Female	$y = -0.06244x + 17.53$, $R^2 = 0.4072$ ***p* < 0.05**	N.S.	***p* < 0.05**
REPLICATE 2	**Male**	$y = -0.08263x + 16.33$, $R^2 = 0.1351$ ***p* < 0.05**	N.S.	N.S.
	Female	$y = -0.06333x + 15.31$, $R^2 = 0.3157$ ***p* < 0.05**	***p* < 0.05**	N.S.

Statistical analysis employed a Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons tests. Significance levels are shown in bold ($\alpha = 0.05$; n = 66)

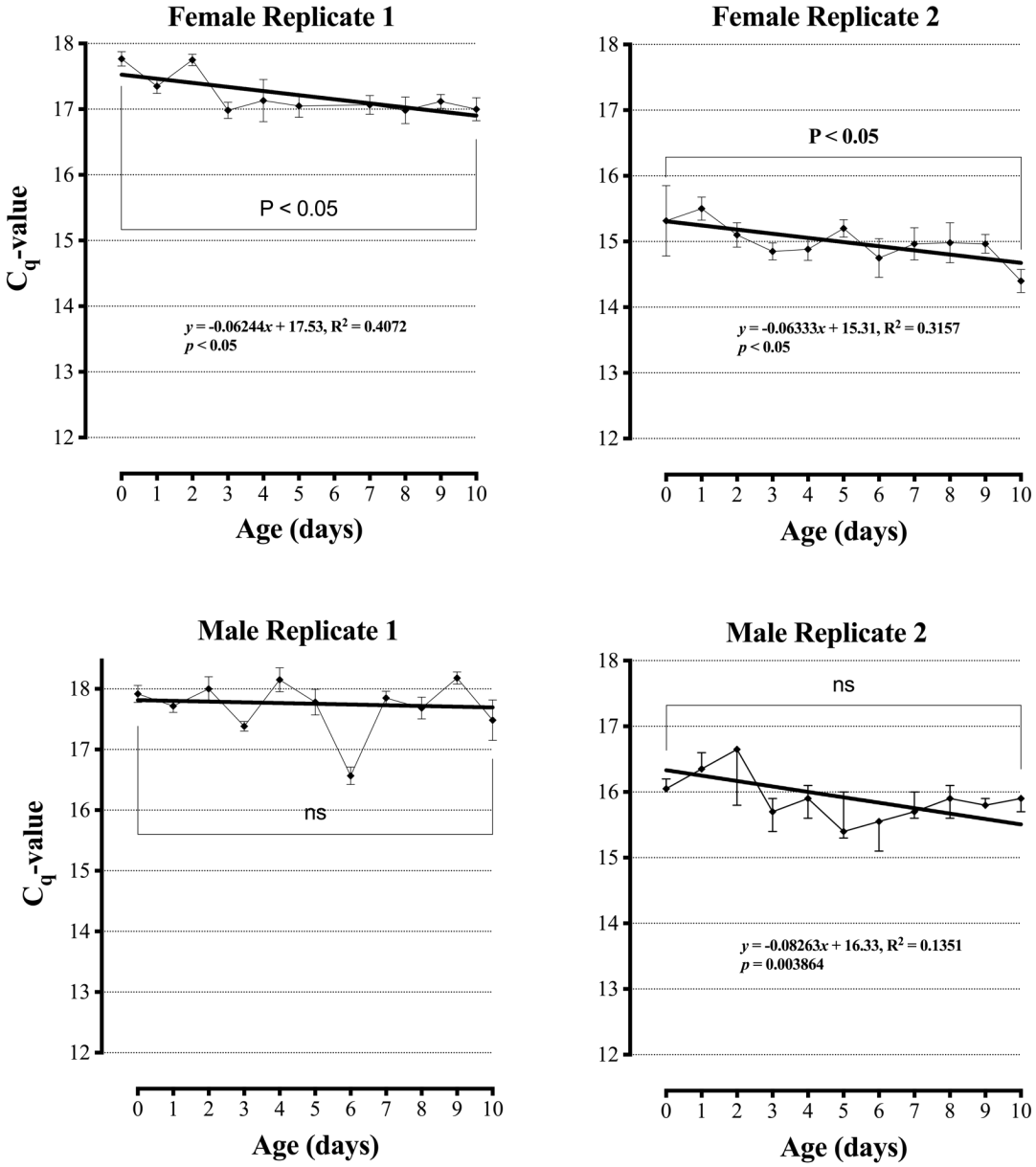

Figure 6. *w*Mel titers within male and female adult *D. melanogaster* with the genotype w; SP/Cyo; Sb/TM6B graphed by age. A lower C_q-value is associated with a higher *Wolbachia* measurement ($\tilde{x} \pm SD$, $n = 6$)

DISCUSSION

Using Critical threshold values (C_q) values as a proxy for titer measurements

Typically, DNA quantification strategies using qPCR either rely on relative measures based on a reference gene or absolute quantification (Pabinger 2014). In general, investigations relying on relative quantification report fold differences in *Wolbachia* titer by computing the ratio between *wsp* (*Wolbachia* surface protein) and the host gene, *rpl*32 (Ribosome Protein L32) (Moreira et al. 2009; Chrostek 2013; Melnikow et al. 2013; Serbus et al. 2015; LePage et al. 2017; Richardson et al. 2019). Good reference genes are housekeeping genes that are constitutively expressed in all cells under standard and pathological conditions (Kozera et al. 2013). While various mathematical models are used to determine an adequate reference gene in relation to a target gene (Hellemans et al. 2007), recent work has indicated that relying on *wsp*/*rpl32* relative counts can be misleading when assessing changes in *Wolbachia* titer in response to diet (Christensen 2019). These results indicate the importance of evaluating more than one reference gene in all conditions of interest when relying on relative quantification to evaluate titer dynamics in *Drosophila*.

In contrast, absolute counts can be derived in one of two ways. One method relies on creating a standard curve by serially diluting DNA standards of known concentrations (Heid et al. 1996). Contrary to qPCR, digital PCR (dPCR) can achieve absolute counts for a target gene without the generation of a standard curve. Instead the reaction components in the PCR tube are separated into thousands to millions of Nano-droplet tubes where the PCR amplification is conducted. A Poisson distribution is generated where counts can be used to quantify the absolute number of DNA molecules in a sample

(Kalinina et al. 1997). There is evidence that suggests that dPCR produces more precise and reproducible absolute counts over qPCR (Marx 2014). Her results indicate that dPCR may provide a more precise method to evaluate changes in *Wolbachia* gene expression in future studies.

Technical limitations precluded the ability to measure *Wolbachia* concentrations using absolute quantification methods outlined above. Genome-wide expression studies have detected variation in gene expression in some tissues of *Drosophila* as a function of age (Carlson et al. 2015), therefore *Wolbachia* measurements between age groups were assessed by comparing C_q values for the *wsp* gene directly. Values of C_q can be directly compared if they are analyzed under the same conditions with the same reagents at the same time (Applied Biosystems 2019). Therefore, all statistical assessments were conducted on values measured from each qPCR plate separately. The consistency of my results under these conditions indicate that C_q values can be used to directly measure of *Wolbachia* dynamics in *Drosophila* during the first ten days after eclosion. It is important to note that lower critical threshold values correspond to a higher wsp count while higher critical threshold values correspond to a lower wsp count. Further, median C_q values between groups that differ by a magnitude of one represent a doubling in *Wolbachia* titer.

Current limitation to this assay do not allow for the comparison in *Wolbachia* titer between female and male flies or between different *Drosophila* populations infected with the same *Wolbachia* strain. I am currently pursuing methods to allow for experimental groups analyzed on separate qPCR plates to be compared with one another (Hellemans 2007).

Replication rates may be influenced by strategies in transovarial transmission

Results in this study indicate that *W*. *riverside* titer does not change with age in male flies while *W*. *melanogaster* shows consistent increases in titer across age in different hosts (Figures 3-6). To maintain consistent *Wolbachia* infection from generation to generation, *Wolbachia* replication rates must be high enough to ensure reliable ovarian transmission without disrupting the hosts normal physiology (McGraw et al. 2002). Further, there are host-dependent mechanisms that facilitate the transfer of *Wolbachia* through oogenesis; moreover, different host-dependent strategies are observed among *Wolbachia* strains (Serbus & Sullivan 2007). For instance, during oogenesis *W*. *melanogaster* localize to the posterior pole of a late stage oocyte using the hosts microtubule-based transport (Serbus & Sullivan 2007). Consequently, the fate of the nuclei within the posterior pole are the future germline cells in the resulting offspring (Serbus et al. 2008). Thus, *W*. *melanogaster* are strategically positioned to be loaded into the next generations germline cells. In contrast, posterior localization is not observed in late stage oocytes infected with *W*. *riverside* (Serbus & Sullivan 2007). Additionally, the *w*Mel strain is maintained at a lower concentration in embryos, while measurements of the *w*Ri strain in embryos is higher (Veneti et al. 2004).

Based on the bacterial dynamics and titer measurements within the germline described above, I hypothesized that *w*Ri replication rates should be higher than rates exhibited by *w*Mel. The results obtained in my experiment appear to conflict with prior assessments (Figures 3-6; Serbus & Sullivan 2007, Serbus et al. 2008). However, it has been suggested that *Wolbachia* replication rates can vary between different host tissues (McGraw et al. 2002). Thus, if bacterial replication rates between the germline and the

somatic tissues are different, it is possible that the lower infection levels observed in the germline of *w*Mel-infected flies allows for a steady increase in *w*Mel titer as the adult fly ages. This interpretation predicts that *w*Mel replication should stabilize to replacement levels at a certain age of the host, suggesting that assays evaluating *Wolbachia* levels in older flies may be warranted.

***Wolbachia* fission may be influenced by host factors**

Wolbachia replication is controlled by complex interactions between host and bacterial components (McGraw et al. 2002). For example, trans-infection experiments show that *D. simulans* carry higher *Wolbachia* levels than do *D. melanogaster* (Boyle 1993). In the same study, it was shown that by selecting for female flies that exhibited high cytoplasmic incompatibility (CI), the average CI of the population increased with each generation. Subsequent measurement of *Wolbachia* titer using an egg a fluorescence assay, also revealed that this selection corresponded to a higher generation *Wolbachia* titer. Effectively, female individuals with the capacity to carry higher *Wolbachia* loads were selected for, suggesting the variability in the hosts genetic background may influence *Wolbachia* replication rates. More recent work by Singh (2019), also show a similar correlation between *Drosophila* lines selected for increased levels of CI and *Wolbachia* titer. Similar to the effect host components have on *Wolbachia* titer, different *Wolbachia* strains exhibit different infection dynamics (McGraw et al. 2002). For example, *Wolbachia popcornia* replication rates are high enough to increase the mortality rate in *Drosophila* (Min & Benzer 1997), while other *Wolbachia* strains in the same *Drosophila* host do not share the same density patterns .

In my experiments the *w*Ri measurements in female *D. simulans* showed an increase in *Wolbachia* titer with age in experimental replicate one; in contrast, *w*Ri titer decreased with age in experimental replicate two (Figure 3). Variability in the genetic background of the host population could explain these disparate results. Further, *w*Ri levels in males were stable with age in both experimental replicates (Figure 3). Multiple interpretations for these results can be made. For instance, either the genetic variability in the host that influences *w*Ri replication rates in females is sex-linked (Clancy et al. 1998) or those genetic effects are exclusive to the germline tissues in female flies. These results support the complex interactions involved between host and bacterial factors described above (Boyle et al. 1993; Min & Benzer 1997; McGraw et al. 2001; Singh 2019), opening other avenues of inquiry that aim to determine the mechanistic basis for variable *Wolbachia* dynamics and replication rates. Furthermore, *w*Mel levels increased with age in female flies with multi-chromosome balancers (Figure 6). However, preliminary results imply that *w*Mel titer does not change with age in males with the same genetic load (Figure 6). These results suggest that preventing recombination, stabilizes *w*Mel replication rates to replacement levels in males and implicate females as more permissive of high *w*Mel titers than males, reinforcing interpretations from other model systems used in this study.

Common density patterns between *Wolbachia* and other endosymbionts

Wolbachia bacterial dynamics observed in this study is analogous to patterns observed in several *Spiroplasma* infected *Drosophila* (Haselkorn et al. 2013). Like the results obtained in my experiments (Figure3-6), the bacterial titer among several *Spiroplasma* infect *Drosophila* species increased with age. However, *Spiroplasma* titer changes varied

with sex, the life cycle of the host, as well as the *Drosophila* and *Spiroplasma* species (Haselkorn et al. 2013). For instance, citri- & poulsonii-type *Spiroplasma* titer did not increase with age in male *D. hydei*, while they did increase with age in females. This pattern was not observed in *D. mojavensis* hosts, however, bacterial levels were much lower (Haselkorn 2013). Similar titer dynamics shared between *Wolbachia* and *Spiroplasma* indicates that bacterial dynamics may be sex specific and dependent on the hosts genetic background.

Considering the hosts genetic background in future experiments

Multiple avenues of inquiry are required to quantify *Wolbachia* replication rates. For instance, I am currently evaluating *Wolbachia* levels from embryo through all stages of the *Drosophila* life cycle before eclosion (Figure 7). To date *Wolbachia* infection dynamics during these stages have not been published that I know of. However, experiments intended to evaluate the effects of tetracycline on cytoplasmic incompatibility in *D. simulans* resulted in asymmetric results between females and males (Clancy & Hoffmann 1998). Several interpretations are possible, for instance, bacterial replication in female larvae could be greater than in male larvae; or binary fission by *Wolbachia* could be higher during larval-ovarian development as opposed to oogenesis in adult flies (Clancy & Hoffmann 1998). *Wolbachia* titer during this period of the hosts development can contextualize the results obtained in this study. Further, the host-dependent variability in *Wolbachia* titer suggests that more biological replicates are necessary to achieve more precise mean values at different age groups.

Precise measurements of *Wolbachia* concentrations are required to definitively measure their binary fission in vivo using qPCR. *Wolbachia* replication rates can be

quantified by computing the absolute copy number ratio between the origin and terminus of replication (ori:ter_{qPCR}), as was established by Haugan et al. (2018). Interpretation of the data presented here (Figure 3-6) and results described in other work (Boyle et al. 1993; Min & Benzer 1997; McGraw et al. 2001) predict that *Wolbachia* of the same strain may replicate at different rates dependent on the hosts genetic background and the tissue the bacteria occupies.

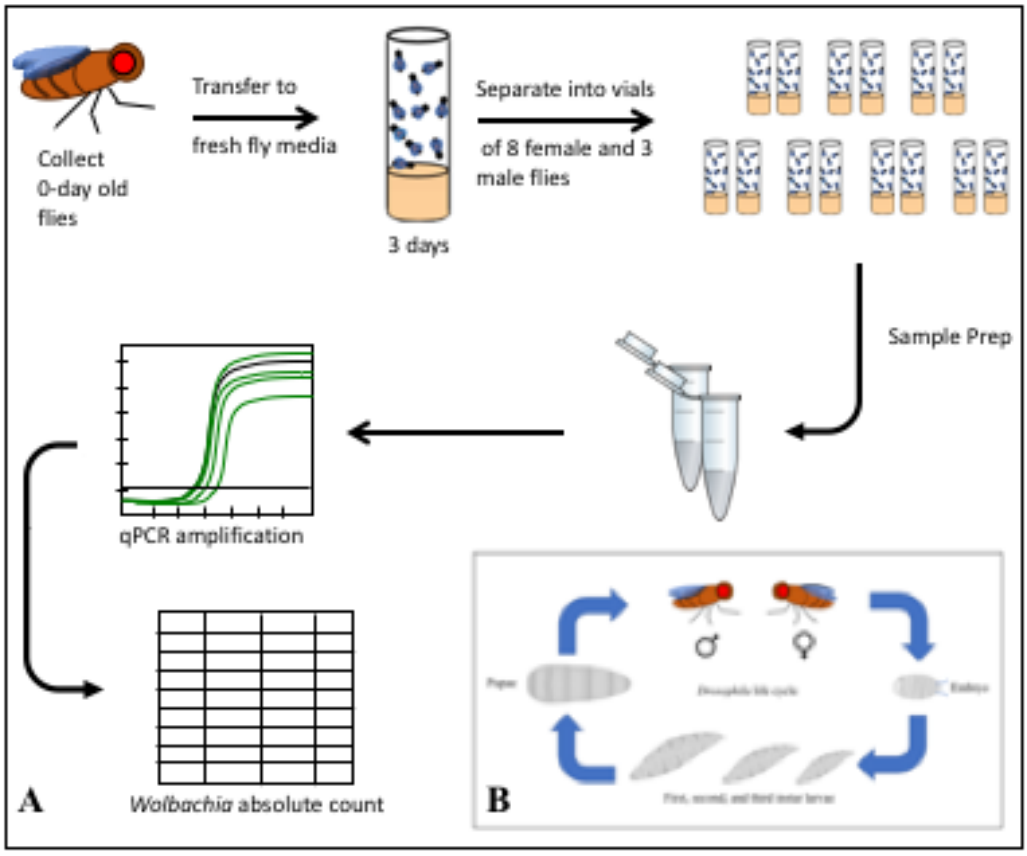

Figure 7. A) Proposed approach to measure *Wolbachia* titer from embryo to one-day old fly in Ore-R *w*Mel and *D. simulans w*Ri, B) Schematic of *Drosophila* life cycle.

Characterizing *Wolbachia* infection dynamics through the entire life cycle of the host, helps inform investigations aimed at understanding the cellular basis of *Wolbachia* regulation. For example, cytological studies evaluating temporal changes in bacteria

within germline cells of *Drosophila* allowed Serbus & Sullivan (2007), to identify the hosts cytoskeletal components involved in regulating titer within the *Wolbachia* germline. Similarly, experiments that evaluate titer dynamics within somatic tissues can form the foundational underpinnings to identify the cellular basis for *Wolbachia* titer control in somatic tissues. For instance, body wide investigations into bacteria titer rely on assays utilizing genetic tools; however, the data presented here suggests the host's genetic background and tissue should be considered when interpreting data. This implies that understanding what factors influence *Wolbachia* dynamics is important to be able to answer other biologically relevant questions.

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