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Trans-Generational Symbiont Transmission Reduced at High Temperatures in a West Nile Virus Vector Mosquito Culex quinquefasciatus

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The influence of environmental factors on the efficacy of the endosymbiont *Wolbachia* used in mosquito and pathogen control are poorly characterized and may be critical for disease control. We studied the vector mosquito *Culex pipiens quinquefasciatus* (Say) to determine the effect of temperature on the composition of the relative abundance of *Wolbachia* spp. and the microbiome, as well as key immune genes of interest in the Toll and IMD pathways. 16S barcode sequencing was used to determine the microbiome composition and qPCR was used to determine the relative abundance of *Wolbachia* spp. based on the highly utilized marker *Wolbachia* surface protein (*wsp*) gene. We found no effect of temperature within a single generation on the relative abundance of *Wolbachia* or immune gene expression, nor on the alpha or beta diversity of the microbiome. However, there was a significant difference in the abundance of *Wolbachia* between generations at high temperatures (≥ 28°C), but not at lower temperatures (≤ 23°C). These results support the idea that *Wolbachia* are reduced at higher temperatures between generations, which has an influence on the establishment of pathogens including West Nile Virus (WNV). Modulation of the Toll or IMD mosquito immune pathways was not indicated. *Wolbachia* endosymbiosis and trans-generation transmission appears especially sensitive to high temperatures, which may have implications for *Wolbachia*-based vector control strategies under climate change scenarios.

Keywords: climate change, culex quinquefasciatus, disease ecology, vector-borne disease, *Wolbachia*

INTRODUCTION

Culex mosquitoes are responsible for spreading numerous arboviruses and parasites, such as West Nile Virus (WNV) and lymphatic filariasis, respectively (1–4). *Culex pipiens quinquefasciatus* (Say) is part of the overall *Culex pipiens* complex, and can be found broadly distributed across the world, typically in lower altitude areas between latitudes of 30°N and 30°S (5, 6). In light of global climate change and anticipated changes in temperature particularly, the range of vector mosquitoes has been increasing into areas previously uninhabitable by these insects. The expansion of mosquito ranges globally is, in turn, likely to lead to an increase in vector-borne disease transmission (5, 7–11).

In response to the growing threat of mosquito-borne disease expansion due to climate change, there has been great interest in using microbial methods to control mosquito populations, and subsequently the spread of those vector-borne pathogens and parasites (12–20). The use of *Wolbachia* has been particularly attractive, as this *Rickettsia-like* bacterium spreads easily through mosquito populations (due to selective advantage of hosts with infections), induces cytoplasmic incompatibility, and effectively blocks the establishment of some viruses (DENV and CHIKV) in mosquito hosts (17, 18, 21–25). *Wolbachia* can either be maternally inherited or can be artificially introduced, frequently by transinfection, to insect populations. *Wolbachia* infection in mosquitoes can lead to cytoplasmic incompatibility, male embryo feminization, selective male killing, and parthenogenesis (26, 27). Cytoplasmic incompatibility (CI) is of particular interest as an incompatible insect technique (IIT) to reduce vector populations. CI occurs when sperm and egg cannot successfully form a viable zygote, which is bidirectional in many mosquitoes. Generally, when male mosquitoes are infected and females are uninfected (or infected with a dissimilar strain), no viable offspring are produced. When *Wolbachia*-infected females mate with uninfected males, they will produce hybrid offspring that carry and perpetuate the spread of the bacterium (21, 23, 24, 28–30). *Wolbachia* also acts competitively with and against members of the mosquito microbiome to structure community assemblage and influence pathogen invasion. This influence on pathogen and disease dynamics has led to increased interest in the role of microbiome community dynamics in mosquitoes (21, 18, 23–25, 30–35).

While after nearly a century of research on *Wolbachia* it is clear that *Wolbachia* could be immensely advantageous given the correct conditions for disease control, *in situ* applications remain complicated and require a better understanding of factors influencing efficacy in application (19, 27). For instance, Dodson et al. (36) found that *Wolbachia* infections in *Culex tarsalis* did not impede, but actually enhanced, infection of WNV in the mosquito host (37) also found that *Wolbachia* infection in *Culex tarsalis* did not inhibit Rift Valley fever virus. Hughes et al. (38) found that introduction of *Wolbachia* into *Anopheles* mosquitoes induced higher mortality in hosts and was inhibited by the native microbiome. Novakova et al. (39) found that *in situ* mosquitoes of the *Culex pipiens* complex had lower abundances of *Wolbachia* at higher temperatures, which corresponded with higher infection rates with WNV. Additional studies have reported impacts of temperature on Wolbachia, with several reporting similar reductions of *Wolbachia* abundances at higher temperatures (40–44). It has become increasingly clear that *Wolbachia* does not respond equally effectively in terms of control across mosquito species or environmental conditions, indicating that a better understanding of these factors is necessary to gain the maximum benefit from the use of *Wolbachia* for vector-borne control.

One factor that also can aid in pathogen transmission reduction is *Wolbachia's* manipulation of the mosquito host's immune response. *Wolbachia* has the ability to alter mosquito innate immune gene expression in response to pathogen invasion of the host (45–50). While the effect of *Wolbachia* on the mosquito immune system is well-documented in *Aedes* mosquitoes, less is understood about these effects in *Culex* mosquitoes (48, 51). The Toll, IMD (Immune Deficiency), and JAK-STAT pathways, in addition to other pathways, play an essential role in pathogen inhibition in mosquitoes (51–55). While gene expression may be altered at many stages within these pathways, two particular genes of interest that are altered with *Wolbachia* infection are *Rel1* (homologue to *Drosophila dorsal*), an integral part of the Toll pathway that aids in the transcription of innate immune factors (like antimicrobial peptides), and *Def1*, a gene in both the Toll and IMD pathways (48, 51). Interestingly, Ant et al. (30) analyzed various *Wolbachia* strains for effective infection and cytoplasmic incompatibility in *Culex quinquefasciatus* and found that immune genes, including *Rel1* and *Def1* as well as others associated with the Toll, IMD, and JAK-STAT pathways, were neither up- or down-regulated in relation to any *Wolbachia* infection. Given the importance of these pathways in mosquito immunity, this lack of change to immune gene expression in *Culex quinquefasciatus* with *Wolbachia* infection warrants further investigation.

Environmental conditions affect both mosquito hosts and endosymbiotic bacteria like *Wolbachia*. Given the implications of global climate change on global temperature and alterations of localized climate patterns, understanding the impact of changing environmental factors on vector control is of the utmost importance (10, 56–58). Boukal et al. (59) found that insects are particularly susceptible to the effects of prolonged increased temperatures at the individual and community levels, due to stress to insect physiological systems, behavior, body size, and, importantly, spatiotemporal distribution. They also found that these changes led to a restructuring of insect communities. Harvey et al. (60) further elaborated on this in their discussion of our current, and very limited, understanding of insect tolerance of temperature extremes due to climate change. Mordecai et al. (58) analyzed the thermal biology of numerous vector mosquito species and found that *Culex quinquefasciatus* had a thermal optimum for transmission of West Nile Virus (WNV) at 25.2°C, with a range for transmission between 19.0°C to 31.8°C. Several recent studies have indicated that WNV transmission tends to be greater under high temperature field conditions, which may be due to a decrease in naturally-occurring *Wolbachia* infection efficacy in mosquitoes (39, 42). Indeed, *Wolbachia* transmission is thermally sensitive in a number of systems including

mosquitoes and *Drosophila* (44, 61–63). In addition, *Wolbachia* colonization can increase thermal sensitivity of host mosquitoes and other insects (64, 65). To ensure that novel methods of mosquito control will remain functional (or may be adapted to maintain function) under new climate regimes, experiments exploring the impacts of temperature on the survival and efficacy of control microbes, like *Wolbachia*, are imperative.

To better understand the effects of environmental temperature on host and *Wolbachia* endosymbionts, we analyzed the relative abundance of native *Wolbachia* (presumed to be *wPip*) in *Culex quinquefasciatus* across upper thermal optima for the transmission of WNV in this species (39, 58). We hypothesized that *Wolbachia* abundances would be reduced at higher temperatures in line with climate change predictions, and that, similarly to *Aedes* mosquitoes and in-line with current thought in the field, *Wolbachia* abundances in *Culex quinquefasciatus* would influence immune genes, particularly in the Toll and IMD pathways (39, 46–48, 50, 51). The overarching goal of this study was to understand if, across a range of temperatures and across generations, *Wolbachia* abundance would be altered and if the mosquito host's immune gene expression would be altered in response to potential differences in *Wolbachia* abundance.

METHODS

Laboratory Mosquito Husbandry

Culex quinquefasciatus were ordered from Benzon Research (Carlisle, PA, USA) as 1st instar larvae and were co-housed at the same 25°C temperature in large 1L sterile glass containers in sterile pond water until 2nd instar. Larvae were fed autoclaved fish food mixed into sterile pond water and added at 2mL to each large 1L larval container until used in the experiment. Second instar larvae were then added along a temperature gradient beam and were used in the transgenerational temperature experiment.

Specifically in the transgenerational temperature experiment, mosquitoes were reared through two generations (with adults collected at generation 0 and 1) and were bloodfed using a membrane-style feeder with pig intestine casing to encase single donor human whole blood in sodium heparin purchased from Innovative Research, Inc (Novi, MI, USA). Mosquitoes had 10% sucrose withheld 24 hours prior to blood feeding. Second generation eggs were hatched by bubbling nitrogen gas through sterilized tubing into water containing eggs to reduce oxygen levels through displacement and trigger hatching. Hatch rate was estimated to be approximately 75%.

Experimental Design-Temperature Gradient Beam

A temperature gradient was designed to house both individual and groups of mosquito larvae along an insulated aluminum beam using Peltier devices to control heating and cooling. Automated temperature measurements along the beam were recorded hourly for the duration of the experiment, and water temperatures of vessels (without larvae) in the beam were measured daily to verify similar temperature measurements. The average temperature for each zone (A-E) was calculated by averaging all temperature measurements for that zone over the duration of the experiment (**Figure 1**). Temperature range was established based on the thermal optima of West Nile virus transmission in *Culex quinquefasciatus* presented in Mordecai et al. (58). Primary containment over the glass vessels consisted of sterilized mesh enclosing the top of the vessel with a sterile tie and secondary containment consisted of a mesh enclosure around the aluminum temperature gradient beam.

Culex larvae were housed individually (5mL water) and in groups of 5 (25mL water) in glass vessels filled with sterile pond water and were fed sterile fish food in sterile pond water per larvae. Water was replenished as needed and was replaced simultaneously from all vessels to maintain relatively consistent levels of oxygenation. The light cycle was 12:12 and larvae were fed every 3-4 days. Larvae were added to the glass vessels simultaneously with all larvae at the second instar stage. Within eight hours of eclosion, mosquito adults were aspirated for collection out of the glass vessels and frozen at -20°C.

Experimental Design-Transgenerational Temperature Experiment

Incubators (Percival I-36VL incubator (Perry, Iowa, USA) and Conviron Gen1000 incubator (Pembina, North Dakota, USA)) were set up in a biosecure insectary with 14:10 light/dark cycles, 65% relative humidity, and set temperatures of 23°C and 30°C, with both incubators reduced 3°C during dark cycles (**Figure 1**). Temperatures in the 30°C chamber were reduced to 28°C prior to the hatching of generation 1 to reduce the potential for mortality, though 28°C is still considered in the same high range, high potential transmission of WNV for this mosquito species (58).

Mosquitoes were reared over two generations with adults collected from generations 0 and 1. Larvae were co-housed in large glass beakers with the non-sterile water in which they arrived from Benzon Research (Carlisle, Pennsylvania, USA) and had sterile pond water added to reduce density. *Culex quinquefasciatus* second instar larvae were placed in large 1L glass beakers with sterile pond water in three sterilized mesh cages in each incubator. Larvae were fed sterile fish food *ad libitum* and upon emergence were fed a 10% sucrose solution using soaked cotton balls. Adults were collected from each generation and were frozen at -20°C. Adult females in generation 0 were blood fed and eggs were hatched as described above. Generation 1 larval housing was covered by sterile mesh to prevent any remote potential for escape into the parent generation prior to the removal of any remaining parents.

Specimen Storage and Extraction

Culex quinquefasciatus samples from both experimental designs were frozen at -20°C and had 100uL of 1X DNA RNA Shield (Zymo Research, Irvine, CA, USA) added after being freeze-killed. Samples were frozen at -20°C until extraction. Samples had wings, legs, and heads removed and carcasses were macerated in DNA RNA Shield. Samples were then extracted using Quick DNA/RNA MagBead kits (Zymo Research, Irvine, CA, USA) and frozen at -80°C. Sample total nucleic acid concentration following extraction was 0.5ug/uL.

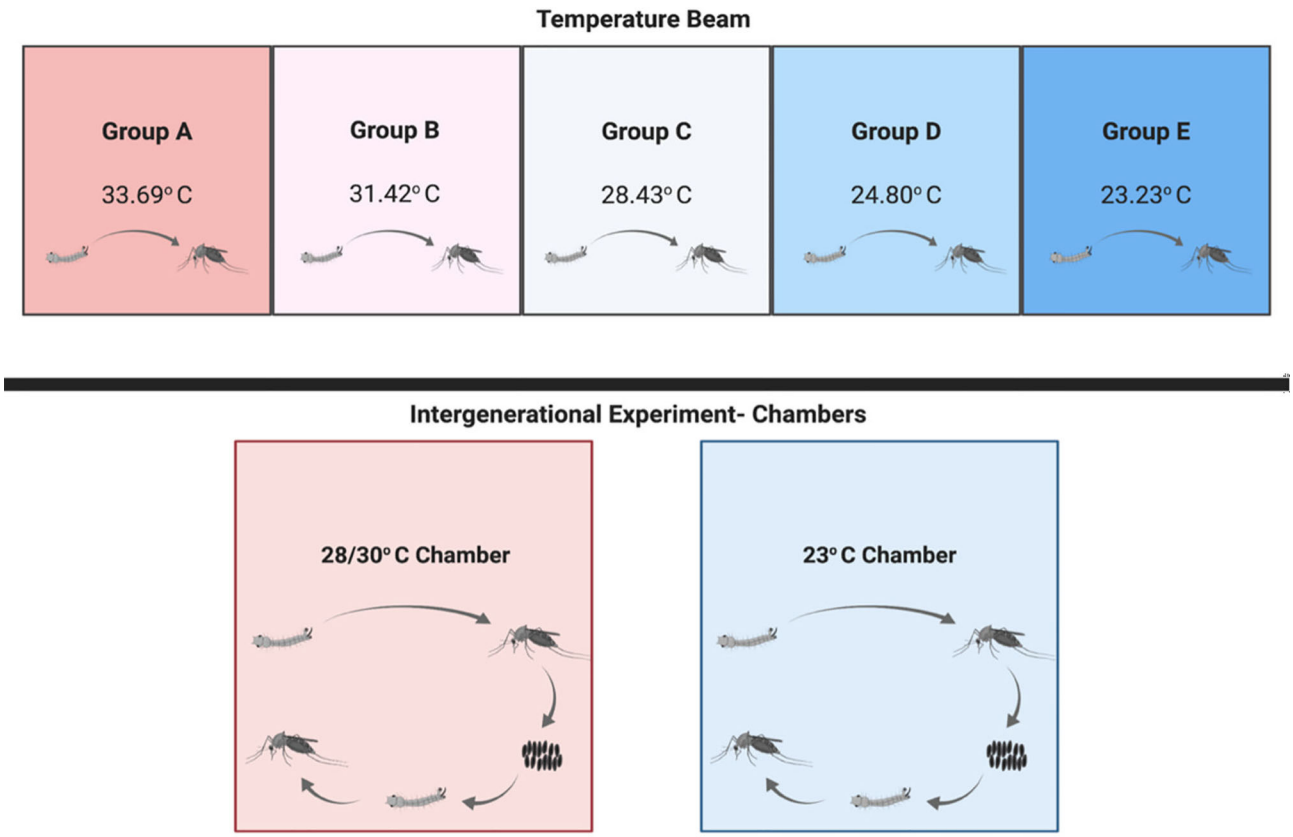

FIGURE 1 | The experimental design for temperature gradient beam and transgenerational temperature experiments in *Culex quinquefasciatus*. The temperature gradient beam ran from 33.69-23.23°C with mosquitoes (n = 30 at setup) reared through eclosion at each of the five temperature groups (A-E) along the beam. Adult mosquitoes were harvested within 8 hours of eclosion. The transgenerational experiment was run in two enclosed chambers with several replicate populations (n = ~200) at each temperature (28/30 and 23°C) reared through generation 0 and the eclosion of generation 1 with adult mosquitoes (n=5 for each group for qPCR) sampled from each generation.

qPCR of Relative *Wolbachia* Abundance (*wsp* gene) and Immune Genes *Rel1* and *Def1*

Quantitative PCRs were performed in duplicate on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) to analyze the relative abundance of *Wolbachia* and key immune genes. For *Wolbachia* qPCRs (n = all surviving adult mosquitoes across groups), the *wsp* gene was targeted as a proxy for relative abundance and using slightly modified primers forward-5'-AGATAGTGTAACAGCRTTTTCAGGAT- 3' and reverse-5'-CACCATAAGAACCAAAATAACGAG- 3' from (66). The reaction mix for the qPCRs was: 5.1uL Milli-Q Ultrapure water, 10uL 2X AzuraQuant Green Fast qPCR Mix LoRox (Azura Genomics, Raynham, MA, USA), 0.8uL of 10uM forward primer, 0.8uL of 10uM reverse primer, 0.3uL 20mg/mL bovine serum albumin, and 3uL template DNA per well. Cycling conditions were: 1 cycle at 95°C, then 40 cycles of 5 seconds at 95°C and 31 seconds of 60°C. Transcript-free negative controls were used in every qPCR run for *Wolbachia*, 18S, and immune genes.

DNase I (Zymo Research, Irvine, CA, USA) was used to degrade genomic DNA from a subsample of the total nucleic acid extractions. Complementary DNA (cDNA) for use establishing active transcription of immune genes was then created from extracted RNA using SuperScript IV VILO Master Mix (Invitrogen, Carlsbad, CA, USA). Concentrations of cDNA were measured using a Nanodrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA) and were diluted to 2ng/uL in the final reaction mix. For the qPCRs of immune genes and the 18S gene (used here as a housekeeping gene, as done in 30), primers from (30) were used (**Table 1**). The same PCR reaction mix (though with 8.1uL Milli-Q water and 1uL of template) and cycling conditions as described above were used.

Microbiome Sequencing and Preparation

The microbiomes of *Culex quinquefasciatus* (n = 68; all surviving adults) from the temperature gradient beam experiment were analyzed using 16S V4 rRNA gene region barcoding following the Earth Microbiome Protocol (EMP) (67–72). The 515F (with barcode) and 806R primers were used for amplification, standard 25uL/well reaction PCR recipe and cycling conditions were followed, but 5Prime HotMasterMix (Quantabio, Beverly, MA,

TABLE 1 | Primers from (30) used in qPCRs to analyze the relative abundance of immune genes *Rel1* and *Def1*, as well as the 18S gene utilized for housekeeping and standardization.

Primer name	5′–3′ sequence
Rel1-F	GCGACTTTGGCATCAAGCTC
Rel1-R	GTTCGACCGGAGCGTAGTAG
Def1-F	GGTCCAATACTTCGCCAATAC
Def1-R	GATTGGGCGTCAACGATAGT
18S rRNA-F	CGCGGTAATTCCAGCTCCACTA
18S rRNA-R	GCATCAAGCGCCACCATATAGG

USA) was used for PCR in place of the EMP recommended master mix. Post-PCR samples were normalized using a Mag-Bind Pure Library Normalization Kit (Omega Bio-Tek, Norcross, GA, USA). Normalized samples were pooled into a library, quantified using a Qubit 2 Fluorometer (Invitrogen, Carlsbad, CA, USA), and appropriately diluted to follow EMP protocols (73, 74). The library was sequenced using an Illumina MiSeq v2 300-cycle kit (Canton, MA, USA) with the EMP recommended PhiX addition (Illumina, Canton, MA, USA).

Bioinformatic and Statistical Analyses

Mosquito survival across temperature groups in the temperature beam experiment were analyzed using a Chi-Square test in Microsoft Excel. Relative abundances of *Wolbachia*, *Rel1*, and *Def1* were calculated in Microsoft Excel by standardizing the average qPCR Cq value for each sample and each gene by its corresponding housekeeping 18S Cq value (30). Average Cq values were then scaled by the relative percent change between 18S housekeeping values to adjust and further account for any variance in the starting concentration of DNA or cDNA in each sample. These corrected Cq values were then analyzed in SPSS (IBM SPSS Statistics, v. 26) by testing for normality using Levene's test and subsequently analyzing the data using either one-way ANOVAs or Kruskal-Wallis tests when data did not meet the criteria for parametric statistics. We used linear regressions to test for correlation of *Wolbachia* abundance, *Rel1*, or *Def1* with generation, temperature and sex.

Microbiomes of the temperature gradient beam mosquitoes were analyzed using QIIME2. Microbiome data were demultiplexed, deblurred, quality filtered to q-scores of 20, and rarefied to 2500 sequences per sample in QIIME2. Taxonomy was matched using the 2019.7 SILVA pre-trained QIIME2 classifier (75, 76). ANOSIMs and Kruskal-Wallis tests were performed to analyze differences in beta (Bray Curtis dissimilarity matrices) and alpha (sOTU Richness, Shannon Diversity Index) diversity between groups, respectively.

RESULTS

Larval Survival Across the Temperature Gradient Beam

Percentage survival at emergence of *Culex quinquefasciatus* larvae reared on the temperature gradient beam was determined for each temperature group, as represented by that group's average temperature over the length of the experiment (**Figure 2**). Temperature groups with an average temperature over 30°C had markedly lower survival (16.7%) through eclosion when compared to the lower temperatures. The lowest temperature group had the highest survival rate through eclosion, at 83.3% survival. A Chi Square test indicated that there was a significant difference in survival across the temperature groups (X^2 = 55.07; df =4; p<0.001).

Microbiome Diversity Across the Temperature Gradient Beam: sOTU Richness,Shannon Diversity, and Bray Curtis Dissimilarity

Alpha diversity was analyzed using. Kruskal-Wallis tests. There were no significant differences in sOTU richness when analyzed using Kruskal-Wallis tests across temperature groups (n=66, df=4, H=5.057, p= 0.410), sexes (n=65, df=1, H=0.810, p= 0.667), or whether larvae were reared solitary or in groups (n=62, df=1, H= 0.018, p= 0.895). There were also no significant differences in Shannon Diversity index values for the microbiomes between temperature groups (n=66, df=4, H=5.176, p=0.365), sexes (n=65, df=1, H=0.374, p=0.829), or larval grouping conditions (n=62, df=1, H=1.351, p=0.245).

ANOSIMs were run to determine differences between groups in terms of beta diversity. There were no significant differences in Bray Curtis dissimilarity when analyzed using ANOSIMs for temperature groups (n=66, df=4, Test Statistic= 0.067, p= 0.092), sexes (n=65, df=1, Test Statistic= 0.052, p= 0.095), or solitary or grouped larvae (n=62, df= 1, Test Statistic=0.011, p= 0.364). Negative controls (extraction and PCR blanks) were significantly different in pairwise comparisons across all experimental groups and tests than experimental samples, though negative controls were removed prior to running Kruskal-Wallis tests within experimental groups. Prominent genera across all samples, in order of relative frequency, included: *Wolbachia*, unspecified Enterobacteriaceae, *Massilia, Pseudomonas, Bacteriodetes, Aeromonas*, unspecified Burkholderiaceae, *Elizabethkingia, Pedobacter*, and *Flectobacillus* (**Supplementary Figure 1**).

Wolbachia and Immune Genes Across Experiments: Temperature Gradient Beam Experiment

Kruskal-Wallis tests were used to analyze differences between Cq values across temperature groups and sexes independently for *Wolbachia relative abundance (in the form of the wsp gene), Rel1*, and *Def1*. Levene's test for homogeneity indicated the need for non-parametric test use. Kruskal-Wallis tests were run in iterations both including Cq values of zero as well as with zeros removed. Tests analyzing temperature groups including the Cq zero values were non-significant overall for *Wolbachia* (n=68, Test Statistic=1.525, df=4, p=0,822), *Rel1* (n=68, Test Statistic=0.283, df=3,p=0.991), and *Def1* (n=68, Test Statistic=0.330, df=4, p=0.988). Kruskal-Wallis test iterations with zero values removed also indicated that there was no

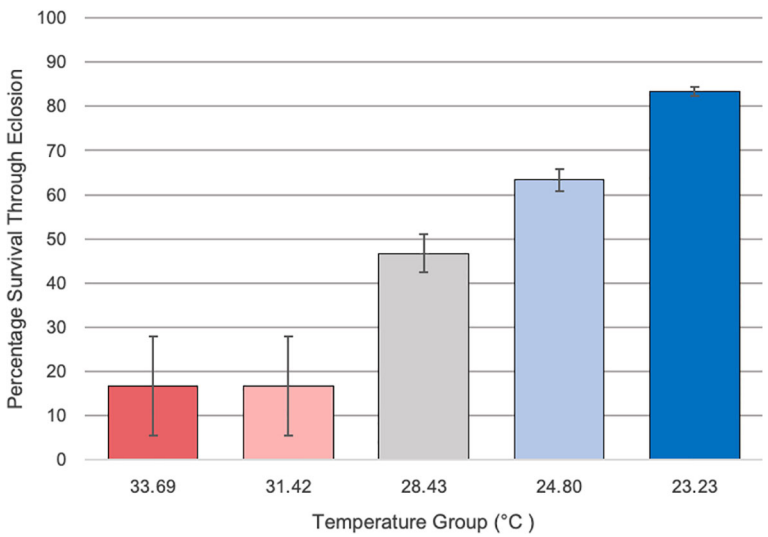

FIGURE 2 | The percentage survival of *Culex* larvae from second instar through emergence along the various temperature groups on the temperature gradient beam. Temperatures ranged from 33.69 to 23.23°C across sections along the temperature gradient beam. Temperatures over 30°C showed a marked reduction in survival (16.7% survival) through emergence compared to the other groups (83.3% in lowest temperature group). Original experimental setups began with n = 30 and error bars indicate standard error. A Chi Square test indicated that there was a significant difference in survival across the temperature groups (X^2 = 55.07; df =4; p<0.001).

significant difference between any temperature groups in regard to *Wolbachia* abundance (n=64, Test Statistic=3.662, df=4, p=0.454; **Figure 3**), the expression of *Rel1* (n=62, Test Statistic=3.496, df=4, p=0.478; **Figure 4**), or the expression of *Def1*(n=62, Test Statistic=3.826, df=4, p=0.430; **Figure 5**). Test iterations with zeros removed also indicated that there was no significant difference between mosquito sex and *Wolbachia* abundance (n=64, Test Statistic=0.451, df=1, p=0.502), *Rel1* expression (n=62, Test Statistic=1.075, df=1, p=0.3), or *Def1* expression (n=62, Test Statistic=1.018, df=1, p=0.313). These collectively indicated that there were no differences between temperature groups or sexes for relative *Wolbachia* abundance, or the relative expression of *Rel1* and *Def1*.

Wolbachia and Immune Genes Across Experiments: Transgenerational Temperature Experiment

One-way ANOVAs were run with *post-hoc* Tukey's HSD tests for pairwise comparisons (where appropriate) to analyze differences in *Wolbachia* and immune genes *Rel1* and *Def1* within and between generations at 23 and 30/28°C (n = 5 randomly and blindly sampled per group). Zero values were removed from analyses, which only removed one sample from Generation 0 at 23 degrees. The ANOVA for *Wolbachia* abundance (in the form of the *wsp* gene) was significantly different across temperature groups (n=19, df=2, F=5.110, p=0.019), which the Tukey's HSD pairwise comparison indicated was driven by the only pairwise significant difference, which was between the 28- and 30-degree temperature groups (n=19, St. Error=0.928, p=0.017, 95% CI=0.5003-5.2877). There was no significant difference between temperature groups for either *Rel1* gene expression (n=19, df=2, F=1.022, p=0.382) or *Def1* gene expression (n=19, df=2, F=1.072, p=0.366). There was also no significant difference between generations 0 and 1 for *Rel1* (n=19, df=18, F=0.525, p=0.479) or *Def1* (n=19, df=18, F=0.492, p=0.493), but there was a significant difference in *Wolbachia* between generations (n=19, df=18, F=5.738, p=0.028).

Additional one-way ANOVAs were run to determine differences between joint temperature groups across generations. Tests indicated that there was no difference between temperature groups and generations for *Rel1* expression (n=19, df=3, F=0.680, p=0.578) or *Def1* expression (n=19, df=18, F=0.734, p=0.548), but that there was a significant difference across the groups in regard to *Wolbachia* abundance (n=19, df=18, F=3.326, p=0.048). Interestingly, the Tukey's HSD *Post-Hoc* multiple comparisons indicated that there was a significant reduction in *Wolbachia wsp* gene expression between Generation 0 and Generation 1 at 30/28°C (n=19, St. Error=0.95, p=0.037). There were no significant differences in *Wolbachia* within generation 0 between temperatures. Linear regression analysis also indicated that *Wolbachia* abundance is significantly correlated with generation (n=19, R^2 = 0.273, F=6.371, p=0.022). However, *Rel1* expression (n=19, R^2 = 0.048, F=0.401, p=0.676) and *Def1* expression (n=19, R^2 = 0.044, F=0.372, p=0.695) was not correlated with generation, temperature, or sex.

Moreover, when mosquito sex was analyzed by ANOVA, there was no significant difference for *Rel1* (n=19, df=18, F=0.555, p=0.467) or *Def1* (n=19, df=18, F=0.538, p=0.473), but there was a significant difference in *Wolbachia* abundance by sex, with greater

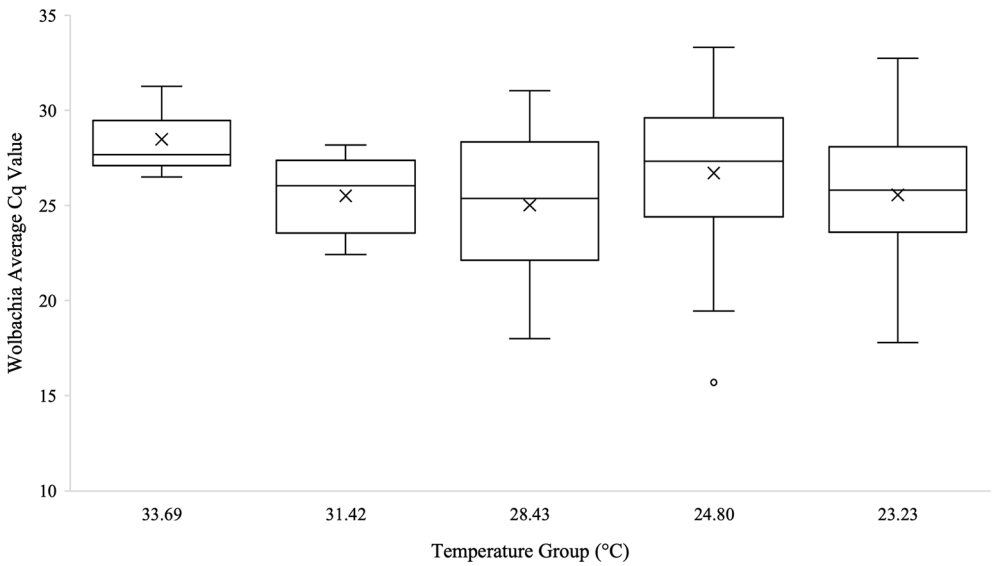

FIGURE 3 | Box and whisker plot of the *Wolbachia* Cq values from qPCR across temperature groups on the temperature gradient beam. *Wolbachia* (*wsp* gene) technical replicates were averaged and all biological replicates that amplified during qPCR are shown across the temperature groups. There was no significant difference in *Wolbachia* Cq values, which are representative of the *Wolbachia* abundance, across the temperature beam groups as analyzed by Kruskal-Wallis test (n=64, Test Statistic=3.662, df=4, p=0.454). Mean Cq values for each temperature group are indicated on the plots with an "X".

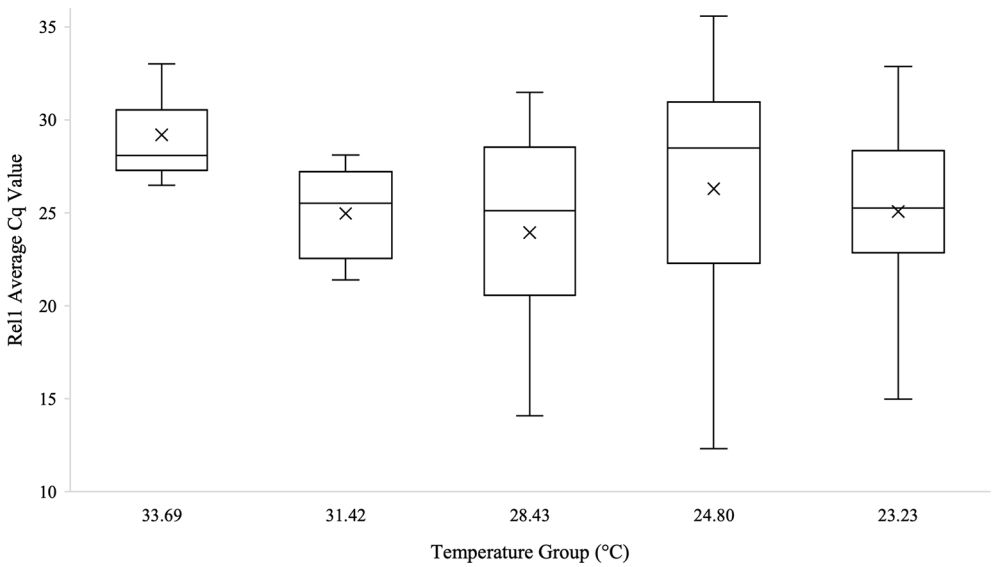

FIGURE 4 | Box and whisker plot of the *Rel1* Cq values across temperature groups on the temperature gradient beam. *Rel1* expression technical replicates were averaged and all biological replicates that amplified during qPCR are shown across the temperature groups. There was no significant difference in *Rel1* Cq values across the temperature beam groups as analyzed by Kruskal-Wallis test (n=62, Test Statistic=3.496, df=4, p=0.478). Mean Cq values for each temperature group are indicated on the plots with an "X".

abundance occurring in males (n=19, df=18, F=6.017, p=0.025). A linear regression further indicated that there was a correlation between mosquito sex and *Wolbachia* abundance (n=19, df=18, F=4.170, p=0.035). Additionally, there were no significant differences in *Wolbachia* abundance (n=14, df=13, F=0.415, p=0.826), *Rel1* expression (n=14, df=13, F=0.659, p=0.665), or *Def1* expression (n=14, df=13, F= 0.741, p=0.614) among mosquito rearing cage positions in the incubators.

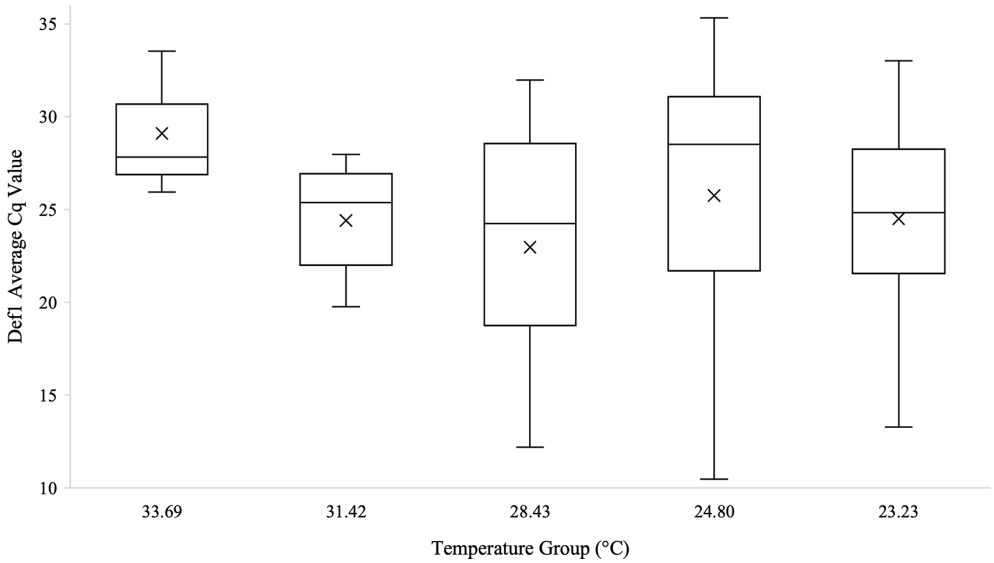

FIGURE 5 | Box and whisker plot of the *Def1* Cq values across temperature groups on the temperature gradient beam. *Def1* expression technical replicates were averaged and all biological replicates that amplified during qPCR are shown across the temperature groups. There was no significant difference in *Def1* Cq values across the temperature beam groups as analyzed by Kruskal-Wallis test (n=62, Test Statistic=3.826, df=4, p=0.430). Mean Cq values for each temperature group are indicated on the plots with an "X".

DISCUSSION

Across both the temperature gradient beam and transgenerational experiments in *Culex quinquefasciatus*, our results indicated that *Wolbachia* and immune genes *Rel1* and *Def1* did not differ with temperature within a single generation. Interestingly, *Wolbachia* relative abundances differed significantly between generations, but only at high temperatures above 28°C, and with no significant change in immune gene expression. Rel1 *and Def1* expression did not differ between any experimental groups, but *Wolbachia* abundance differed by generation and by sex. Differences in *Wolbachia* abundance by sex were likely due to cytoplasmic compatibility (21, 23, 28, 30). Female mosquitoes and mosquitoes in generation 0 had higher average abundance of *Wolbachia*. Furthermore, there was no difference in microbiome diversity across mosquitoes in the temperature beam experiment.

Reductions in *Wolbachia* abundance at high temperatures are fairly well documented in *Aedes* species (41, 42, 44), particularly in *Aedes albopictus* where *Wolbachia* was markedly reduced at 37°C (40). Novakova et al. (39) found that *in situ* mosquitoes of the *Culex pipiens* complex had lower abundances of *Wolbachia* at higher temperatures, and that a reduction in *Wolbachia* also negatively correlated with West Nile virus (WNV), which is also supported by other works (40, 77, 78). Our results further support both of these findings in *Culex pipiens* complex mosquitoes, indicating that *Wolbachia* abundance is generally reduced at high temperatures, similar to findings in a meta-analysis of insect microbiomes (79). Novakova et al. also hypothesized that this reduction of *Wolbachia* in the *Culex pipiens* complex differed across generations and may have been influenced mosquito immune function, which is consistent with findings in *Aedes* mosquitoes (22, 47, 51, 80). Our findings support the reduction of *Wolbachia* abundance at high temperatures across generations (**Figure 6**), but do not provide support that *Wolbachia* influenced immune function in *Culex pipiens quinquefasciatus*, as we observed no differences in key Toll and IMD pathway genes at varied temperatures.

Rel1 (homologue to *Drosophila dorsal*) is an integral part of the Toll pathway that aids in the transcription of innate immune factors (like antimicrobial peptides) and *Def1* is part of both the Toll and IMD pathways (48, 51). While no genes of the JAK-STAT pathway were analyzed here, based on the findings of Ant et al. (30), we would not expect to see any changes in this pathway either. They found that immune genes across multiple pathways were neither up- nor down-regulated in relation to *Wolbachia* infection in *Culex quinquefasciatus* (30). Their results, taken together with our results and Novakova et al. (39), indicate that temperature has an impact on the abundance of *Wolbachia* in *Culex pipiens quinquefasciatus* (Say) over generations but *Wolbachia* appears unlikely to be altering immune gene expression in turn.

We propose further study of the tripartite interactions between mosquito host, microbiome, and pathogens to help elucidate the mechanism by which high temperatures alter the

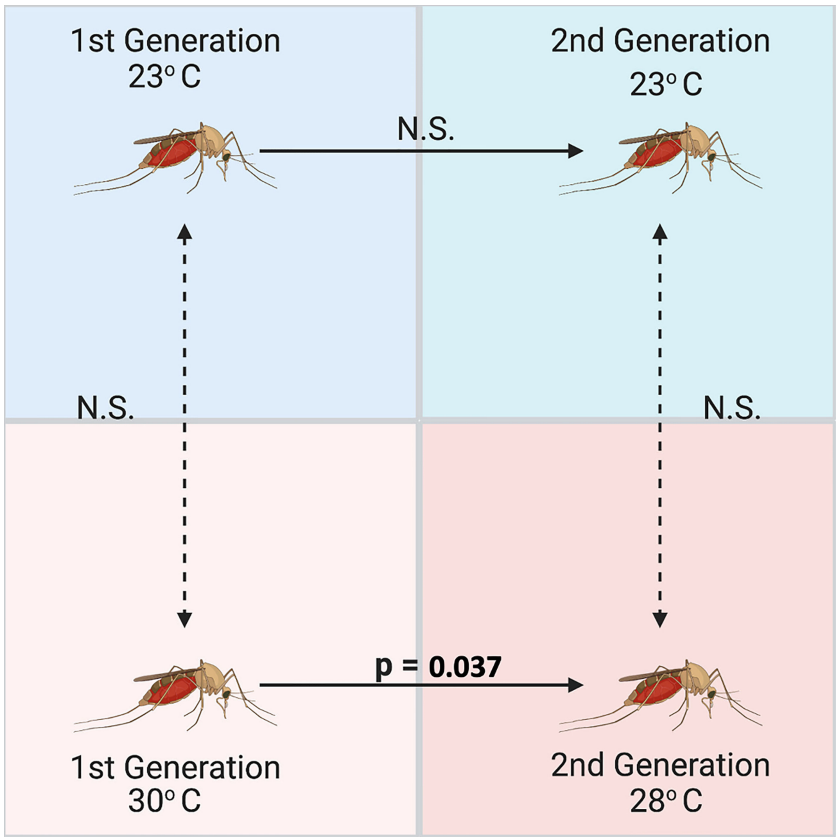

FIGURE 6 | Significant differences across generations between relative abundances of *Wolbachia* (*wsp* gene) in *Culex quinquefasciatus* measured using qPCR and standardized with 18S as a housekeeping gene (n = 5 mosquito adults per group). *Culex* were reared over two generations at a low (23°C) and high (30/28°C) temperature, but *Wolbachia* was only significantly different between generations at the high temperature treatment based on an ANOVA comparing the *Wolbachia* abundances and a subsequent Tukey's HSD multiple comparisons test (n=19, St. Error=0.95, p=0.037).

abundance of *Wolbachia* (and subsequently, infection with pathogens like WNV). We also propose further investigation into the potential impacts of temperature variations (*i.e.* seasonal, daily) on the abundance of *Wolbachia* in *Culex* mosquitoes. Additionally, we anticipate that there would be interactions between other host factors that have not been accounted for in this study, including competition within the microbiome at varied temperatures, influence of other immune pathways, and potential changes in other signal pathways due to changes in environmental factors (13, 38, 39, 81). Furthermore, we recommend that temperature, weather, and climate conditions be considered when timing the deployment of *Wolbachia*-based control methods in the field.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA809017.

AUTHOR CONTRIBUTIONS

AT-P and DCW conceived, planned, and acquired funding for this study. AT-P, JJ, MF, and SL carried out the experiments. AT-P and JP analyzed the data. AT-P wrote the manuscript with input from the other authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2022.762132/full#supplementary-material

Supplementary Figure 1 | Taxa plot of adult *Culex quinquefasciatus* microbiomes following emergence across the temperature gradient beam temperature groups. Group A is 33.69°C, Group B is 31.42°C, Group C is 28.43°C, Group D is 24.80°C, and Group E is 23.23°C. The taxa legend includes the twenty highest relative frequency sOTUs at the Genus level. There were no significant differences in sOTU richness by Kruskal-Wallis test (n=66, df=4, H=5.057, p= 0.410), in Shannon diversity by Kruskal-Wallis test (n=66, df=4, H=5.176, p=0.365), or in beta diversity based on Bray-Curtis dissimilarity tested using ANOSIM (n=62, df=4, p= 0.092) between temperature groups.

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