Stress test: Effects of endosymbiotic bacteria on thermal tolerance of a montane leaf beetle

By

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Date: June 29, 2018

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Abstract

Insects are a diverse group of organisms found in most terrestrial and aquatic ecosystems. Because insects are ectotherms, they are frequently exposed to variation in environmental temperature and thus variation in body temperature, the latter of which may cause physiological stress. Insects host a variety of microbes, including intracellular and extracellular bacteria, endosymbiotic or parasitic, which may alter how the host responds to environmental temperature variation. Physiological, biochemical and molecular responses to thermal variation have been well characterized for many species of insects, yet few studies have examined how microbes alter their response to environmental stress. In this thesis, I examined features of the microbiome of a montane insect, the leaf beetle *Chrysomela aeneicollis*. Prior studies of beetle populations living at high elevation in the Eastern Sierra Nevada mountains of California have shown that adult and larval beetles are exposed to both elevated and sub-zero temperatures during the summer growing season, and that exposure to these thermal extremes impacts survival, performance, and reproductive success. Like most insects, this beetle is host to endosymbiotic bacteria; however, little is known about the composition of this microbiome, or the role endosymbionts play in how beetles respond to thermal extremes. My study pursued three aims: 1) describe the beetle microbiome and determine which microbes are most common; 2) assess the evolutionary relationships between willow beetle microbes and those of other known insect symbionts; and 3) quantify the interacting effects of host genetic variation and microbe composition and abundance on the beetles' response to an environmentally realistic cold stress. To examine the beetle microbiome, I used the metagenomic application Metaphlan, a computational tool used for profiling the composition of microbiome communities. The results of this analysis indicated that *Wolbachia* was the most abundant endosymbiont in Sierra willow beetles, making up 99% of the microbiome. I therefore focused the remainder of my work on that common endosymbiont. To determine the number of *Wolbachia* strains infecting *C. aeneicollis*, I used Multi Locus Sequence Typing (MLST), which is based on five genetic loci known to have appropriate properties for use in distinguishing *Wolbachia* types. To quantify the relationship between *Wolbachia* density and recovery from cold exposure, I performed quantitative PCR (qPCR) on beetles exposed to either control conditions, or an ecologically relevant sub-lethal cold exposure. I used restriction digestion and SNP genotyping to determine the haplotype of *cytochrome oxidase II* (*COII*) and genotype of *phosphoglucose isomerase* (*PGI*), two genes coding for proteins of central metabolism, for each beetle. MLST analysis determined the presence of at least three strains of *Wolbachia*; two of these belonged to *Wolbachia* Supergroup A and one belonged to *Wolbachia* Supergroup

B. Results from qPCR analysis showed that *Wolbachia* density was greater in non-stressed individuals than those exposed to cold stress, and that *Wolbachia* density was related to both *PGI* and *COII* genotype. I found that running speed measured directly after field collection of beetle adults was related to nuclear and mitochondrial genotype, and negatively related to the density of *Wolbachia* B. Running speed after cold exposure was also related to nuclear and mitochondrial genotype, and was negatively correlated with the density of *Wolbachia* A. These results implicate both host genetics and pathogen density in mediating a response to thermal stress. Thus, the microbiome of Sierra willow beetles may influence the ability of these insects to live in a rapidly changing thermal environment.

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Introduction

Insects are regarded as the most abundant and diverse group of animals on the planet (Rahman *et al.* 2018). They perform critical roles in most terrestrial communities, acting as pollinators, biological control agents, facilitators of nutrient cycling, and major remodelers of both natural and agricultural environments (Losey and Vaughan 2006; Edburg *et al.* 2012, Hicke *et al.* 2012, Harvey *et al.* 2013; Rahman *et al.* 2018). The central role of insects in most ecological communities means that it is crucial that we understand how insects respond to environmental change, especially as human-caused climate change accelerates (Bale *et al.* 2002, Hegland *et al.* 2009). Rapid, daily changes in air temperature, precipitation and humidity can cause physiological stress, which may affect growth rate, performance and components of fitness (Bowler and Terblanche 2008, Terblanche *et al.* 2010, Kingsolver *et al.* 2013). To survive, insects have evolved ways to combat environmental stressors. For example, during episodes of cold stress, the fruit fly *Drosophila melanogaster* and other insects use cryoprotective compounds such as glycerol to "cold harden", so that they become tolerant of previously sub-zero, lethal cold temperature (Lee *et al.* 1987). During episodes of exposure to high temperature, *D. melanogaster* (and other insects) express stress-inducible molecular chaperones to refold denatured proteins and prevent formations of cytotoxic aggregates (Feder and Hofmann 1999, Clark and Worland 2008, Shilova *et al.* 2018). These and many other studies have effectively characterized how insects respond to thermal variation in nature and in the laboratory.

Insects are associated with diverse intracellular or extracellular microbes that may influence whole animal metabolism and reproductive success, and therefore may play a role in how insects respond to thermal stress (Kikuchi 2009, Salem *et al.* 2015). If a microbe benefits its host, the interaction is mutualistic, while if they harm the host, the interaction is parasitic. Either way, both mutualisms and parasitic infections are geared towards maximizing the microbe's likelihood of survival (Toft and Andersson 2010). As such, microbes must balance the need to acquire enough resources from the host to survive and reproduce, but not take so much from the host that the host fails to survive. In some cases, such as the microbes infecting aphid guts, metabolic integration of host and endosymbiont is so complete that the relationship becomes an obligate symbiosis (Oliver *et al.* 2010). However, even obligate endosymbionts may act selfishly in some situations, that is, be considered parasites. Thus, the relationship between obligate endosymbionts and their host may fluctuate between mutualism and parasitism, depending on the life stage of the host or the environmental conditions experienced (Toft and Andersson 2010).

Prior studies have shown that the mutualistic interactions between endosymbiotic bacteria and insect hosts positively affect a wide variety of host life history traits and are especially important for allowing insects to thrive in nutrient poor environments or on specialized diets, like blood, sap, or toxic plants (Wang *et al.* 2011, Weiss and Aksoy 2011). In recent decades, a growing body of evidence suggests that endosymbionts may protect their insect hosts from thermal stress. For example, the endosymbiont *Buchnera aphidicola* produces high levels of heat shock protein GroEL,

which is then excreted for use by their aphid host during episodes of thermal stress (Fares *et al.* 2002). Not surprisingly, bacterial endosymbionts within the same genus have a range of thermal tolerance characteristics distinct from their hosts (Lee and Lim 2001, Montllor *et al.* 2002, Grégoire *et al.* 2017); thus, endosymbiont abundance and microbiome composition may be negatively affected by exposure to high or low temperatures (Parish and Bale 1991, Wernegreen 2002, Zientz *et al.* 2004, Anbutsu *et al.* 2008). For example, exposure to thermal extremes results in a reduction of endosymbiont densities in *Drosophila nebulosa* and *Drosophila melanogaster* (Anbutsu *et al.* 2008, Moghadam *et al.* 2017). Under some conditions an endosymbiont may affect the fitness consequences of allelic variation in the host nuclear or mitochondrial genome, partly because of competition for nutritional resources between the host and its symbionts, and partly because the host's metabolic machinery is influenced by the energetic cost of supporting populations of microbes. One might further predict that intracellular microbes would be likely candidates for altering the fitness effects of host genetic variation. As such, it is important to understand effects of thermal stress on endosymbiont densities in insects, and how this could influence the interactions between endosymbionts and insect nuclear and mitochondrial genes. In this manner, endosymbionts can mediate natural selection and the adaptive significance of genetic variation in their hosts.

One of the most well-understood insect endosymbionts is *Wolbachia*, a genus of intracellular bacteria that also infects nematodes and other arthropods (Hilgenboecker *et al.* 2008). *Wolbachia* is estimated to infect more than 65% of all species of insects

worldwide (Hilgenboecker *et al.* 2008, Werren *et al.* 2008). Additionally, *Wolbachia* is known to manipulate host reproductive systems for its own gain. Feminization, parthenogenesis, male killing, and cytoplasmic incompatibility (CI) are means by which *Wolbachia* optimizes its vertical transmission at a cost to host fitness (Werren *et al.* 2008). In the butterfly *Eurema hecabe*, *Wolbachia* interferes in the butterfly sex-determination pathway, causing genetic males to develop as females, and in the moth *Ostrina scapulalis*, *Wolbachia* preferentially kills males during embryogenesis (Werren *et al.* 2008, Sugimoto and Ishikawa 2012). In contrast, in some insects, the presence of *Wolbachia* leads to an increase in host survival and reproductive success relative to non-infected hosts (Werren *et al.* 2008). For example, *Wolbachia* has been found to protect the yellow fever mosquito *Aedes aegypti* from RNA viruses, as *Wolbachia* suppresses the viral titer by as much as 10,000-fold, as well as reducing the pathogenicity of the virus (Teixeira *et al.* 2008). In bedbugs, *Wolbachia* supplies the host with a vital micronutrient, Vitamin B (Hosokawa *et al.* 2010). While much is known about how *Wolbachia* may benefit or harm insect hosts, little is known about how *Wolbachia* might alter insect performance during environmental stress.

Prior studies have shown that both high and low temperatures negatively affect *Wolbachia* densities in insects (Jia *et al.* 2009, Bordenstein and Bordenstein 2011), suggesting that *Wolbachia* itself may be vulnerable to thermal stress. Previous studies in the parasitoid wasp *Nasonia vitripennis* suggest that exposure to heat reduces *Wolbachia* density in insects (Bordenstein 2011). High temperature seems to induce *Wolbachia* phage (WO, a virus that infects *Wolbachia*) to undergo lytic cycle, thus killing

Wolbachia (Bordenstein 2011). Low temperatures, on the other hand, have been shown to result in WO remaining in its lysogenic cycle inside *Wolbachia;* thus, low temperatures do not seem to reduce *Wolbachia* density in these insects (Bordenstein 2011). However, low temperature does seem to negatively affect *Wolbachia* density, by slowing down its growth *in situ* (Bordenstein 2011). Given that both high and low temperatures seem to alter *Wolbachia* density, and that *Wolbachia* is known to manipulate host genetic machinery for its own survival, *Wolbachia* may interact with host stress machinery to bolster its own defense against temperature extremes, either increasing host resistance to stress or reducing host fitness by obtaining resources from the host. This hypothesis, however, has yet to be thoroughly tested.

High elevation montane environments pose unique, stressful challenges to insects. Montane insects experience short growing seasons and extreme weather conditions, both during the growing season and during winter dormancy, as well as physical barriers to dispersal. These features of montane life both pose challenges to survival and reproduction, and opportunities for adaptation to local conditions (Hodkinson 2005, McMillan *et al.* 2005). Furthermore, most insects are small ectotherms, meaning their body temperatures are primarily driven by environmental temperatures; thus, even small changes in the external environment may lead to large changes in rate of metabolic processes and stability of cellular structures (Hochachka and Somero 2002, Willmer *et al.* 2009). *Wolbachia* endosymbionts that live inside insects in montane environments, where the mountainous geography limits insect movement away from thermally stressful environments, may be especially important

for modulating the insect host response to physiological stress caused by environmental thermal variation. One such montane insect is the willow leaf beetle *Chrysomela aeneicollis*. This leaf beetle lives on willows that grow in discrete patches in moist habitats across Western North America (Brown 1956, Dellicour *et al.* 2014, Smiley and Rank 1991). In the Sierra Nevada mountains of California, beetles are restricted to habitats between 2400 and 3600 meters in elevation (Rank 1992, Dahlhoff and Rank 2000, Dahlhoff *et al.* 2008), where beetles experience both high daytime (>32°C) and low nighttime (< -5°C) temperatures during the summer growing season (Rank and Dahlhoff 2002). Prior studies of Sierra populations of these beetles have suggested that natural selection to temperature acts on metabolic genes such as *phosphoglucose isomerase* (*PGI*) (Dahlhoff *et al.* 2008, Neargarder *et al.* 2003). In general, beetles with *PGI* 1-1 genotypes recover better from a single thermal stress, whereas individuals with 4-4 genotypes are more tolerant of extreme or repeated stress. This effect may be influenced by differences in expression of a 70-kDa heat shock protein (Hsp70) between different *PGI* genotypes (Dahlhoff and Rank 2000, Rank and Dahlhoff 2002, Dahlhoff and Rank 2007). *Wolbachia*, by interacting with genes such as *PGI* could both alter its susceptibility to temperature extremes while also having an impact on the beetle's response to temperature stress.

Preliminary research indicates that *Wolbachia* is widely present in populations of Sierra willow beetles. Given that these beetles are confined in patches of isolated drainages separated by high-elevation ridges, their ability to escape environmental stress is limited; thus, changes in the thermal environment within each drainage could

alter beetle body temperature rapidly, which would affect both the physiology of both the beetle host and its *Wolbachia* endosymbiont. Furthermore, exposure to environmental stressors could upset the *Wolbachia*-host relationship as both struggle to cope with the stress. It is possible that *Wolbachia* is benign or even beneficial to its insect host under benign conditions, but is detrimental to the host under stressful conditions, where it acts to maximize its reproductive success at the expense of the host. To date, nothing is known about how stress affects this host-endosymbiont relationship in Sierra willow beetles.

With my project, I examined three aspects of the *Wolbachia-C. aeneicollis* relationship. First, I analyzed the beetle microbiome and determined the most common microbes. Next, I examined the evolutionary relationships between microbes living within willow beetles and other known insect symbionts. Finally, I assessed the role *Wolbachia* plays in how beetles respond to an environmentally realistic cold stress and determined if this effect depended on host genetic variation. To examine beetle (host) microbiome, I used the metagenomic application Metaphlan, a computational tool used for profiling the composition of microbe communities in organisms. To determine the number of *Wolbachia* strains infecting *C. aeneicollis*, I used Multi Locus Sequence Typing (MLST), which is based on five genetic loci known to have appropriate properties for use in distinguishing *Wolbachia* types (Baldo *et al.* 2006). To quantify the relationship between *Wolbachia* density and recovery from cold exposure, I performed quantitative PCR (qPCR) on beetles exposed to either control conditions or a single sub-lethal cold exposure. I used restriction digestion and SNP genotyping to determine the haplotype of

cytochrome oxidase II (*COII*) and genotype of *phosphoglucose isomerase* (*PGI*), two genes coding for proteins of central metabolism, for each of these beetles. Both *COII* and *PGI* were previously found to play an important role in beetle's response to thermal stress (Rank *et al.* 2007, Heidl 2013).

Methods

Analysis of beetle microbiome

Study populations used were based on prior work in Sierra Nevada populations of *Chrysomela aeneicollis* (e.g. Rank 1992; Dahlhoff and Rank 2000; Dahlhoff and Rank 2007). Samples collected from Big Pine Creek (BP) and Bishop Creek (BC) populations were used for genetic analysis (Table 1); specifics about these study sites are detailed in Roberts (2016). DNA was extracted following the methods in Roberts (2016). Extracted DNA was quantified using a Qubit Fluorimeter (Life Technologies, Grand Island, NY) with hsDNA kits (Thermofisher Scientific, Waltham, MA). Samples were combined at equimolar concentration (> 25 ng/µL) into seven genomic libraries. To construct each genomic library, 12 beetles from each site in BP and 50 from BC were used. Genomic data from four sites in BP were combined into one library; genomic data for the three sites in BC were kept as separate libraries. Each library contained greater than 3 µg total DNA. Library preparation (200-300 base pair insertion size), barcoding, and full genome sequencing (Illumina HiSeq2000, 100PE) was performed by The Beijing Genomics Institute (Sacramento, CA). The genome of *C. aeneicollis* is estimated to be around 600Mb; using 100PE HiSeq2000 sequencing, the coverage was expected to be roughly 50 times for the entire genome per lane. All sequencing was completed in six lanes,

which means that about one billion base pairs of data from 198 beetle genomes were analyzed.

After sequencing, several stages of file preparation were implemented before data analysis. PCR duplication removal was completed using clonefilter (Catchen *et al.* 2013). Adapters were removed in the forward and reverse direction using BBduk2, which was also used to remove low confidence base pairs with a phred quality score below 10. A *de novo* assembly was created (kmer = 64, bubble size = 2000) using pooled DNA sequence data (pool-seq data) from 50 individuals collected in 2011 from High Stream Crossing and assembled using CLC genomics workbench (CLC Bio-Qiagen, Aarhus, Denmark). Using the pool-seq data, a metagenomics computational tool, Metaphlan2 (metagenomic phylogenetic analysis, Huttenhower lab, Harvard University) was used to obtain preliminary data for all species of endosymbionts living within *C. aeneicollis*. Metaphlan2 is used to examine genomics data from microbe and viral organisms from pool-seq files and refers to its bank of 1 million clade-specific marker genes to identify microbes at the species level (Segata *et al.* 2012). Excel tables detailing microbial abundance from these two drainages were assembled and average *Wolbachia* abundance from each drainage was determined.

Identification of *Wolbachia* strains

First instar larval families were collected on 28 June 2014 near Bluff Lake in southern Bishop Creek (37° 10' 36.7" N; 118° 32' 58.0" W; 3200 m elevation) and reared in the laboratory of University of California's White Mountain Research Center (WMRC; Bishop, CA). At the 18th day of development, third instar larvae, one per family, were

flash frozen on dry ice, immediately transported to the laboratory at Sonoma State University and held at -80 °C until analysis. DNA from one larva per family was extracted from abdomen following the "genomic DNA from tissue" protocol in the Nucleospin Tissue Kit (E&K Scientific, Santa Clara, CA, USA).

To identify *Wolbachia* strains from these DNA extractions, the multilocus sequence typing (MLST) method was used. Multilocus sequence typing is a system that uses the sequences of five conserved genes to genotype *Wolbachia* strains. These five genes are known to be housekeeping genes with strong stabilizing selection within *Wolbachia* and are used to indicate the identity of the *Wolbachia* strain. For each gene, each sequence is assigned a unique allele number. For sequence typing, each strain is characterized by a unique combination of five integers, with the specific allele combination defining its allelic profile. Each allelic profile is then identified as a sequence type, and a number assigned to it (Baldo *et al.* 2006).

To characterize presence of *Wolbachia* infection by *Wolbachia* from the A and B supergroups, PCR was performed on five MLST genes: *coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpA* using an automated PCR machine (Applied Biosystems 2720 Thermocycler). Both forward and reverse strands were sequenced for each sample using degenerate primers (see Table 2) for *Wolbachia A* (WolA) and *Wolbachia B* (WolB) supergroups. After confirming that WolA and WolB strains were present, species-specific, gene specific primers were designed using contigs determined through pool-seq analysis as detailed above (Table 2). Sequences were manually curated using Geneious R9 (www.geneious.com) to obtain a consensus sequence between forward and reverse

sequences. Double nucleotide peaks were found along the sequences of *hcpA* and *ftsZ* of WolA and none in WolB. All curated sequences were compared to the PubMLST database (https://pubmlst.org/*Wolbachia*/). New alleles discovered in this study were curated and added to the PubMLST database.

Phylogenetic relationships to known *Wolbachia* strains

The same DNA sequences used in the MLST analysis were also used to construct a phylogeny of *Wolbachia* strains. Two types of phylogenetic trees were made for the analysis presented here- one based on individual MLST genes (individual gene tree) and another based on a combination of all five MLST genes (composite gene tree). Both types of trees were made following protocols detailed in (Duplouy and Brattstrom 2017), with some changes in the compilation of DNA sequences used to make the composite tree. For each individual gene tree, at each gene where there were different alleles, a new tree was constructed for each allele to determine whether the alleles produce different topologies. For the composite gene tree, all DNA sequences were combined for all five MLST genes into one sequence. The order of the gene sequences remained constant for all *Wolbachia* taxa to ensure a correct phylogenetic tree was made. A phylogenetic tree making website (Phylogeny.fr) was then used to make all phylogenetic trees. The one-click mode was used with default settings for all trees. One-click mode builds a maximum likelihood tree by using PhyML with MUSCLE to align inputted sequences. All five MLST gene markers were used to build the composite *Wolbachia* tree. Similarly, MLST sequences from six additional *Wolbachia* strains previously determined to belong to the A, B, and D *Wolbachia* supergroups were used

to determine the validity of MLST results obtained previously. *Wolbachia* from nematode *Brugia malayi w*Bm was added as an outgroup for all phylogenetic trees.

Genetic and environmental effects on *Wolbachia* density and beetle performance

Beetle collection and experimental procedure- *Wolbachia* infection levels of adult Bishop Creek beetles were measured after exposure to cold stress. Protocol for the cold exposure experiment followed methods detailed in Rank *et al.* (2007). Briefly, adult beetles were collected from Bluff Lake in Bishop Creek in June 2013 and held in temperature-controlled incubators at WMRC (20°C, 14h d; 4°C, 10h d). Beetle running speeds were measured 24 hours after collection. Beetles were randomly assigned to either a cold exposure group or control group. Exposure experiments were conducted at night (2:00 a.m. – 6:00 a.m.) to mimic timing of cold exposure in nature. Beetles were subjected to minus 6°C cold stress for 4h. This temperature is slightly warmer than LT_{50}, so it was expected to induce non-lethal stress (Boychuk *et al.* 2015). Running speeds were measured at 20°C one hour after completion of the cold exposure treatment. After measuring running speed, beetles were weighed, flash frozen on dry ice and stored in an Ultracold freezer (-80 °C) until analysis. For this study, 64 individuals were randomly selected from the original sample of 304 experimental beetles.

DNA Extraction-DNA was extracted following methods of Roberts (2016). Briefly, beetles were dipped in liquid nitrogen and crushed with glass pestles (Kontes Duall #20, DWK Life Sciences, Millville, New Jersey) in 1.5ml microcentrifudge tubes (Thermofisher Scientific, Waltham, MA). After pulverization, 180 µL of buffer ATL (Qiagen, Hilden, Germany), 20 µL of Proteinase K (Qiagen, Hilden, Germany) and 4 µL of RNase A

(Thermofisher Scientific, Waltham, MA) were added to each tube; vortexed briefly (Vortex Genie 2 VMR) and left to incubate overnight at 56°C in a digital dry bath incubator (Boekel Scientific, Feasterville-Trevose, PA). The following day all tubes were inverted three times and DNA was extracted as per manufacturer instructions in the DNeasy Blood & Tissue Handbook (Qiagen, Hilden, Germany).

Determination of Cytochrome Oxidase II (COII) haplotype- A polymerase chain reaction (PCR) was performed using custom designed primers (Table 3) to amplify a 650 base pair region of the mitochondrial gene *cytochrome oxidase II* (*COII*). PCR reaction temperatures were as follows: 95 °C for 2 min, 34 cycles of 95°C for 30 sec, 51°C for 1 min, 72°C for 1 min followed by 72°C for 7 min. Each reaction contained: 22.2 µL H_2O, 8 µL 5X GoTaq Green Buffer (Promega, Madison, WI), 3.2 µL 2.5mM dNTP, 2.4 µL 25mM $MgCl_2$, 1 µL forward/reverse primer and 0.2 units of Taq Polymerase (New England Biolabs, Ipswich, MA). Each reaction also contained 2 µL of DNA template. PCR reactions were verified on a 1% agarose gel to check for presence of the amplified product. Post verification, restriction digest reactions were run for each sample: one sample of digestion mix contained: 2.25 µL of D.I. water, 1.5 µL of Buffer 1.1 (New England Biolabs, Ipswich, MA), 0.5 µL of BSA (10mg/ml) (New England Biolabs, Ipswich, MA), 0.25 µL AIu1, 0.25 µL Sau3AI to distinguish between mitochondrial haplotypes.

Determination of Phosphoglucose isomerase (PGI) genotype- To determine SNP variation at the *phosphoglucose isomerase* gene, a single non-synonymous substitution in the coding region of the gene was selected. For *PGI* amplification, Custom TaqMan Genotyping Assay Non-Human Small Scale 40X Concentration was used. The assay was

diluted to a working 20X concentration with 1X TE buffer, pH 8.0 (Thermo Fisher Scientific, see Table 3 for DNA template). For reaction mastermix, TaqMan Genotyping MasterMix was used (Thermofisher Scientific, Waltham, MA). For this analysis individuals from the cold-stress experiment were genotyped using 12.5 µL of TaqMan Genotyping MasterMix (Thermofisher Scientific, Waltham, MA), 1.25 µL of Custom TaqMan Genotyping Assay (Thermofisher Scientific, Waltham, MA), 1 µL of DNA and 10.25 µL of Nuclease-free water (Qiagen, Hilden, Germany) for a total reaction volume of 25 µL.

To amplify DNA, the standard reaction module on 7900HT Fast Real-Time PCR system as used, using a MicroAmp Optical 96well Reaction Plate with Barcode (Applied Biosystems, Foster City, CA). PCR amplification was as follows: 10 min of hotstart at 95°C, 3 min of denaturation at 95°C, 1 min of annealing/elongation at 60°C. For *PGI* allele identification, the protocol on Allelic Discrimination Getting Started Guide for 7900HT Fast Real-Time PCR system was followed. In order to equate *PGI* genotypes (GG, AG, AA) with published electromorphs from allozyme electrophoresis (1-1, 1-4, 4-4; see Rank 1992), 25 individuals with known electromorphs were SNP genotyped. This verified that the 1-1 electromorph matched with the PGI genotype GG, the 1-4 electromorph matched with the PGI genotype AG and the 4-4 electromorph matched with the PGI genotype AA.

Quantification of *Wolbachia* density- To examine differences in *Wolbachia* gene expression between stress treatment groups, quantitative PCR was performed to characterize densities of *Wolbachia* A and *Wolbachia* B. For genes of interest, the MLST

gene with the lowest nucleotide variation was selected. I selected a 100 base pair region of *hcpA* where there was only one SNP in the middle of the amplicon. Primers for qPCR were designed using PrimerQuest tool under Gene Expression tab from the Integrated DNA Technologies website (Integrated DNA Technologies, Coralville, IA). The sequence of primers used is shown in Table 3.

All qPCR reactions were performed using 7900HT Fast Real-Time PCR System with standard block module (Thermofisher Scientific, Waltham, MA). Initial primer efficiency tests were performed for two genes of interest and three reference genes were within the 90 - 110% primer efficiency range (see Table 4) at 62°C. qPCR conditions were as follows: 3 min step at 95°C, 40 cycles of 15 sec at 95°C, and 30 sec at 62°C. A melting curve analysis was conducted at the end of the primer efficiency test to check for primer dimers and nonspecific amplifications. During experimental runs of qPCR, all five genes were run on the same microamp optical 96 well reaction plate with barcode sealed with MicroAmp™ Optical Adhesive Film (Thermofisher Scientific, Waltham, MA), along with a plate control. Samples were run in triplicates to reduce measurement error. Densities of WolA and WolB were measured using C_t value of the *hcpA* gene. The C_t value, or value of cycle threshold, is defined as number of cycles required for fluorescent signal to cross a detection threshold above background fluorescence. A low C_t value for a gene means that the number of cycles it takes for the fluorescence to cross the threshold level is lower and there are more copies of that gene in the sample, while higher values indicate the presence of less DNA in the sample. C_t values for *hcpA* gene for both *Wolbachia* were measured. Reference genes, whose expression level does

not change with experimental treatment, were also measured (ribosomal protein S18 [*Rps18*], ribosomal protein S3 [*Rps3*] and cytochrome oxidase I [*COI*]). These housekeeping genes served to normalize gene expression results of genes of interest. *Rps18* and *Rps3* were used as reference genes due to these genes being stably expressed in all cells (Zhong *et al.* 2013). *COI* was chosen because it was the reference gene in a study that used quantitative PCR for the identification of *Wolbachia* (Sumi *et al.* 2017). However, the amount of *COI* depends partly on the number of mitochondria in the cell, which can be environment-dependent. Therefore, to normalize *hcpA* C_t values the geometric mean of the nuclear genes *Rps18* and *Rps3* were calculated without including *COI*.

Each 96 well plate included a plate control that serves to normalize each plate. The difference between each plate and the first plate was found by dividing each subsequent plate by the first plate. For each plate, the C_t values of all genes tested (*hcpA*, *Rps18*, *Rps3*, *COI*) were then divided by that number to obtain plate normalized C_t values. The average of each gene was then multiplied by efficiency of their respective primers. Final C_t values were corrected for primer efficiency and normalized to reference genes using the equation:

$$\Delta C_t \textit{ Wolbachia}_{\text{normalized}} = C_t \textit{ Wolbachia}_{\text{P.E.corrected}} - C_{t\text{geometric mean(Rps18, Rps3)}}$$

Preliminary results indicated that the error variable from the ΔC_t analysis was not normally distributed. Thus, ΔC_t values were Box-Cox transformed before final statistical analysis. This transformation requires that values be greater than zero; therefore ΔC_t values were adjusted to positive numbers by subtracting the column

minimum from each value and adding 0.00000001 to it. Finally, to graphically represent *Wolbachia* density values on a natural rather than inverted scale (because ΔC_t is inversely related to the amount of DNA), Box-Cox transformed values were subtracted from the integer 10 for graphical display (Figs 4, 5).

Statistical analyses- A mixed model analysis was conducted to analyze Box-Cox transformed ΔC_t values. "Beetle" was specified as a random effect in a mixed models analysis that tested for differences in *Wolbachia* A and B level, which allows for experimental effects to be tested over error terms with the appropriate degrees of freedom. For some analyses, multiple treatments were applied to each beetle and these included nested within-subjects factors (Moser 2004). To identify the best model, the best covariance structure was determined by comparing Akaike information criterion (AIC) values for typical covariance structures: unstructured, autoregressive, and compound symmetry (with or without heterogeneous variances). The co-variance structure with the lowest AIC value was selected before comparing the 'full' saturated model with all main effects and interaction terms to reduced models using the maximum likelihood option (ML) in SAS version 9.2 (Littell *et al.* 1998; Kincaid 2005). The model with lowest AIC value was selected for final analysis and run using the restricted maximum likelihood option (REML in SAS) to test fixed effects and interactions.

Running speed before and after cold stress-To analyze relationships between *Wolbachia* level and performance, analyses of covariance that included the grouping factors of *PGI* genotype, *COII* haplotype, and beetle sex along with Box-Cox transformed *Wolbachia* density were conducted. To analyze interactions between these factors and cold

exposure, we added exposure treatment as a grouping factor and used the difference between running speed before and after the exposure treatment as the dependent variable. Preliminary analyses indicated that the error variable for the difference in running speed was not normally distributed; thus, a Box-Cox transformation [$(\Delta Runspeed^{1.819} - 1) / 1.82$] was conducted before analysis. For these analyses, general linear models were used because only one *Wolbachia* density value per beetle was used. The best model was identified by comparing Akaike information criterion (AIC) values between models with the fewest possible interaction terms. Post cold exposure running speed values were not normally distributed so were Box-Cox transformed. To analyze whether *Wolbachia* and other main effects influenced beetle's post cold exposure running speed, analysis of covariance models were used. The best model was identified by comparing AIC values among models.

Results

Analysis of beetle microbiome

Data from Metaphlan analysis of pool-seq population samples indicates that *Wolbachia* was the most important microbe in all sampled populations. Over 99 percent of microbial sequences detected in beetles from both Bishop Creek and Big Pine Creek were identified as *Wolbachia* (Table 1).

Identification of *Wolbachia* strains

Two *Wolbachia* supergroup B (WolB) alleles in *coxA* and *gatB* were found that did not previously exist in the MLST database (https://pubmlst.org/*Wolbachia*/), whereas the sequence of the other three genes (*hcpA*, *fbpA* and *ftsZ*) matched existing MLST profiles.

With respect to *Wolbachia* supergroup A (WolA), two new WolA alleles in *ftsZ* were found, with the remaining four genes matching existing MLST profiles. All new alleles were added to the MLST database as part of this study.

Number of *Wolbachia* strains inside each beetle

Amplification of five MLST genes using primers specific for WolA and WolB resulted in distinct, solid bands for each individual beetle on electrophoresis gels, which suggests that each beetle was infected by *Wolbachia* from supergroups A and supergroup B (Fig 1). When examining the DNA sequences of each MLST gene from each beetle sampled, the chromatograms revealed presence of double peaks within WolA genes *hcpA* and *ftsZ*. This indicates that two to four strains of *Wolbachia* A could possibly be present in each beetle.

Phylogenetic relationships to known *Wolbachia* strains

Results of the phylogenetic analysis revealed that *Wolbachia* strains in *C. aeneicollis* are genetically distinct from previously investigated strains. *C. aeneicollis*-*WolA* strain is a sister group to other known *Wolbachia* A strains, and all four taxa form a monophyletic group with strong bootstrap support (Fig 2). Comparison to known *Wolbachia* group A strains in the MLST database revealed that the closest known relative to the WolA strain in *C. aeneicollis* occurs in the European leaf beetle *Oreina cacaliae* (Montagna *et al.* 2014). The phylogenetic tree shows that the WolB strain in *C. aeneicollis* also forms a monophyletic group with known strains in *Wolbachia* B, but the strain in *C. aeneicollis* is also distinct from them with a high level of bootstrap support (Fig 2). Comparison to known *Wolbachia* group B strains revealed that the closest known relative to WolB is

found in the parasitoid wasp *Leptopilina clavipes* (Pannebakker *et al.* 2004). The phylogenetic trees for each individual MLST locus show substantial similarity to the composite tree. The topology of the *Wolbachia* B lineage is identical for each gene tree and each tree shows that the WolB found in *C. aeneicollis* is a sister group to the known *Wolbachia* B strains. The branching patterns for *C.aeneicollis*-*WolA* were less consistent, though they did all provide support for the monophyly of *Wolbachia* A strains (Figs 2 and 3). For *fbpA* and *ftsZ*, *C.aeneicollis*-*WolA* is nested as a sister taxon to the other known supergroup *Wolbachia* A taxa, while in the trees for *coxA*, *gatB* and *hcpA*, one of the known *Wolbachia* taxa is a sister group to *C.aeneicollis*-*WolA* and the other two known *Wolbachia* taxa (Fig. 3).

Experimental and genetic factors influencing *Wolbachia* density and beetle performance

Factors influencing Wolbachia density- The cold exposure experiment suggested that both genetic and environmental factors influenced *Wolbachia* density. The statistical model that yielded the lowest AIC indicated that *Wolbachia* type (WolA vs WolB), mitochondrial haplotype, *PGI* genotype, and exposure temperature all related to *Wolbachia* density, and there were several significant interactions among these factors (Table 5). *Wolbachia* density was greater with increasing values of *COI* ΔC_t, but this positive relationship depended on temperature and sex (Table 5). Specifically, in females, higher *Wolbachia* density was associated with higher *COI* ΔC_t values, but in males there was no relationship between *COI* ΔC_t and *Wolbachia* density in the cold exposure treatment (data not shown).

Individuals with *COII-3* (Southern) mitochondria had higher *Wolbachia* densities than those with *COII*-1 (Northern) mitochondria (Fig. 4) and WolB density was higher than WolA density (Fig 4; Table 5). The effect of cold exposure on *Wolbachia* density differed among mitochondrial *COII* haplotypes (Table 5). Individuals with the *COII*-1 haplotype showed little difference between the cold exposure treatment and the control, but densities of WolA and WolB in *COII*-3 beetles were lower in the cold exposure group than the controls (Fig 4). *Wolbachia* densities were also related to *PGI* genotype. Individuals with the Northern (GG) genotype had the highest *Wolbachia* densities, while individuals with the AA genotype had the lowest densities (Fig 5; Table 5). The relative position of the AG heterozygote differed between the sexes. For AG males, *Wolbachia* density was intermediate between the homozygotes, a pattern consistent with incomplete dominance. In contrast, for females, AG heterozygote values were closer to one of the homozygotes, suggesting that either *PGI* allele may be dominant with respect to *Wolbachia* density in females (Fig 5).

Factors influencing running speed after field collection- *PGI* genotype was related to running speed after field collection. Females with the Southern (AA) genotype had the fastest running speed, and those with the GG genotype the slowest. For males, the opposite is true; GG males were fastest, AA males slowest (Fig. 6, Table 6). WolB density was also negatively related to pre-exposure running speed (Fig. 8A, Table 6).

Factors influencing running speed after cold exposure treatment- Beetles exposed to control treatment (4°C) increased their running speed between the initial and final measurement, while those exposed to a non-lethal, sub-zero temperature (-6°C) ran at

approximately the same speed (Fig 7). Beetles exposed to 4°C ran significantly faster than beetles exposed to -6°C (Table 6, Fig 7). Mitochondrial haplotype was related to recovery from cold exposure (Table 7). Within cold exposed beetles, only females with *COII*-1 (Northern) mitochondrial haplotypes increased their running speed between the initial and final measurement (Fig 7). In this analysis, pre-exposure running speed was negatively related to the change in running speed ($m = -0.206 \pm 0.066$, $n = 64$; Table 7; Fig 8A), and WolA density was negatively related to it (Table 7, Fig 8B). *PGI* genotype and WolB density did not relate to the change in running speed (Table 7). For beetles in both the control and cold exposure groups, there were more variability in WolA than WolB in terms of *Wolbachia*'s effects on beetles running speed (Fig 8).

Discussion

This study revealed that *Wolbachia* is an important part of the willow beetle microbiome that appears to influence beetle metabolism and response to thermal stress. Multiple strains of *Wolbachia* were found belonging to *Wolbachia* supergroup A (WolA) and one strain belonging to *Wolbachia* supergroup B (WolB); phylogenetic analysis further shows that the strains of *Wolbachia* identified in *Chrysomela aeneicollis* fall into known taxonomic groups and were sister groups to known *Wolbachia* strains. *Wolbachia* A and B densities related positively to mitochondrial concentration and recent thermal history. For all beetles sampled, *Wolbachia* densities related to *PGI* genotype (GG > AG > AA), but the relationship depended on sex and exposure history. Changes in *Wolbachia* densities post cold exposure varied depending on the mitochondrial haplotype of the host beetle. *PGI* genotypes were also found to relate

differently to beetle running speed before cold exposure. In addition, running speed after cold exposure differed among mitochondrial haplotypes and sex, a result that has been observed in prior experiments on Sierra willow beetles (Dahlhoff and Rank 2007, Heidl 2013). Finally, the two groups of *Wolbachia* (WolA and WolB) had distinct effects on running speed; WolB was negatively correlated with running speed before cold exposure, while WolA was negatively correlated with running speed after cold exposure. The results of this study suggest a link between *Wolbachia* density and host metabolic genes. It could be that *Wolbachia* may influence the beetle host response to thermal stress by interacting with host metabolism.

Within the *Wolbachia* that colonized Sierra willow beetles, *Wolbachia* A (WolA) was found to be closely related to *Wolbachia* infecting the European leaf beetle *Oreina cacaliae* and *Wolbachia* B (WolB) was most closely related to *Wolbachia* found in a parasitoid wasp *Leptopilina clavipes*. These results suggest that in Sierra willow beetles, WolA could be an older infection than WolB. Prior studies have found that bacterial microbiome communities are significantly more similar among closely related species in other groups of insects (Jones *et al.* 2013). Since WolA infecting the willow beetle was found to relate closer to WolA infecting the European leaf beetle, WolA could be a strain of *Wolbachia* that infects all leaf beetles in the family Chrysomelidae. In contrast, WolB is related to a strain of *Wolbachia* that infects a parasitoid wasp. This is because within the WolA genome, two MLST genes (*hcpA* and *fbpA*) were found to be polymorphic; in contrast, no polymorphic MLST genes were found in the WolB genome. Low sequence diversity across pathogen populations supports the idea that a single pathogen type

recently spread through host populations (Carpenter *et al.* 2007, Desprez-Loustau *et al.* 2007). These findings support the notion that WolB seems to be a younger infection than WolA in *C. aeneicollis*. One way to test this hypothesis would be to analyze *Wolbachia* in related willow beetles in other regions of the world. If *Wolbachia* in these beetles were found to relate to WolA and not WolB, then it would suggest WolA might be an infection native to willow beetles, and that WolB may be a *Wolbachia* that invaded willow beetle populations via horizontal transfer.

WolB was generally more abundant than WolA, and females had higher *Wolbachia* density than males. Prior research has found that *Wolbachia* is found in both insect reproductive and somatic tissues, and that *Wolbachia* density may vary between host tissues depending on the sex of the host (Liao *et al.* 2001, Zouache *et al.* 2009). Differences observed in *Wolbachia* density here may be caused by differential tissue infection, if say WolB infects primarily the reproductive tissue while WolA infects primarily somatic tissue. Given that there is more reproductive than somatic tissue in the abdomen, the result would be that more WolB is detected overall than WolA, especially in females which have the largest fraction of reproductive tissue. Another possibility is that WolB outcompetes WolA for host resources inside the cell. *Wolbachia*, with its reduced genome is highly dependent on host-derived metabolites (Moreira *et al.* 2009, Wu *et al.* 2004). In fruit flies and mosquitos, *Wolbachia* seems to successfully outcompete intracellular pathogens for vital nutritional resources such as cholesterol, which *Wolbachia* must obtain from the host (Caragata *et al.* 2013). The same

intracellular competition that exists between *Wolbachia* and other intracellular microbes might exist between different *Wolbachia* strains as well.

Both WolB and WolA were detected in every beetle sampled in this study. In prior research, multiple strains of *Wolbachia* are found to coexist within the same host individual (Sicard *et al.* 2014). Some *Wolbachia* are known to possess cytoplasmic incompatibility (CI) genes, which results in a delay in the nuclear envelope breakdown in sperm and egg during embryogenesis, causing unsuccessful development when females and males are infected with different CI-inducing *Wolbachia* strains (Tram and Sullivan 2002). Thus, when multiple CI-inducing *Wolbachia* strains are present in the same insect population, it would be most advantageous for *Wolbachia* if the host were infected by all strains. This way, multi-infected females can produce offspring with all males in the population (Sicard *et al.* 2014). Previous studies have also found that *Wolbachia* lacking the genes that cause CI may also persist in insect populations by having significant survival and fecundity effects on insect hosts (Fry *et al.* 2004). Within *C. aeneicollis*, multiple CI-inducing WolA strains were found, while WolB lacked CI genes. Given what is currently known about CI-inducing *Wolbachia*, it is likely that beetle tissues are also infected by multiple strains of WolA. WolB, lacking any CI genes, may persist in willow beetles by influencing the beetles' fecundity or survival, although this is currently unproven. However, one of the limitations of this study is that it cannot determine whether WolA and WolB infect the same tissues, so it is not known whether any direct interactions are occurring between WolA and WolB. More work is needed to fully

understand *Wolbachia* infection in the willow beetle and in particular, any direct interactions between WolA and WolB.

In this study, *Wolbachia* was found to be positively related to mitochondrial density, as measured by copy numbers of *cytochrome oxidase* subunit I gene (*COI*). Prior studies have found weak or no relationships between mitochondrial density and *Wolbachia* density (Ballard and Melvin 2007). Studies in the parasitic wasps *Leptopilina heteroma* and *Asobara tabida* have found that while mitochondrial density is higher than *Wolbachia* density in both males and females, *Wolbachia* does not seem to affect mitochondrial density (Mouton *et al.* 2009). Thus, the positive association between *Wolbachia* and mitochondria in willow beetles may be due to another factor, such as nutrition level. Because both *Wolbachia* and mitochondria reside in the cytoplasm, increased levels of nutrients could result in higher densities of both *Wolbachia* and mitochondria. Future experiments could focus on effects of different levels of nutrition on *Wolbachia* and mitochondrial densities to test this hypothesis.

Wolbachia densities were found to be lower in beetles exposed to a sub-zero cold treatment than for beetles in held under control conditions. Low temperatures may have killed off *Wolbachia*, alternately, low temperature may have caused a slowdown in *Wolbachia* metabolism, reducing its growth. As stated earlier, previous studies have found that while high temperatures actively kill off *Wolbachia*, low temperatures seem to only inhibit *Wolbachia* growth (Bordenstein 2011). As such, the effect of cold exposure on *Wolbachia* density may be due to increased *Wolbachia* density in the control group, rather than a decrease in the cold exposure group. Low temperatures

may slow down *Wolbachia* metabolism, resulting in a slower rate of *Wolbachia* replication. The results from this study corroborates with existing data from current literature and reinforces the idea that cold exposure seems to slow down, but not kill, *Wolbachia*. General differences in *Wolbachia* densities between beetles with different mitochondrial haplotypes hint at the presence of an interaction between *Wolbachia* and host mitochondria. Future experiments in Sierra willow beetles could examine the differences heat and cold stress may have on *Wolbachia* density to address this hypothesis.

Variation in host metabolic genes was found to relate to *Wolbachia* density post cold exposure. Beetles with the southern mitochondrial haplotype of the *cytochrome oxidase II* gene (*COII*-3) were more sensitive to cold exposure than beetles with the northern mitochondrial haplotype (*COII*-1). Furthermore, *Wolbachia* densities were higher in beetles with the northern *phosphoglucose isomerase* (*PGI*) genotype GG, than beetles with the southern *PGI* genotype AA. *Wolbachia* densities also differ between beetle mitochondrial and nuclear genotypes. Whereas *Wolbachia* density is higher in beetles with the southern mitochondrial haplotype than beetles with the northern mitochondrial haplotype, *Wolbachia* density is lower in beetles with the southern *PGI* genotype than beetles with the northern *PGI* genotype. Previous studies have found that beetles with the northern *PGI* genotype appeared to have a higher rate of survival than those with the southern *PGI* genotype after cold exposure; and that larvae with the southern mitochondrial haplotype ran faster post heat exposure than larvae with the northern mitochondrial haplotype (Dahlhoff *et al.* 2018, Dahlhoff and Rank 2007). As

such, different alleles of mitochondrial and nuclear genes seem to increase beetle resistance to thermal stress post exposure; and that *Wolbachia* may be benefiting from this effect. However, the exact relationship between *Wolbachia*, mitochondria and beetle nuclear genes remains unclear and is under active investigation.

In this study, different *PGI* genotypes related to beetle pre-cold exposure running speed differently, depending on sex. In males, beetles with the Southern (AA) *PGI* genotype ran the slowest and beetles with the Northern (GG) *PGI* genotype ran the fastest. In females, beetles with the AA *PGI* genotype ran the fastest and beetles with the GG *PGI* genotype ran the slowest. Previous studies have found that the GG genotype is better adapted to a single round of thermal exposure whereas the AA genotype is better adapted to repeated rounds of thermal exposure (Rank *et al.* 2007). Previous studies have also found the most common behaviors in females are sitting and feeding, whereas for in males it is walking (Rank *et al.* 2006). These behaviors suggest that females are focused on energy intensive, biosynthetic (anabolic) metabolic processes such as egg development and digesting large amounts of food, whereas and males are focused on locomotive (catabolic) metabolism- searching for mates. Thus, the stress resulting from differences in lifestyles between males and females could negatively affect beetle physiological performance in distinct ways. More studies are needed, however, to fully elucidate the reason why male and female beetles with different *PGI* genotypes exhibit different running speeds.

Mitochondrial haplotype was related to beetle running speed post cold exposure. After an episode of cold exposure, only female beetles with the *COII*-1

haplotype ran faster compared to all other cold exposed beetles. As such, beetles with the *COII*-1 haplotype seem to be, on average, more tolerant of low temperatures compared to beetles with the *COII*-3 haplotype. This is consistent with the data on the relationship between *Wolbachia* density and mitochondrial haplotype after cold exposure, where *Wolbachia* density was the same between cold exposure and control groups in beetles with *COII*-1 mitochondrial haplotype. These results suggest that the *COII*-1 haplotype seems to be more adapted to function at lower temperatures compared to *COII*-3. Unfortunately, the exact underlying mechanism of how *COII*-1 haplotype adapted to low temperatures remains unknown.

Wolbachia density is not only related to beetle metabolic genes, but is also negatively related to beetle running speed before and after cold exposure. *Wolbachia* is known to influence insect gene expression and physiological traits in many insects (Saridaki and Bourtzis 2010, Kageyama *et al.* 2017, Bi *et al.* 2018). As a bacterium living in beetle cells, both *Wolbachia* and the host may rely on similar resources to maintain cellular functions. This means that both *Wolbachia* and host cells may have to share the pool of available nutrients at certain times; thus, the more *Wolbachia* there are inside a cell, the less nutrients will be available for the host cells to use. Given that *Wolbachia* is the most abundant microbe within beetle cells, higher *Wolbachia* density means fewer nutrients available for beetle to use. Less available nutrients for host cells to use means weaker physiological performance, such as slower running speed or less robust stress response. In particular, WolA was found to negatively relate to beetle running speed post cold exposure. More work is still needed, however, to fully understand the

underlying mechanism of how *Wolbachia* seems to negatively influence beetle physiology, before and after cold exposure.

The results presented here demonstrate that *Wolbachia* seems to interact intimately with beetle metabolic genes and this may affect beetle physiology, before and after exposure to sub-zero temperature. Furthermore, the findings here suggest intracellular microbes may play a critical role in affecting aspects of insect physiology during episodes of thermal stress, and that these interactions may influence insect survival in nature. Future research may want to not only focus on examining the underlying mechanisms of how *Wolbachia* interacts with host nuclear and mitochondrial genes, but also on whether other intracellular microbes with known effects on host physiology, such as microbes in the *Spiroplasma* genus, exhibit similar interactions. Ultimately, a thorough understanding of the role intracellular microbes play in influencing the physiological response of a montane beetle during periods of thermal stress should shed light into how montane insects living in a geographical landscape with high barriers to dispersal might survive in an environment with increasingly severe temperature fluctuations.

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Table 1. Study populations and *Wolbachia* abundance. Each population in Bishop Creek consisted of a genetic library made from pooled DNA from multiple beetles, and the Big Pine Creek populations were pooled into a single genetic library.

Population	Latitude (37°N)	Longitude (118°W)	Elevation (meters)	*N*	*Wolbachia* abundance (%)
Bishop Creek					
High Stream Crossing	09' 33.8''	33' 46.5''	3205	50	99.85
Pipeline Site	10' 41.4''	33' 10.0''	3135	50	99.89
La Hupp	10' 53.7''	33' 29.2''	2870	50	99.93
Big Pine Creek					
Falls Site	07' 40.3''	28' 41.4''	2995	12	-
Black Lake	07' 56.4''	29' 27.1''	3197	12	-
Pond Bog	07' 22.9''	29' 36.7''	3125	12	-
Sam Mack Lake	07' 05.3''	30' 36.6''	3544	12	-
Pooled BP					99.83

Table 2. Sequences for primers used in MLST analysis. Degenerate primer sequences used can be found on the *Wolbachia* database (https://pubmlst.org/*Wolbachia*/) as well as Baldo *et al.* 2006. The letter "F" indicates the forward primer and the letter "R" indicates the reverse primer.

Primer Name	Sequence
coxA A Primer F	5’-CACATAGTTGCAATCCAG-3’
coxA A Primer R	5’-ATCACAGGTAATGTCCCA-3’
gatB A Primer F	5’-GCAAGACGCAGGAAAGAG-3’
gatB A Primer R	5’-CGGAAGTAATGATATTTGC-3’
hcpA A Primer F	5’-AATATGGCTGGTCATTCAC-3’
hcpA A Primer R	5’-CTCTTTATCGCTAATTTC-3’
fbpA A Primer F	5’-ACCCTGATGCTTATGAC-3’
fbpA A Primer R	5’-CCACCAGAGAAAACTACTAT-3’
ftsZ A Primer F	5’-CGACGCTCAAGCACTAGAAA-3’
ftsZ A Primer R	5’-CATCTACTTCTTCACGCACTCT-3’
fbpA B Primer F	5’-GGTGGAACTGGCAAGTTAGT-3’
fbpA B Primer R	5’-TTACCACCGCCTTGCTTAAT-3’

Table 3. Sequences for primers used in quantitative PCR analysis, *PGI* genotype and *COII* haplotype determination. Primers were named after gene and organism.

Primer Name	Sequence
hcpA WolA qPCR F	5'-AGGCCCATGACCTTCATATTG-3'
hcpA WolA qPCR R	5'-CGCCTTCGCTCTGCTATATT-3'
hcpA WolB qPCR F	5'-CGTCTTCGCTCTGCTATCTTT-3'
hcpA WolB qPCR R	5'-GGTCCGCAGCCTTCATATT-3'
RpS18 AEN qPCR F	5'-GCCCACAGAGGCATGAG-3'
RpS18 AEN qPCR R	5'-GACACACCCACAGTCCTAC-3'
Rps3 AEN qPCR F	5'-CCTACCTGACAATGTTGCTGTG-3'
Rps3 AEN qPCR R	5'-GGAATAGCCAAAGGTGCATCAA-3'
COI AEN Forward	5'-CATACGGCCTGAAGGGATAAA-3'
COI AEN Reverse	5'-GGTAGAGATAGCAGTAGCAGAATG-3'
PGI AEN Forward	5'-TGCTGTCTTGCACATAGCCC-3'
PGI AEN Reverse	5'-AGGGCTTCAGTGACCATTAGTG-3'
COII AEN Forward	5'-TACAGGATAGAGCCTCACCTT-3'
COII AEN Reverse	5'-TTTGGTTTAATCGACCTGGGG-3'

Table 4. Genes used in quantitative PCR analysis and primer efficiency at 61.5°C, the optimized temperature used for quantitative PCR experiments.

Gene of interest/ Temperature	60 ° C	**61.5 ° C**	62 ° C
hcpA WolA	330.74%	**100.41%**	86.7%
hcpA WolB	89.74%	**96.46%**	91.21%
Rps18	105.70%	**104.39%**	108.8%
Rps3	99.76%	**95.07%**	100.4%
COI	N/A	**92.87%**	101.38%

Table 5. Three-way analysis of co-variance for the relationship between *Wolbachia* density, *Wolbachia* type, *COII* haplotype, sex and *PGI* genotype. Significance of each interaction indicated as follows: + $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Source	DF_{num}	DF_{Den}	*F*
Categorical Factors			
COII Haplotype (H)	1	45	5.6^{*}
Sex (S)	1	45	7.4^{**}
PGI (G)	2	45	8.3^{***}
ExposureTemp (E)	1	45	2.8^{+}
*Wolbachia*Type (W)	1	55	181.2^{***}
H x E	1	45	6.6^{**}
H x S	1	45	4.6^{*}
H x G	2	45	0.55
H x W	1	55	2.7
S x E	1	45	3.7^{+}
G x S	2	45	2.4^{+}
E x W	1	55	0.36
S x W	1	55	6.3^{*}
H x E x S	1	45	1.5
H x E x W	1	55	0.7
H x S x W	1	55	0
E x W x S	1	55	0.5
E x W x S x H	1	55	0.1
Covariates			
Delta*COI* (D)	1	45	14.1^{****}
D x S	1	45	0.35
D x E	1	45	5.2^{*}
D x S x E	1	45	7.1^{**}

Table 6. Three-way analysis of co-variance for the relationship between pre-exposure running speed and WolA density, *PGI* genotype and sex. Symbols represent statistical significance as shown in Table 5.

Source	df	SS	*F*
Sex (S)	1	0.008	0.1
PGI Genotype (G)	2	0.1728	0.6
S x G	2	1.6005	5.1^{**}
DeltaCTWolA (A)	1	0.111	0.7
DeltaCtWolB (B)	1	0.6627	4.2^{*}
S x A	1	0.0669	0.4
S x A x G	2	0.8127	2.6^{+}
Error	52	8.166	

Table 7. Three-way analysis of co-variance for the relationship between change in running speed, experimental groupings, *COII* haplotype and sex. Symbols represent statistical significance as shown in Table 5.

Source	df	SS	*F*
COII haplotype (H)	1	0.0569	1.3
Treatment [°C] (T)	1	7.677	178.7****
Sex (S)	1	0.1993	4.6*
H*T	1	0.1375	3.2^{+}
H*S	1	0.3348	7.8**
T*S	1	0.0008	0
H*T*S	1	0.0039	0.1
pre exp Running Speed [cm/sec]	1	0.4238	9.9**
DeltaCTWolA	1	0.2412	5.6*
Error	53	2.276	

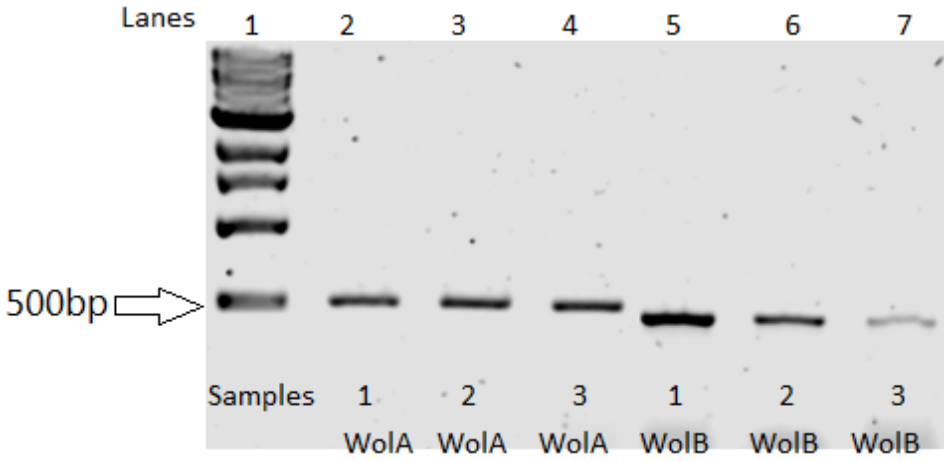

Fig 1. Sample agarose gel of PCR amplification of *gatB* gene for *Wolbachia* A and B. Lane 1 is 500 base pair ladder; PCR amplifications with degenerate primers for *Wolbachia* A are shown for 3 individuals on lanes 2-4, those for *Wolbachia* B on lanes 5-7. Sequences for degenerate primers are reported in Baldo *et al.* (2006).

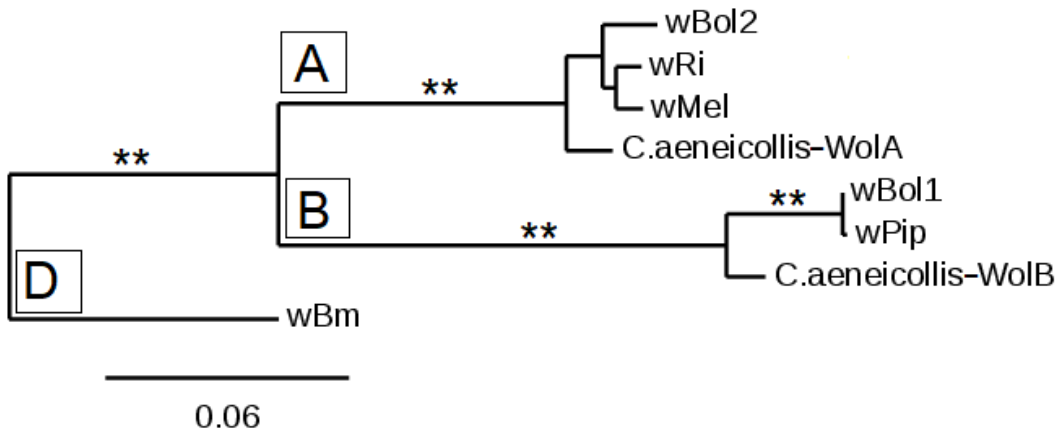

Fig 2. Phylogenetic relationships between *Wolbachia* strains found in *Chrysomela aeneicollis* and published reference strains. Reference strains belong to *Wolbachia* groups A, B, and D (each group identified on figure). Rooted composite phylogram based on combined DNA sequences from five *Wolbachia* Multi Locus Sequence Typing (MLST) genes, corrected by differences in sequence length, amplified from *C. aeneicollis* and reference strains (Duplouy 2017), with PhyML aLRT-based branch support values. The line at the bottom represents a genetic change of 0.06. Bootstrap values of 0.8-0.9 are indicated by one asterisk *, 0.9-1.0 by two asterisks **.

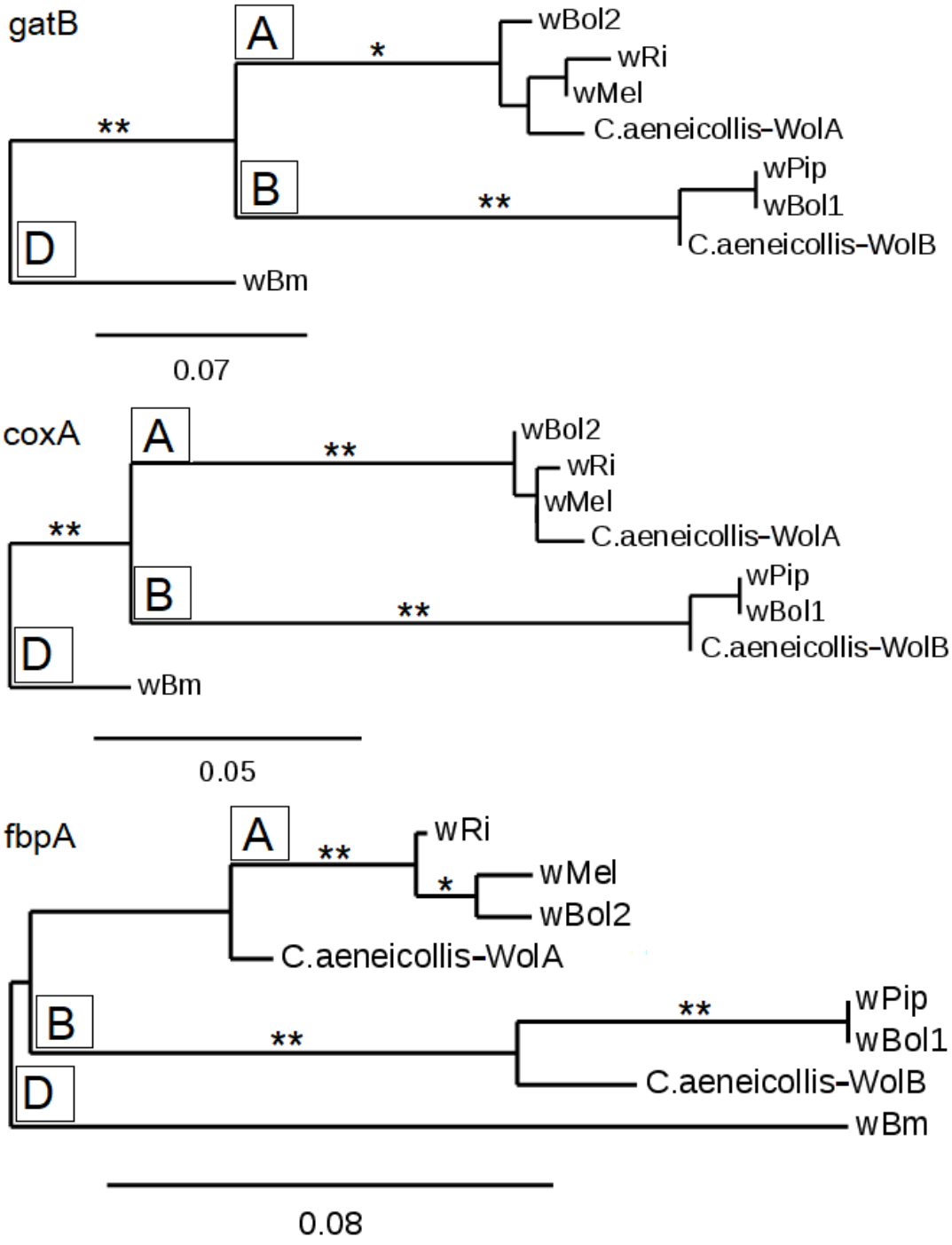

Fig 3A. Phylogenetic trees based on MLST genes. Based on *gatB*, *coxA* and *fbpA*. Topology of tree is same for *gatB* and *coxA*; *fbpA* has a different topology to all other MLST genes. Details of phylogenetic tree shown in Fig 2.

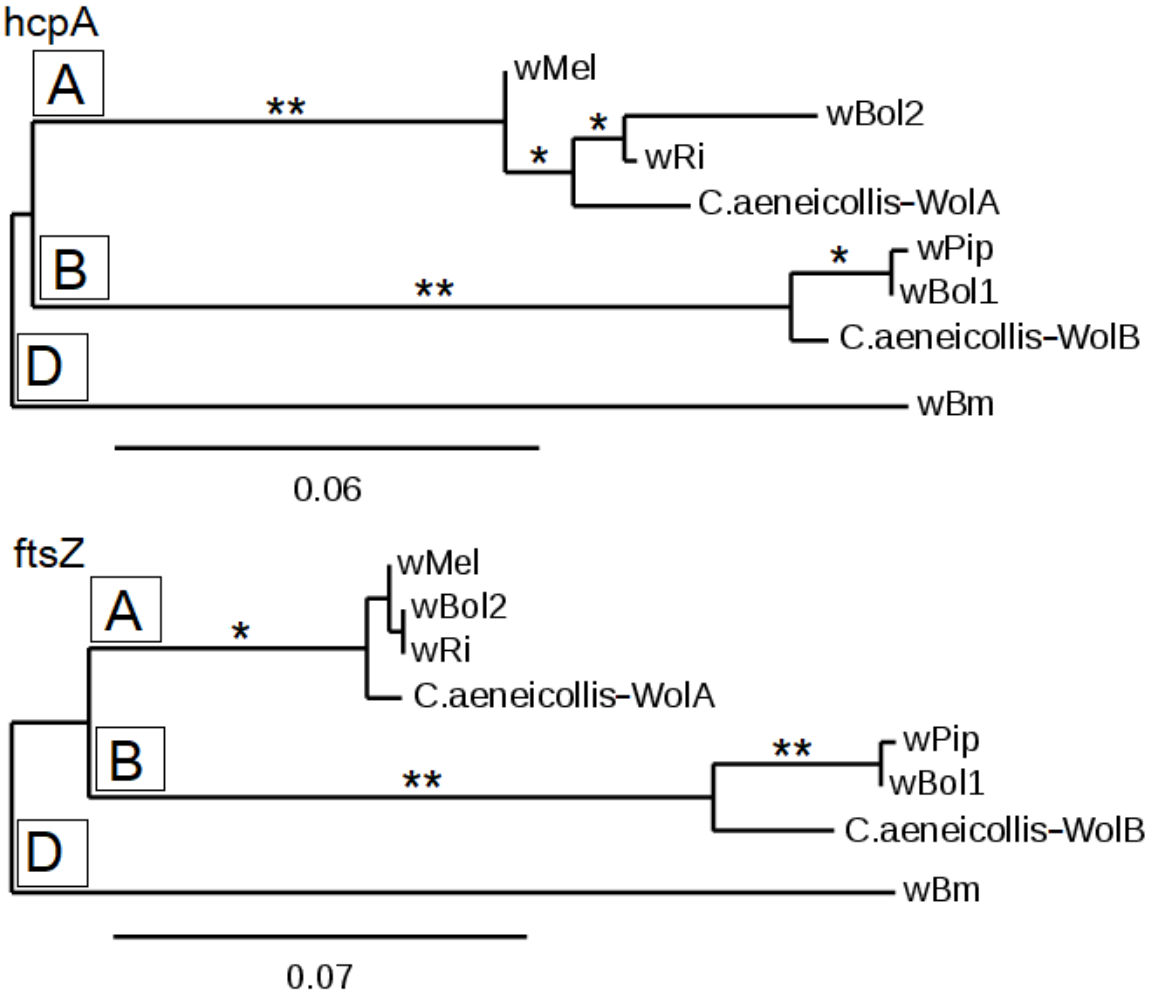

Fig 3B Phylogenetic trees based on MLST genes. Based on *hcpA* and *ftsZ* genes. Topology of tree is same for both genes. Details of phylogenetic tree shown in Fig 2.

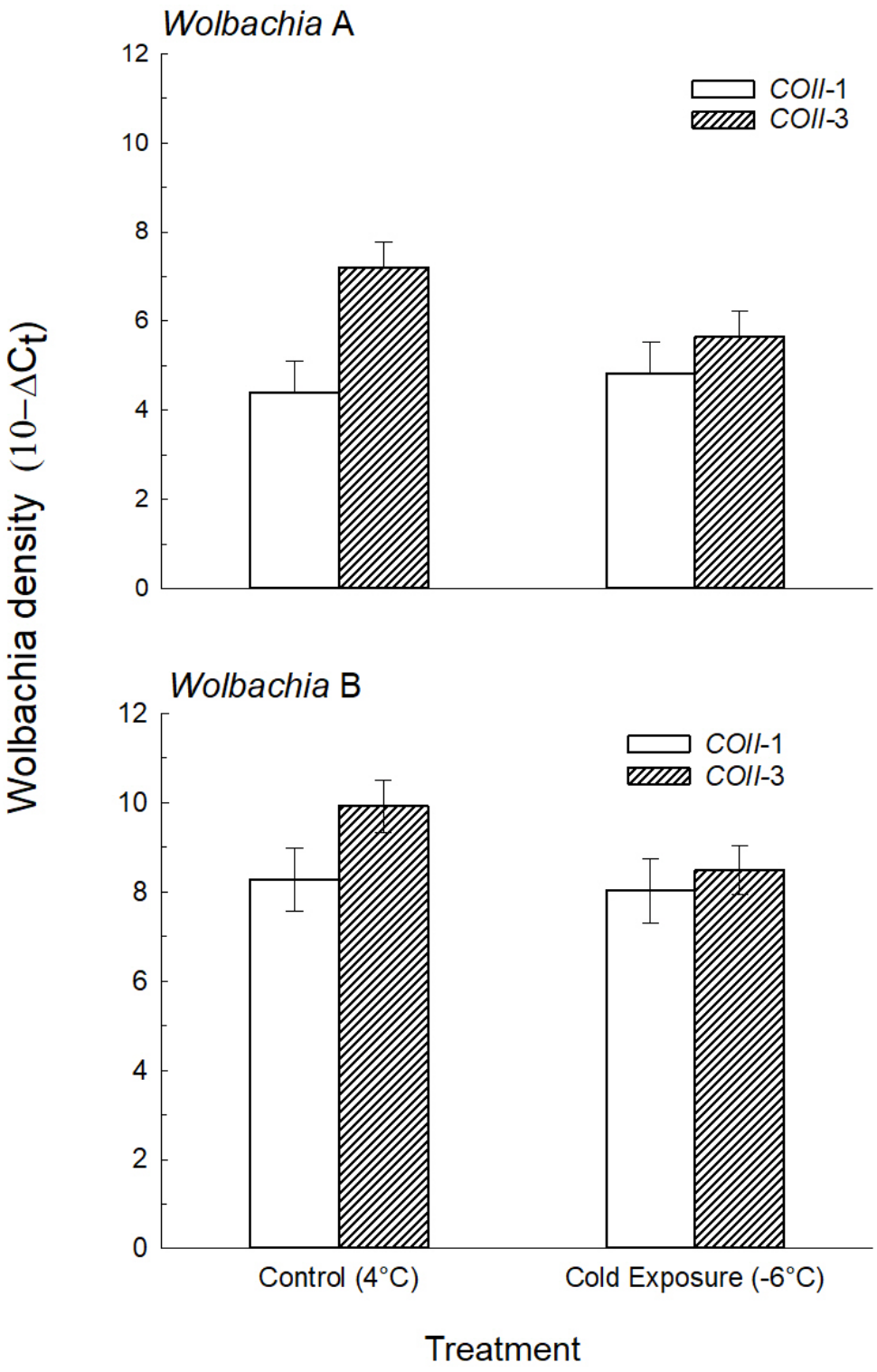

Fig. 4. Two *Wolbachia* strains differ in density in adult *Chrysomela aeneicollis* and are altered by exposure to stress. Data shown are *Wolbachia* density values for 64 beetles collected from Bishop Creek (see Table 1). *Wolbachia* density is reported as 10 minus Box-Cox transformed ΔC_t for clarity. Statistical analyses shown in Table 5.

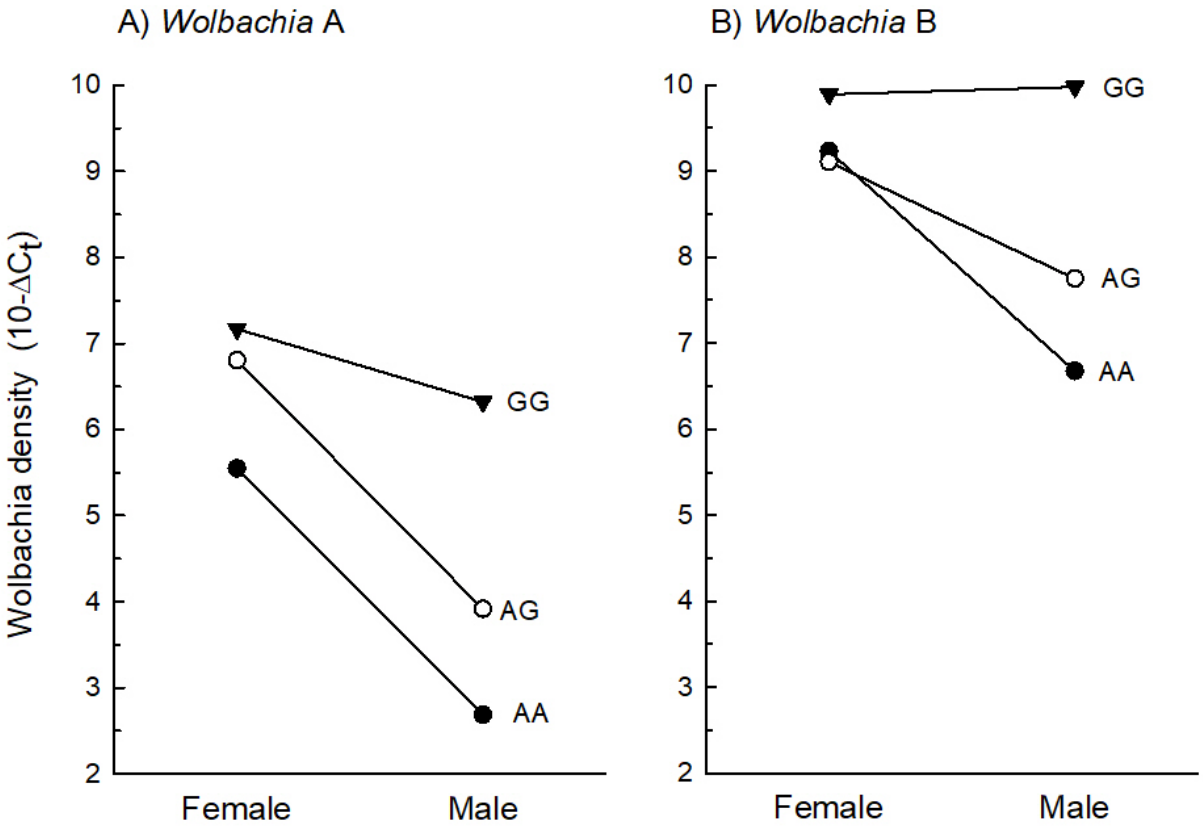

Fig 5. *Wolbachia* A and B densities differ among sexes and *PGI* genotype. Data shown are *Wolbachia* density values (reported as 10- Box-Cox transformed ΔC_t) for *N* = 64 beetles collected from Bishop Creek. Sample sizes are as follows: Females: AG = 18, AA = 2, GG = 13; Males: AA = 1, GG = 13, AG = 17. Additional statistical analysis shown in Table 6.

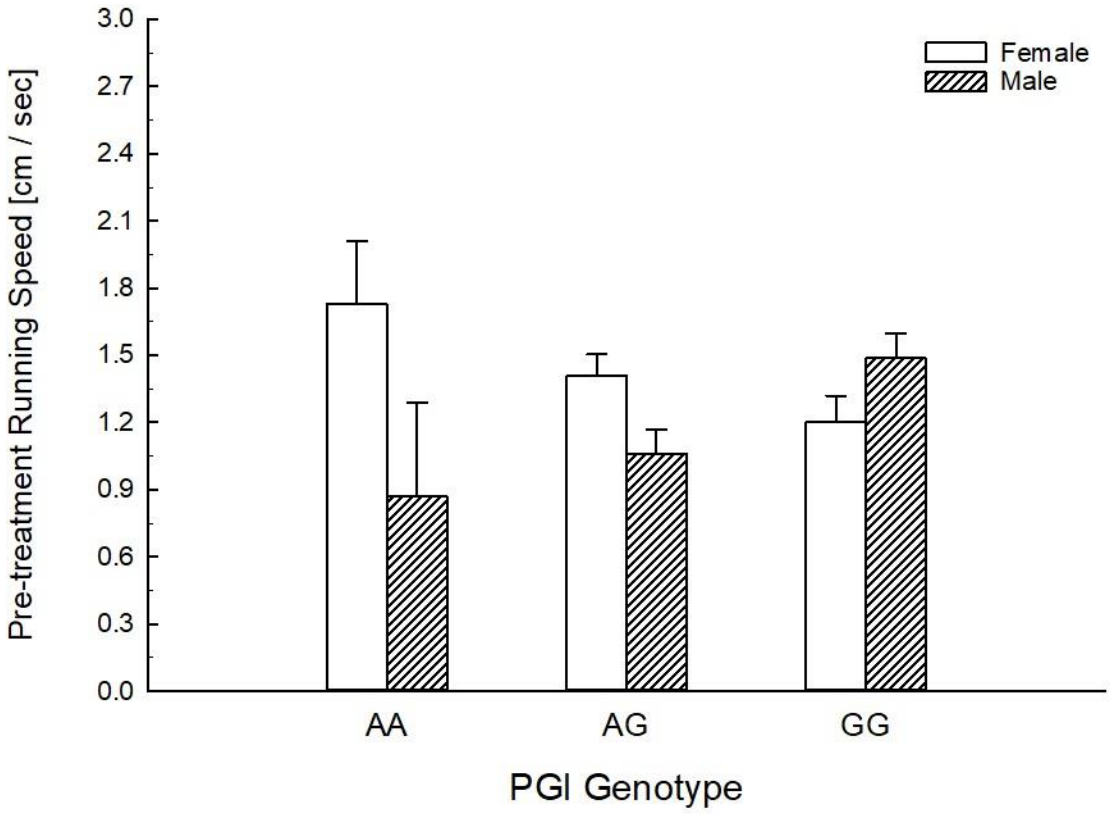

Fig 6. Female and male beetle running speeds differ before exposure to cold stress. Data shown are pre-treatment running speeds. Sample sizes as shown in Fig 5. Additional statistical analyses shown in Table 6.

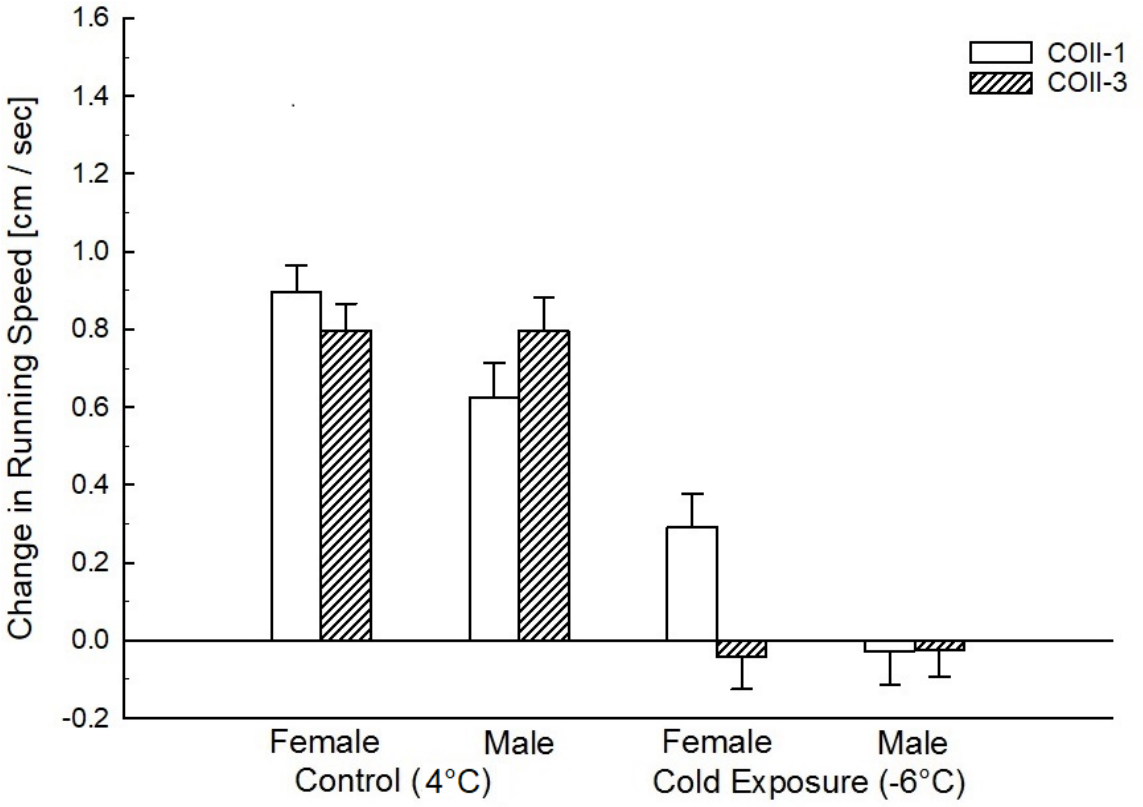

Fig 7. Females with Northern (*COII*-1) mitochondrial haplotypes ran faster after cold exposure than those with Southern (*COII*-3) haplotypes. Data shown are Box-Cox transformed mean post-treatment running speeds. Sample sizes as shown in Fig 5. Additional statistical analyses shown in Table 7.

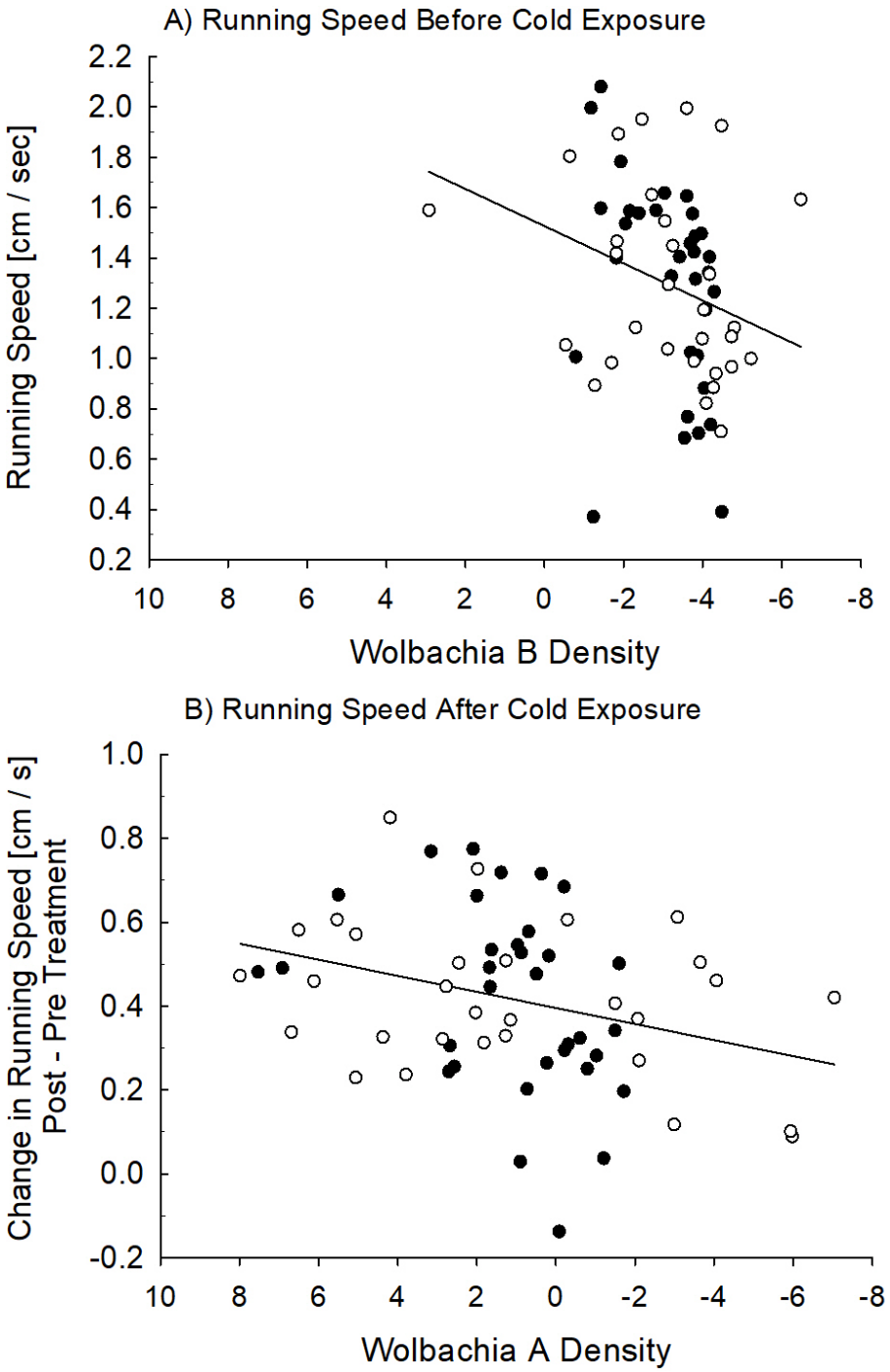

Fig 8. Relationships between *Wolbachia* A and B density and beetle running speed before and after stress. For *Wolbachia* B, *N* = 64, *P* = 0.04; for *Wolbachia* A, *N* =64, *P* = 0.02. Data shown are leverage plots of Box-Cox transformed running speeds as dependent variable adjusted for the contributions of other factors shown in Table 5 versus Box-Cox transformed *Wolbachia* densities (described in Fig 6 and 7). Females are represented by filled symbols, males by open symbols. Additional statistical analyses shown in Tables 6 and 7.