2018

Seasonal shifts in the insect gut microbiome are concurrent with changes in cold tolerance and immunity

Laura V. Ferguson
Western

Pranav Dhakal
York

Jacqueline E. Lebenzon
Western

David E. Heinrichs
The University of Western Ontario

Carol Bucking
York

See next page for additional authors

Seasonal shifts in the insect gut microbiome are concurrent with changes in cold tolerance and immunity

Laura V. Ferguson[1,4*], Pranav Dhakal[2], Jacqueline E. Lebenzon[1], David E. Heinrichs[3], Carol Bucking[2] & Brent J. Sinclair[1]

[1]Department of Biology, University of Western Ontario, London, ON, N6A 5B7, Canada

[2]Department of Biology, York University, Toronto, ON, M3J 1P3, Canada

[3]Department of Microbiology and Immunology, University of Western Ontario, London, ON, N6A 5B7, Canada

[4]Present Address: Department of Biology, Acadia University, Wolfville, NS, B4P 2R6

*Corresponding author: Department of Biology, Acadia University, Wolfville, NS, B4P 2R6
Email: lfergus9@uwo.ca; tel: 902-799-9726

Key words: bacteria, cold, cricket, symbiosis, winter

Abstract

1. Seasonal changes in the environment, such as varying temperature, have the potential to change the functional relationship between ectothermic animals, such as insects, and their microbiomes. Our objectives were to determine: a) whether seasonal changes in temperature shift the composition of the insect gut microbiome, and b) if changes in the microbiome are concomitant with changes in the physiology of the host, including the immune system and response to cold.
2. We exposed laboratory populations of the spring field cricket, *Gryllus veletis* (Orthoptera: Gryllidae), to simulated overwintering conditions in both a laboratory microcosm and a field-like microcosm containing soil and leaves. In summer, autumn, winter and spring, we extracted and sequenced 16S bacterial genomic DNA from cricket guts, to capture seasonal variation in the composition of the microbiome.
3. The composition of the gut microbiome was similar between microcosms, and overall highly anaerobic. In both microcosms, we captured similar seasonal variation in the composition of the microbiome, where overwintering resulted in permanent changes to these microbial communities. In particular, the abundance of *Pseudomonas* spp. decreased, and that of *Wolbachia* spp. increased, during overwintering.
4. Concurrent with overwintering changes in the gut microbiome, *G. veletis* acquire freeze-tolerance and immune function shifts temporarily, returning to summer levels of activity in the spring. Specifically, haemocyte concentrations increase but survival of fungal infection decreases in the winter, whereas the ability to clear bacteria from the haemolymph remains unchanged.

5. Overall, we demonstrate that the gut microbiome does shift seasonally, and in concert with other physiological changes. We hypothesize that these changes may be linked, and suggest that it will next be important to determine if these changes in the microbiome contribute to host overwintering success.

1 Introduction

Animal biology is shaped by interactions with symbiotic communities of microbes (the 'microbiome'), the large majority of which are housed in the gut (Douglas 2011, 2015; Engel & Moran 2013). The insect gut microbiome includes bacteria, archaea, yeasts and protozoa that may colonise the mucosa of the gut or exist transiently in the food as it passes through the digestive tract (Douglas 2015; Engel & Moran 2013). These microbes contribute to digestion of food, provide essential nutrients, protect the host from colonisation by pathogenic microbes, and communicate with the host through neuroendocrine signaling to regulate host physiology (Douglas 2015; Engel & Moran 2013; Shin *et al.* 2011). Changes in these communities of bacteria can affect a range of host phenotypes (Douglas 2011, 2015), thus it is important to explore how microbiomes may shift in response to changes in the environment. In particular, insects in temperate areas spend prolonged periods of time overwintering (Williams *et al.* 2015) which has the potential to influence the composition of the microbiome and its relationship with the host.

Because microbes are ectotherms, the microbiome of ectothermic animals will be exposed to the same temperature fluctuations as their hosts. These fluctuations have the potential to challenge individual microbe species, modify community interactions, and alter the functional host-symbiont relationship (i.e. the holobiont; Lokmer & Mathias Wegner 2015; Webster *et al.* 2008). Insects that overwinter in temperate environments are exposed to low temperatures for prolonged periods (Williams *et al.* 2015), and undergo profound seasonal changes in feeding (Hahn & Denlinger 2007), gut contents (Olsen & Duman 1997; Olsen *et al.* 1998), immunity (Ferguson & Sinclair 2017), and physiology (Denlinger & Lee 2010). Because the composition of the

67 microbiome depends on the physiological state of the host (Douglas 2015), these seasonal
68 changes in host physiology are also likely to influence the composition of the gut microbiome
69 (Carey & Duddleston 2014). Further, because the microbiome may differ depending on diet
70 (Franzini *et al.* 2016; Maes *et al.* 2016; Wang *et al.* 2011), seasonal changes in food or
71 microbiota in the external environment likely contribute to changes in the insect microbiome
72 (Ludvigsen *et al.* 2015). However, we know little about the seasonality of the insect gut
73 microbiome in the context of low temperatures and overwintering.
74
75 Overwintering influences the composition of the microbiome in other taxa, and these changes
76 can have important physiological consequences. For example, overwintering favours pathogens
77 in the gut microbiome of bullfrogs, *Rana catesbianae*, leading to systemic infection and
78 mortality (Carr *et al.* 1976). In this example, seasonal changes in the immune system likely work
79 in concert with dysbiosis and subsequent host mortality (Maniero & Carey 1997). Insects that
80 physiologically avoid freezing during the winter, such as *Dendroides canadensis*, actively
81 regulate the gut microbiome by voiding or masking bacteria that contribute to ice nucleation
82 (Olsen & Duman 1997). Further, *Drosophila melanogaster* that are reared at high temperatures
83 but receive transplants of gut bacteria from flies reared at low temperatures become more cold-
84 tolerant (Moghadam *et al.* 2017). Thus, we hypothesize that the composition and function of the
85 insect microbiome is related to the physiological processes that allow insects to survive multiple
86 overwintering pressures (e.g. cold and pathogens).
87
88 By understanding concurrent shifts in both the microbial community and host physiology (e.g.
89 immunity, cold tolerance), we can begin to understand the functional links between them, and

90 the potential consequences of climate change for host fitness. If the microbiome is directly
91 regulated by the external temperature, then the warmer or more variable winters predicted for
92 many temperate regions under climate change will modify the microbiome. By contrast, if the
93 microbiome is directly regulated by the host, the host will either continue to regulate the
94 microbiome and thus maintain performance under novel conditions, or the regulation of the
95 microbiome under novel conditions will impair their ability to shift physiology to suit new
96 environments. We hypothesise that the composition and function of the insect gut microbiome
97 could change during overwintering in four, non-mutually-exclusive, ways: 1) low temperatures
98 select for the growth of psychrophilic microbes that outcompete others; 2) cold directly kills
99 those microbes intolerant of low temperatures, thereby increasing the relative abundance of those
100 tolerant of cold; 3) seasonal changes in physiochemical conditions in the gut select for particular
101 microbes; or 4) the host actively manipulates the composition of the microbiome *via* the immune
102 system (Carey *et al.* 2013) or microRNAs (Hussain *et al.* 2011).
103
104 To explore how the microbiome contributes to host success in different seasons, we examined
105 concurrent changes in the composition of a gut microbiome with changes in host physiology
106 during different seasons. We exposed the overwintering stage of a temperate species of field
107 cricket native to Ontario, Canada, *Gryllus veletis,* to simulated overwintering conditions in either
108 a lab or field-like microcosm. During the overwintering period, we characterised the composition
109 of the gut microbiome. Further, we measured seasonal shifts in both immune activity and cold
110 tolerance. We show that the community of gut microbes changes with season, and that these
111 changes are conserved across both microcosms. Further, the microbiome does not reset in the
112 spring, suggesting that winter causes permanent perturbation or a plastic change in function of

the microbiome. In addition, immune activity decreases while cold tolerance increases in *G. veletis* during the overwintering period. Overall, concurrent changes in the composition of the microbiome and host physiology indicate that these changes may be interconnected, and that changes in the microbiome are likely linked to overwintering success.

2 Materials and Methods

2.1 Cricket housing and overwintering conditions

Gryllus veletis were derived from a population collected in Lethbridge, Alberta, Canada in 2010. In 2014-2015, we reared *G. veletis* from egg to 6th instar nymph at 25 °C (14 L:10 D) as described by Coello Alvarado (Coello Alvarado *et al.* 2015). Rearing conditions represented summer conditions. We maintained crickets in groups of approximately 100 individuals in plastic bins (28 × 17 × 15 cm) on *ad libitum* rabbit chow (Little Friends Rabbit Food, Martin Mills, Elmira, ON, Canada) and water with cardboard shelters.

To determine if microbes in the external environment influence the composition and seasonal plasticity of the microbiome, we divided crickets into two bins with the same food, water, and shelter conditions as during rearing (28 × 17 × 15 cm; n = 100 crickets in each bin): a field-like microcosm (FM) and a lab microcosm (LM). We exposed all crickets in a temperature-controlled incubator to a gradual, fluctuating decline in temperature and photoperiod to mimic autumn temperatures and photoperiods in London, ON, Canada until the temperature reached 0 °C (Fig. 1). To determine if changes in the microbiota of the external environment also influence any seasonal changes in the insect gut microbiome, we created both a lab and field-like microcosm.

At the beginning of autumn (i.e. directly after summer samples were collected), we introduced soil and humus collected from local areas where we had previously heard *G. veletis* males calling, thereby creating the field-like microcosm. To mimic the conditions under snow cover (where *G. veletis* nymphs overwinter), we maintained crickets in darkness at 0 °C and under darkness for four weeks. We then gradually increased temperature and photoperiod to mimic conditions in the spring (Fig. 1).

2.2 Gut dissection and DNA extraction

In summer, autumn, early winter, mid-winter and spring (21 – 26 d between sampling points; Fig. 1) we haphazardly selected ten crickets from each microcosm, surface-sterilised them with 70 % ethanol and removed the hindgut [as described by MacMillan and Sinclair (2011)] under sterile conditions in a laminar-flow clean bench. We immediately snap-froze samples in liquid nitrogen in sterile tubes, and stored them at -80 °C until DNA extraction. To choose a method of extraction based on suggestions by Hart et al. (2015), we initially extracted DNA from a test gut sample of *G. veletis* using a QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA), DNeasy blood and tissue kit (QIAGEN, Valencia, CA, USA), and QIAamp DNA Microbiome Kit (QIAGEN, Valencia, CA, USA), according to manufacturer's instructions. We achieved the highest OTU with the DNeasy blood and tissues kit, and proceeded with this extraction method for all samples. We pooled two guts for each replicate (n = 5 per time point, per microcosm) and extracted total bacterial genomic DNA (gDNA) using the DNeasy blood and tissue DNA extraction kit according to the manufacturer's instructions. We only used samples with a 260/280 ratio >2, and confirmed the presence of bacterial DNA using PCR with the universal bacterial DNA primers 27F BacU (AGRGTTTGATCMTGGCTCAG) and 519R BacU

(GTNTTACNGCGGCKGCTG; Integrated DNA Technologies, Coralville, Iowa, USA; Rogers *et al.* 2014).

2.3 DNA sequencing

We pooled extracted bacterial gDNA for each sampling point (n = 5 per microcosm) for a total of one sample per time point, per microcosm. A fragment of the Bacterial 16S rRNA gene, spanning the V1 – V2 hypervariable regions, was amplified by MR DNA (Shallowater, TX, USA 79363) using universal bacteria primers that were modified by adding ligation adaptors and barcodes (sample identification sequences) to the 5`- ends. MR DNA performed PCR using a high fidelity polymerase (HotStarTaq Plus Master Mix Kit, Qiagen, Valencia, CA, USA) and with the following conditions: 94ºC for 3 minutes, followed by 28 cycles of 94ºC for 30 seconds; 53 °C for 40 seconds and 72 °C for 1 minute; after which a final elongation step at 72 °C for 5 minutes was performed. MR DNA performed three PCR reactions per sample before pooling to reduce PCR amplification bias in the library preparation. MR DNA further examined PCR products in a 2% agarose gel to determine the success of amplification and the relative intensity of bands. MR DNA purified the amplicons using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA) and mixed amplicons in equal concentrations (following quantification via Qubit) before sequencing. The pooled samples were sequenced using Illumina MiSeq sequencing at 300 bp reads and >20,000 reads per sample following the manufacturer's protocol by Molecular Research LP (MR DNA, Shallowater, TX, USA 79363). The Truseq Illumina reagent kit (TruSeq Nano DNA LT; Illumina) was used for library preparation and sequencing.

We used Qiime software (Caporaso *et al.* 2010a) on the obtained raw sequences to filter and remove unique barcodes and primers, low quality reads (quality score <25 bp), short and long sequences (< 200 bp; >1000 bp), zero ambiguous base calls, gaps, zero primer sequence mismatch, and sequences with homopolymer runs exceeding 6bp. We checked the resulting filtered sequences for chimeras; subsequently removing them from the dataset using uchime [part of USEARCH v5.2.236; (Edgar *et al.* 2011)]. We then sorted the remaining filtered sequences into OTUs (Operational Taxonomical Units) using USEARCH at a 97 % threshold of sequence similarity through a open-reference OTU picking protocol (Edgar *et al.* 2011). As each OTU may consist of many related sequences, Qiime software was used to pick a representative consensus sequence from each OTU for taxonomic identification and phylogenetic alignment. Taxon identity (kingdom to species level) was assigned to the representative OTU sequences based on the curated GreenGenes database (v. 13.5; DeSantis *et al.* 2006) using the Uclust consensus taxonomy assigner (Edgar 2010). Following identity assignment, we removed all unassigned sequences (i.e. OTUs unidentifiable at the kingdom level) from the data before continuation with further downstream processing. Phylogenetic alignment of the representative OTU sequences was determined against existing alignments using PyNAST (Caporaso et al., 2010b) following filtering (to remove positions that are all gaps and those that are known to be highly variable). This alignment was used for subsequent UniFrac beta diversity measurements.

We examined alpha diversity, or diversity within each community or sample, using Qiime software to calculate Observed species (count of unique species), Chao1 (estimate of species richness), and Shannon Index (estimate of species richness and evenness) metrics for each sample (Caporaso *et al.* 2010a). Rarefaction curves (graphs of each diversity metric vs

sequencing depth) were then generated up to the minimal observed sequencing depth (58, 669 sequences).

We then examined the beta diversity (or diversity between communities or samples) by creating weighted (species abundance based) and unweighted (species identity based) Unifrac matrices, based on the normalized abundance data and phylogenetic alignment created by Qiime (Lozupone *et al.* 2011). These matrices represent the dissimilarity or distance calculated between every pair of community samples and were then used to generate distance histograms and Principal Coordinate Analysis (PCoA) plots. Distance histograms were generated by constructing a bootstrap consensus tree using the UPGMA (Unweighted Pair Group Method with Arithmetic mean) hierarchical clustering method to interpret the distance between each sample. To measure the robustness of the distance histograms, jackknife support for each node was determined (Caporaso *et al.* 2010b; Caporaso *et al.* 2011) by selecting subsamples of the full dataset to generate replicates of the above distance matrices and subsequently generate distance histograms. The jackknifed UPGMA results were then compared to the UPGMA clustering based on all available sequences to determine how frequently a node had the same set of samples. PCoA plots were based on computed principal coordinates (Vázquez-Baeza *et al.* 2013) to statistically identify and compare the bacterial OTU abundance and association between the samples.

2.4 Abundance estimation of *Pseudomonas* and *Wolbachia* spp.

To confirm the largest changes we observed in the abundance of bacterial species (i.e. an increase or decrease of at least 40 % of abundance), we used quantitative real-time PCR and genus-specific primers to amplify bacterial 16s rRNA genes in our samples (Table S2). We then calculated gene copy numbers by coordinating mean Cq (quantification cycle) values with

corresponding copy numbers on a standard curve based on known copy numbers of *Escherichia coli* (Livak & Schmittgen 2001) (see Supplemental methods).

2.5 Seasonal changes in cold tolerance of *G. veletis*

To determine if cold tolerance of *Gryllus veletis* differs between summer and mid-winter, we assessed survival of low temperatures in the laboratory microcosm, following Sinclair et al. (2015). Briefly, we cooled crickets from 0 °C to –10 °C at a rate of 0.25 °C/min and determined the supercooling point from the freezing exotherm (Sinclair *et al.* 2015). Following 4 h at -10 °C, we rewarmed crickets to 0 °C at 0.25 °C/min and recorded survival 24 h following cold exposure. We compared supercooling points using a t-test in R (R Development Core Team 2010).

2.6 Seasonal changes in immune activity of *G. veletis*

To determine if constitutive (i.e. activity present without infection) and realised (i.e. response to infection) immunity change from summer through to spring, we haphazardly selected crickets in the laboratory microcosm in the summer, mid-winter, and spring and measured circulating haemocyte concentrations, melanisation, *in vivo* bacterial clearance, and survival of fungal infection (Ferguson & Sinclair 2017). All crickets were sampled immediately after being removed from their incubation temperature, so that we could avoid or minimise acclimation effects and attempt to capture a snapshot of their seasonal immunity. Due to sample size constraints following mortality during overwintering, we were unable to measure bacterial clearance in the spring. To understand whether or not substrate availability might limit the melanisation response, we also measured hemolymph protein concentration. All statistical

analysis of immune activity was performed in R (version 3.2.2; R Development Core Team 2010).

To measure the concentration of circulating haemocytes, we collected 1 µL of haemolymph and diluted it in 24 µL of anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 6.8) immediately after removing an individual cricket from its incubation temperature, to avoid any effects of a temperature change on haemocyte number. We counted the total number of circulating haemocytes (CHC) in a Neubauer improved hemocytometer (Hausser Scientific, Blue Bell, PA, USA) at 400 × magnification following Ferguson and Sinclair (2017). We compared CHC among groups (summer, winter, & spring) using ANOVA.

We assessed the strength of the melanisation response as described by Ferguson et al. (2016). Briefly, we inserted a nylon filament into the hemocoel of the cricket, removed the filament after 24 h at 25 °C, and measured melanisation as the grey value of the filament. We compared the melanisation response among groups using ANOVA with a log-transformation of the grey value.

We measured clearance of the Gram-positive bacteria, *Staphylococcus aureus*, at 25 °C following Ferguson et al. (2016). Briefly, we injected *G. veletis* with a suspension of streptomycin-resistant *S. aureus* [1×10^7 colony forming units (CFU)/mL] and spot-plated homogenised whole crickets in PBS on an agar plate containing streptomycin (25 µg/mL) either 1 min or 24 h post-injection to capture the remaining bacteria. We compared clearance in summer and winter samples using a t-test.

We infected *G. veletis* with the entomopathogenic fungus *Metarhizium anisopliae* following Marshall and Sinclair (2011). Briefly, we injected 1 µL of a spore suspension (1×10^7 spores/µL)

of *M. anisopliae* diluted in 0.01 % Tween 80 (1 µL of Tween 80 only, for controls) in the membrane under the pronotum using a Hamilton syringe and a 32-gauge needle. We housed infected and control crickets individually in vials (25 mm × 95 mm; 46 cm^3) with *ad libitum* rabbit chow and water and paper shelters at 25 °C. We used the *survival* package in R (version 2.41-3; Therneau & Grambsch 2015) to generate Kaplan-Meier survival curves and compared the fit of generalised linear models to the data using log-likelihood. Based on log-likelihood, we chose a lognormal model and compared survival curves among treatments.

We measured haemolymph protein as described by Ferguson et al. (2016), using a Bicinchoninic Acid assay (BCA; Life Technologies, Carlsbad, CA, USA). We measured absorbance at 562 nm in a microplate spectrophotometer (SpectraMax, Molecular Devices, Sunnyvale, CA, USA). We then converted absorbance to concentration values using a standard curve created from bovine serum albumin. We compared protein in summer, winter, and spring using ANOVA.

3 Results

3.1 The composition of the gut microbiome changes seasonally

We obtained an average of 79026 ± 10 211 (standard deviation) reads per sample, ranging from 58669 to 90453 (Table S1). We assigned reads to 1377 OTUs at 97% sequence identity threshold. There were 906 to 1127 OTUs per sample (Table S1) with an average of 991 ± 59 OTU/sample. The gut microbiome was dominated by Bacteroidetes (the majority of these bacteria in the genera *Bacteroides* and *Parabacteroides*), Firmicutes, and Proteobacteria across all seasons and environmental treatments (Fig. 2 A,B; see Supplementary Figure S3 for coloured bar chart). Due to high overwintering mortality in the field-like microcosm, we did not complete

289 sampling in that microcosm beyond the mid-winter timepoint. We were unable to confirm why
290 mortality occurred in this microcosm.

291 The rarefaction curves of the OTU's approached saturation (Supplementary Fig. S1A),
292 suggesting that we captured the majority of microbial diversity in each sample. Species richness
293 of the gut microbiome (e.g. total number of species) was highest in the autumn (LM) and lowest
294 in early and mid-winter (FM); however, species richness was similar among seasonal time points
295 and between microcosms (Supplementary Fig. S1 B). Autumn and summer (LM) microbiomes
296 had the most diverse bacterial communities (i.e. the abundance of species is distributed evenly
297 among number of species), whereas early- and mid-winter (FM) had the lowest diversity (i.e. the
298 abundance of species is dominated by few species; Supplementary Fig. S1 C).

299 Overall, changes in the composition of the microbiome can be explained by season, as well as by
300 microcosm (i.e. by the addition of soil and leaves). The first axis of the principle coordinates
301 analysis (PCoA; Fig. 3A) explains 42 % of the variation among samples, and is driven largely by
302 season. The second axis describes 31 % of the variation among samples and appears to be driven
303 by microcosm. Jackknifed bootstrap trees (Fig. 3B) lend a confidence level of 75-100 % in
304 support of how samples group by season and microcosm in the PCoA. As such, spring is separate
305 from all other samples, autumn samples cluster together and then most closely with summer
306 samples, and winter samples cluster together based on whether or not soil and leaves were
307 present in the microcosm (Fig. 3B).

308 The trends between the two microcosms were similar across season. As season progressed, the
309 relative abundance of Proteobacteria increased from 5 % to 26 % of the relative abundance of the
310 microbiome from summer to spring in the LM, or 7 % to 26 % from summer to mid-winter in the

311 FM (Fig. 2A,B), whereas the relative abundance of Firmicutes decreased from 47 % to 25 %
312 from summer to spring in the LM and 50 % to 38 % from summer to mid-winter in the FM (Fig.
313 2A,B).

314 The *Wolbachia* sp. present displayed the greatest change across season, increasing from less than
315 50 % to almost 90 % of the Proteobacteria, and from approximately 5 % to 20 % of the total
316 bacterial abundance in mid- and late-winter (Fig. 2C,D); however, the relative abundance of
317 *Wolbachia* sp. decreased back to summer levels, or lower, in the spring (Fig. 2C). By contrast,
318 *Pseudomonas* spp. (several species that we were unable to identify beyond genus) decreased
319 from summer through winter, and remained at low relative abundance in the spring (Fig. 2C).
320 Additionally, *Pragia fontium* appeared to increase in abundance in the spring (Fig. 2C). Within
321 the Firmicutes, the relative abundance of *Blautia* sp. and Erysipelatoclostrichaceae peaked in the
322 winter. In both microcosms, the relative abundance of species in the genus *Clostridium*
323 decreased over the winter (Fig. 2 E,F). Finally, we detected at least three genera of facultative
324 pathogens (e.g. *Serratia, Escherichia,* and *Pseudomonas*) in the gut (Lysenko 1985), as well as
325 potential ice nucleators [e.g. *Pseudomonas* spp. (Lee *et al.* 1993)]. In particular, *Serratia*
326 *marcescens* comprised >1 % of Proteobacteria in summer, but fell below detectable levels in the
327 winter before increasing above 1% in the spring. Similarly, *Pseudomonas* spp. decreased in
328 relative abundance in both microcosms. We confirmed changes in the abundance of both
329 *Pseudomonas* spp. and *Wolbachia* sp. (i.e. the largest changes in abundance) using Q-RT-PCR
330 (Supplementary Figs S2 A,B).

3.2 Cold tolerance increases in the winter

Winter-acclimated crickets were more cold-tolerant than summer-acclimated crickets: four of seven winter-acclimated crickets survived exposure to – 10 °C for 4 h, whereas no summer-acclimated crickets (n=7) survived this exposure. There was no significant change in the supercooling point (winter: -8.7 ± 0.4 °C; summer: -8.1 ± 1.8 °C; $t_{12} = 0.78$, $p = 0.23$).

3.3 Immune activity is differentially affected by overwintering

During the winter, crickets displayed a weaker melanisation response (Fig. 4A, $F_{2,11} = 5.46$, $p = 0.02$; n = 5-10 per season), decreased total hemolymph protein (Fig. 4B; $F_{2, 21} = 24.91$, $p < 0.001$; n = 5-10 per season) and decreased survival of fungal infection (Fig. 4D; winter *vs* summer: $z = -3.41$, $p < 0.001$; n = 10 per season). However, circulating haemocyte counts increased (Fig. 4C; $F_{2,24} = 51.66$, $p < 0.001$; n = 5-16 per season), and the ability to clear bacteria from the haemolymph remained unchanged ($F_{1,11} = 0.8$, $p = 0.39$; n = 5-10 per season). Further, CHC, melanisation, and survival of fungal infection returned to summer levels in the spring.

4 Discussion

Here we show that overwintering affects both the composition of the gut microbiome and physiology of the spring field cricket, *Gryllus veletis.* Regardless of the microbial conditions in the external environment (i.e. presence or absence of soil and leaves), the gut microbiome is similar in summer and autumn, but changes in winter to favour an increase in the relative abundance of Proteobacteria. Immune activity changes during the winter, but the direction of change depends on the type of activity measured. Simultaneously, crickets also increase their

cold-tolerance. These concurrent shifts in the microbiome and host physiology suggest that these changes may be connected, and play a role in the overwintering success of *G. veletis*.

Overall, the microbiome in both the laboratory and field-like microcosm was dominated by anaerobic bacteria across seasons (e.g. *Bacteroides* and *Parabacteroides*), in concordance with a previous study on the hindgut bacteria of *Acheta domesticus* crickets (Santo Domingo *et al.* 1998b). Indeed, the cricket hindgut appears to be fermentative (Santo Domingo *et al.* 1998a), and the bacteria are essential for the digestion of complex plant polysaccharides (Kaufman & Klug 1991). Although the specific function of *Bacteroides* spp. has not been determined in crickets, *Bacteroides* spp. dominate the human gut microbiome and perform carbohydrate fermentation (Wexler 2007). Thus, the predominantly anaerobic nature and dominance of *Bacteroides* and *Parabacteroides* across season indicates that the overarching dietary function of the microbiome is likely conserved across seasons and between microcosms.

Apart from the consistent dominance in abundance of *Parabacteroides* and *Bacteroides* we observed seasonal variation in abundance in a variety of taxa. In particular, the abundance of the endosymbiont *Wolbachia* sp., as well as *Clostridium symbiosum* and *Pragia fontium* increased in the winter. Similarly, *Wolbachia* spp. are more prevalent in the gut tissue microbiome in *Drosophila melanogaster* reared at 13 °C compared to 31 °C (Moghadam *et al.* 2017), which suggests that *Wolbachia* spp. associated with insect guts may be psychrophilic. Conversely, other *Clostridium* species, as well as *Pseudomonas* spp. decreased in relative abundance over the winter. In the spring, although the increase in *Wolbachia* sp. reversed, the overall relative abundance of Proteobacteria (potentially driven by *Pragia fontium*) remained elevated. The composition of bacterial communities can permanently shift when microbial interactions are

disrupted (Coyte *et al.* 2015) and we suggest that, unlike the reversible changes in host immunity, the microbiome of the overwintering, juvenile stage of *G. veletis* is sensitive to disruption via environmental pressures present during overwintering.

We expected that the introduction of soil, leaves, and humus would change the composition of microbes in the external environment and food of the crickets, thereby introducing new microbes into the gut. However, because the overall composition of the microbiome shifts similarly across season, the dominant bacterial taxa do not appear to have been disturbed by a change in microcosm for the host. Further, the conserved changes between microcosms suggests that we were able to minimise the influence of inter-individual variation that is overlooked by pooling samples. Changes in diet can lead to shifts in the composition of the gut microbiome in crickets (Santo Domingo *et al.* 1998a); however, these shifts are likely to be driven by a switch in nutrients available to the microbial community. Further, core microbiota (i.e. those that are consistently found among individuals in a species) are less likely to be perturbed by changes in microbial habitat (Cariveau *et al.* 2014; Sudakaran *et al.* 2012). Thus, it appears that the hindgut of *G. veletis* houses a core group of resident microbiota. Further, the conserved shifts between microcosms suggests that we can predict generalisable shifts in core microbiota following environmental perturbations.

Because the microbiome appears to respond in a consistent way to variation in season, our task becomes to determine the driving force behind these patterns. In the introduction, we suggested four hypotheses and we will address the likelihood of each as the driver of changes in the microbiome of *G. veletis.* We largely focus on the largest changes in abundance of bacterial species to support or refute each hypothesis, as these changes are likely to also represent significant functional shifts in the microbiome.

1) Low temperatures may directly select for psychrophilic bacteria

If low temperatures select for psychrophlic bacteria, we would predict an increase in the abundance of such species. For example, *Pseudmonas* spp. include ice-nucleating bacteria (Lee *et al.* 1993) and psychrophiles that are selected for in the gut of overwintering bullfrogs (Carr *et al.* 1976), and we were able to detect *Pseudomonas* spp. in the gut of *G. veletis* during summer. However, *Pseudomonas* spp. decline to nearly undetectable levels in the winter. Thus, we believe that it is unlikely that changes in the gut microbiome are driven by low temperatures favouring psychrophiles.

2) *Low temperatures directly kill or select against bacteria that are intolerant of cold*

If low temperatures kill or select against bacteria intolerant of cold, we would expect to see decreases in the abundance of such species, and relative increases in cold-tolerant species. Similar to the first hypothesis, we might expect *Pseudomonas* spp. to increase in relative abundance as cold-intolerant bacteria decline; however, these putatively cold-tolerant species decline in abundance during the winter. Further, the abundance of *Clostridium* spp., which are often soil-dwelling in temperate areas (Wobeser *et al.* 1987) declines as winter progresses. Together, the decrease in putatively cold-tolerant species suggests that low temperatures are not directly driving changes in the composition of the microbiome.

3) *Low temperatures indirectly mediate the gut microbiome by modifying the physiochemical environment of the gut.*

Gryllus veletis cease feeding but do not void the gut in the winter (L.V. Ferguson, personal observation), which likely alters available nutrients and selects for bacteria capable of using

these nutrients. For example, the gut microbiome of hibernating *Ictidomys tridecemlineatus* ground squirrels changes to favour bacteria that survive on host-derived mucins (Carey *et al.* 2013). Similarly, the gut could become increasingly anaerobic throughout the winter if insects close their spiracles to reduce water loss (Danks 2000) or maintain a barrier against pathogens (Hajek & Leger 1994) and ice-nucleating bacteria (Olsen *et al.* 1998). Indeed, *Pseudomonas* spp. decrease in the winter, which are the only dominant, obligate aerobes we observed in the microbiome (excluding *Wolbachia,* which are intracellular and less likely to be affected by oxygen gradients within the gut). This suggests that the gut could become increasingly anaerobic in the winter such that the abundance of aerobic bacteria in the gut microbiome declines.

4) The host may directly regulate the microbiome.

Immunity in *G. veletis* may have been seasonally restructured to maintain a constitutive baseline of activity (e.g. phagocytic activity of haemocytes) while suppressing costly immune activity [e.g. melanisation; (González-Santoyo & Córdoba-Aguilar 2012)] to account for energy maintenance and trade-offs during the winter (Ferguson & Sinclair 2017). We do note that increased constitutive activity through an increase in haemocyte numbers may instead be a by-product of haemocytes losing adherence to tissues in the cold, thereby increasing haemocytes in circulation without increasing realised immune responses. Indeed, survival of a fungal infection (a realised immune response) was nonetheless reduced, suggesting that these insects may be vulnerable to infection during overwintering. If hosts are threatened by pathogens that enter *via* the gut (Sinclair *et al.* 2013), while some aspects of systemic immunity are suppressed, then they would benefit from actively reducing populations of potential pathogens in the gut, such as *Pseudomonas* spp. and *Serratia marcescens*. Indeed, we observed a reduction in both

439 *Pseudomonas* spp. and *Serratia marcescens* in the gut during overwintering. Further,
440 *Pseudomonas* spp. are also known ice nucleators and may be regulated by the host to control ice
441 formation (Olsen & Duman 1997). However, *G. veletis* are freeze-tolerant (McKinnon 2015) and
442 may be more likely to benefit from maintaining ice nucleators (Lee & Costanzo 1998).
443 Therefore, we hypothesise that these crickets may either control ice nucleators for an unknown
444 reason, or control potential pathogens in the gut. However, as we did not measure immune
445 activity within the gut itself, we are limited in our understanding of the mechanism that may
446 underlie active control of the microbiome. Further, the pronounced increase in *Wolbachia* spp.,
447 which may become increasingly pathogenic as density increases (Caragata *et al.* 2016) suggests
448 that the host is actually impaired in its ability to control endosymbionts and gut bacteria during
449 overwintering. In either case of control or lack-there-of, it would be likely that changes in the
450 microbiome would impact host overwintering success.

451

452 Seasonal changes in the gut microbiome may also result from variables that are independent of
453 temperature. First, microbiomes can change in composition as insects age (Clark *et al.* 2015),
454 and as our study spanned several weeks, it is possible that age is a contributing factor to seasonal
455 changes in the cricket microbiome. However, age-related changes in the microbiome are likely
456 functional shifts linked to metamorphosis to a new instar (Chen *et al.* 2016) or dysbiosis as an
457 insect approaches death (Clark *et al.* 2015). Prolonged exposure to low temperatures (e.g. during
458 this study) should instead slow the aging process (Le Bourg 2007), we sampled all crickets
459 within the same instar, and as these crickets were juveniles, they would be unlikely to be
460 approaching aging-related death. Second, the density of crickets did decline during our study,
461 which could reduce potential social transmission of microbes (Lombardo 2007). We suggest that

462 this may be a relatively small contribution to changes in microbial composition in our study as
463 we used confined spaces and were primarily concerned with changes during a period of
464 dormancy. However, overwintering could increase aggregation (Copp 1983) or decrease
465 population density through overwintering mortality, leading to changes in the social transmission
466 of microbes and resulting composition of microbiomes. Overall, we suggest that seasonal
467 changes in the microbiome are complex, and likely to arise from several, integrated variables that
468 must be teased apart to understand their relative contributions to the microbiome and its stability
469 under phenomena such as climate change.

470

471 We did observe mortality in both microcosms, which was more pronounced in the field-like
472 microcosm, but we were unable to confirm the cause. We did not observe any outward signs of
473 infection (e.g. growth of fungal spores) but can not rule out the role of pathogens in the gut or
474 surrounding environment. Mortality may have been higher in the field-like microcosm due to
475 inoculative freezing *via* ice nucleators in the soil, leading to lethal freeze-thaw cycles for the
476 crickets. Dehydration and starvation are also possible explanations for winter mortality
477 (Williams *et al.* 2015), although both food and water were continuously available so these seem
478 unlikely causes. Overall, it appears that *G. veletis* do experience overwintering mortality in
479 conjunction with changes in physiology and the composition of the microbiome, so it will next
480 be important to determine whether or not these events are linked.

481

482 5 Conclusions

483

Overall, the gut microbiome of *Gryllus veletis* changes during overwintering, and these changes correlate with changes in host physiology. Further, the patterns of change in both host physiology and microbiome composition support host-driven changes (passive or active) in microbial community composition, as opposed to independent, temperature-driven changes. Concurrent changes in immunity and composition of the microbiome imply that insect hosts may be faced with pathogen pressure within the microbiome during overwintering. It will next be important to move towards a functional understanding of these shifts in the microbiome, as their role may be an important contribution to insect overwintering success that we have previously ignored.

Acknowledgements

We would like to thank Drs. Yolanda Morbey, Graham Thompson, Barb Katzenback, Beth MacDougall-Shackleton, Rodney Dekoter and several anonymous reviewers for comments on an earlier draft of the manuscript. We thank the Natural Sciences and Engineering Research Council for Discovery Grants to BJS and CB, and a graduate scholarship to LVF. We have no conflicts of interest to declare.

Author Contributions

LVF, BJS, CB and DEH conceived the ideas and designed the methodology; LVF and JEL collected the data; LVF and PD analysed the data; LVF and BJS led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data Accessibility

We have uploaded sequence files to Pubmed and will provide submission number upon acceptance of the manuscript.

References

Caporaso JG, Bittinger K, Bushman FD, *et al.* (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26**, 266-267.

Caporaso JG, Kuczynski J, Stombaugh J, *et al.* (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**.

Caporaso JG, Lauber CL, Costello EK, *et al.* (2011). Moving pictures of the human microbiome. *Genome Biology* **12**.

Carey HV, Duddleston KN (2014). Animal-microbial symbioses in changing environments. *Journal of Thermal Biology* **44**, 78-84.

Carey HV, Walters WA, Knight R (2013). Seasonal restructuring of the ground squirrel gut microbiota over the annual hibernation cycle. *American Journal of Physiology* **304**, R33-42.

Cariveau DP, Elijah Powell J, Koch H, Winfree R, Moran NA (2014). Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*). *The ISME Journal*, 1-11.

Carr AH, Amborski RL, Culley DDJ, Amborski GF (1976). Aerobic bacteria in the intestinal tracts of bullfrogs (*Rana catesbeiana*) maintained at low temperatures. *Herpetologica* **32**, 239-244.

Chen B, Teh BS, Sun C, *et al.* (2016) Biodiversity and activity of the gut microbiota across the life history of the insect herbivore *Spodoptera littoralis*. *Scientific Reports* **6**, 29505.

Clark RI, Salazar A, Yamada R, *et al.* (2015) Distinct shifts in microbiota composition during *Drosophila* aging impair intestinal function and drive mortality. *Cell Rep* **12**, 1656-1667.

Coello Alvarado LE, MacMillan HA, Sinclair BJ (2015). Chill-tolerant *Gryllus* crickets maintain ion balance at low temperatures. *Journal of Insect Physiology* **77**, 15-25.

Copp NH (1983) Temperature-dependent behaviours and cluster formation by aggregating ladybird beetles. *Animal Behaviour* **31**, 424-430.

Coyte KZ, Schluter J, Foster KR (2015). The ecology of the microbiome: networks, competition and stability. *Science* **350**, 663-666.

Danks HV (2000). Dehydration in dormant insects. *Journal of Insect Physiology* **46**, 837-852.

Denlinger DL, Lee RE, Jr. (2010) *Low temperature biology of insects* Cambridge University Press, Cambridge, United Kingdom.

DeSantis TZ, Hugenholtz P, Larsen N, *et al.* (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied Environmental Microbiology* **72**, 5069-5072.

Douglas AE (2011). Lessons from studying insect symbioses. *Cell Host Microbe* **10**, 359-367.

545 Douglas AE (2015). Multiorganismal insects: diversity and function of resident microorganisms.
546 *Annual Review of Entomology* **60**, 17-34.
547 Douglas AE, Bouvaine S, Russell RR (2011). How the insect immune system interacts with an
548 obligate symbiotic bacterium. *Proceedings of the Royal Society B* **278**, 333-338.
549 Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*
550 **26**, 2460-2461.
551 Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011). UCHIME improves sensitivity
552 and speed of chimera detection. *Bioinformatics* **27**, 2194-2200.
553 Engel P, Moran NA (2013). The gut microbiota of insects - diversity in structure and function.
554 *FEMS Microbiology Reviews* **37**, 699-735.
555 Ferguson LV, Heinrichs DE, Sinclair BJ (2016). Paradoxical acclimation responses in the
556 thermal performance of insect immunity. *Oecologia* **181**, 77-85.
557 Ferguson LV, Sinclair BJ (2017). Insect immunity varies idiosyncratically during overwintering
558 *Journal of Experimental Zoology* **327**, 222-234.
559 Franzini PZ, Ramond JB, Scholtz CH, Sole CL, Ronca S, Cowan DA. (2016). The gut
560 microbiomes of two *Pachysoma* MacLeay desert dung beetle species (Coleoptera:
561 Scarabaeidae: Scarabaeinae) feeding on different diets. *PLoS One* **11**, e0161118.
562 González-Santoyo I, Córdoba-Aguilar A (2012). Phenoloxidase: a key component of the insect
563 immune system. *Entomologia Experimentalis et Applicata* **142**, 1-16.
564 Hahn DA, Denlinger DL (2007). Meeting the energetic demands of insect diapause: nutrient
565 storage and utilization. *Journal of Insect Physiology* **53**, 760-773.
566 Hajek AE, Leger RJS (1994). Interactions between fungal pathogens and insect hosts. *Annual*
567 *Review of Entomology* **39**, 293-322.
568 Hart ML, Meyer A, Johnson PJ, Ericsson AC (2015). Comparative evaluation of DNA extraction
569 methods from feces of multiple host species for downstream next-generation sequencing.
570 *PLoS One* **10**, e0143334.
571 Hussain M, Frentiu FD, Moreira LA, O'Neill SL, Asgari S (2011). *Wolbachia* uses host
572 microRNAs to manipulate host gene expression and facilitate colonization of the dengue
573 vector *Aedes aegypti*. *Proceedings of the National Academy of Sciences* **108**, 9250-9255.
574 Kaufman MG, Klug MJ (1991). The contribution of hindgut bacteria to dietary carbohydrate
575 utilization by crickets (Orthoptera: Gryllidae). *Comparative Biochemistry and Physiology*
576 **98A**, 117-123.
577 Le Bourg E (2007) Hormetic effects of repeated exposures to cold at young age on longevity,
578 aging and resistance to heat or cold shocks in Drosophila melanogaster. *Biogerontology*
579 **8**, 431-444.
580 Lee RE, Jr., Costanzo JP (1998). Biological ice nucleation and ice distribution in cold-hardy
581 ectothermic animals. *Annual Review of Physiology* **60**, 55-72.
582 Lee RE, Jr., Lee MR, Strong-Gunderson JM (1993). Insect cold-hardiness and ice nucleating
583 active microorganisms including their potential use for biological control. *Journal of*
584 *Insect Physiology* **39**, 1-12.
585 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time
586 quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408.
587 Lokmer A, Mathias Wegner K (2015). Hemolymph microbiome of Pacific oysters in response to
588 temperature, temperature stress and infection. *The ISME Journal* **9**, 670-682.
589 Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R (2011). UniFrac: an effective
590 distance metric for microbial community comparison. *The ISME Journal* **5**, 169-172.

Ludvigsen J, Rangberg A, Avershina E, *et al.* (2015). Shifts in the midgut/pyloric microbiota composition within a honey bee apiary throughout a season. *Microbes and the Environment* **30**, 235-244.

Lysenko O (1985). Non-sporeforming bacteria pathogenic to insects: incidence and mechanisms. *Annual Review of Microbiology* 673-695.

MacMillan HA, Sinclair BJ (2011). The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. *Journal of Experimental Biology* **214**, 726-734.

Maes PW, Rodrigues PA, Oliver R, Mott BM, Anderson KE (2016). Diet-related gut bacterial dysbiosis correlates with impaired development, increased mortality and *Nosema* disease in the honeybee (*Apis mellifera*). *Molecular Ecology* **25**, 5439-5450.

Maniero GD, Carey C (1997). Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. *Journal of Comparative Physiology* **167**, 256-263.

Marshall KE, Sinclair BJ (2011). The sub-lethal effects of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*. *Journal of Experimental Biology* **214**, 1205-1212.

McKinnon AH (2015). Freeze tolerance in the spring field cricket *Gryllus veletis*, Western University, London, ON, Canada.

Moghadam NN, Thorshauge PM, Kristensen TN, De Jonge N, Bahrndorff S, Kjeldal H, Nielsen JL (2017). Strong responses of *Drosophila melanogaster* microbiota to developmental temperature. *Fly*, doi: 10.1080/19336934.2017.1394558.

Olsen TM, Duman JG (1997). Maintenance of the superooled state in overwintering pyrochroid beetle larvae, *Dendroides canadensis*: role of hemolymph ice nucleators and antifreeze proteins. *Journal of Comparative Physiology B* **167**, 105-113.

Olsen TM, Sass SJ, Li N, Duman JG (1998). Factors contributing to seasonal increases in inoculative freezing resistance in overwintering fire-colored beetle larvae *Dendroides canadensis* (Pyrochroidae). *Journal of Experimental Biology* **201**, 1585-1594.

R Development Core Team (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Santo Domingo JW, Kaufman MG, Klug MJ, Holben WE, Harris D, Tiedje JM (1998a). Influence of diet on the structure and function of the bacterial hindgut community of crickets. *Molecular Ecology* **7**, 761-767.

Santo Domingo JW, Kaufman MG, Klug MJ, Tiedje JM (1998b). Characterization of the cricket hindgut microbiota with fluorescently labeled rRNA-targeted oligonucleotide probes. *Applied Environmental Microbiology* **64**, 752-755.

Shin SC, Kim S-H, You H…Lee W-J (2011). *Drosophila* microbiome regulates modulates host development and homeostasis via insulin signaling. *Science* **334**, 670-674.

Sinclair BJ, Coello Alvarado LE, Ferguson LV (2015). An invitation to measure insect cold tolerance: Methods, approaches, and workflow. *Journal of Thermal Biology* **53**, 180-197.

Sinclair BJ, Ferguson LV, Salehipour-shirazi G, MacMillan HA (2013). Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and immune stress in insects. *Integrative and Comparatove Biology* **53**, 545-556.

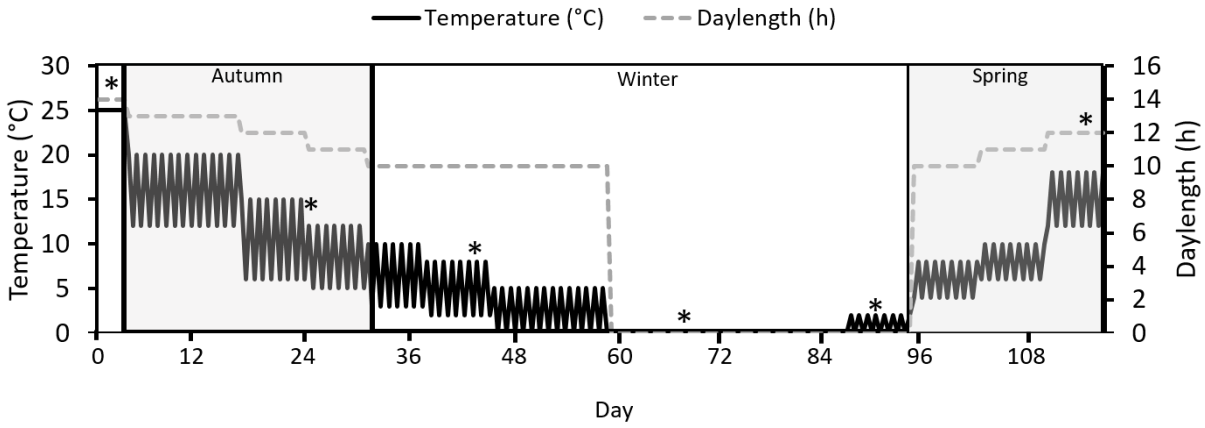
Sudakaran S, Salem H, Kost C, Kaltenpoth M (2012). Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae). *Molecular Ecology* **21**, 6134-6151.

Therneau TM, Grambsch PM (2015). A Package for Survival Analysis in R.

637 Vázquez-Baeza Y, Pirrung M, Gonzalez A, Knight R (2013). EMPeror: a tool for visualizing
638 high-throughput microbial community data. *GigaScience* **2**, 16.
639 Wang Y, Gilbreath TM, 3rd, Kukutla P, Yan G, Xu J (2011). Dynamic gut microbiome across
640 life history of the malaria mosquito *Anopheles gambiae* in Kenya. *PLoS One* **6**, e24767.
641 Webster NS, Cobb RE, Negri AP (2008). Temperature thresholds for bacterial symbiosis with a
642 sponge. *The ISME Journal* **2**, 830-842.
643 Wexler HM (2007). Bacteroides: the good, the bad, and the nitty-gritty. *Clinical Microbiology*
644 *Reviews* **20**, 593-621.
645 Williams CM, Henry HA, Sinclair BJ (2015). Cold truths: how winter drives responses of
646 terrestrial organisms to climate change. *Biological Reviews* **90**, 214-235.
647 Wobeser G, Marsden S, MacFarlane RJ (1987). Occurrence of toxigenic *Clostridium Botulinum*
648 Type C in the soil of wetlands in Saskatchewan. *Journal of Wildlife Diseases* **23**, 67-76.

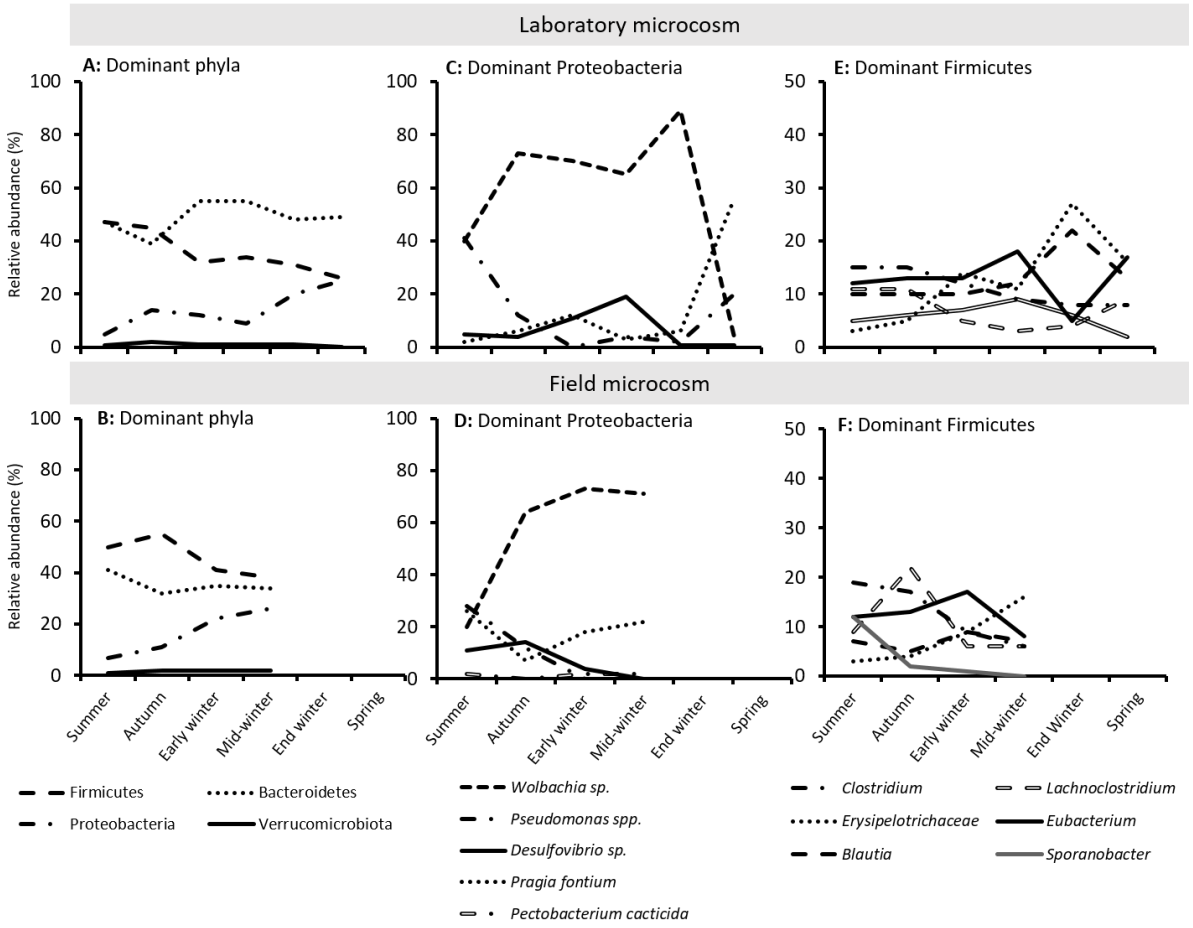
649

650



651

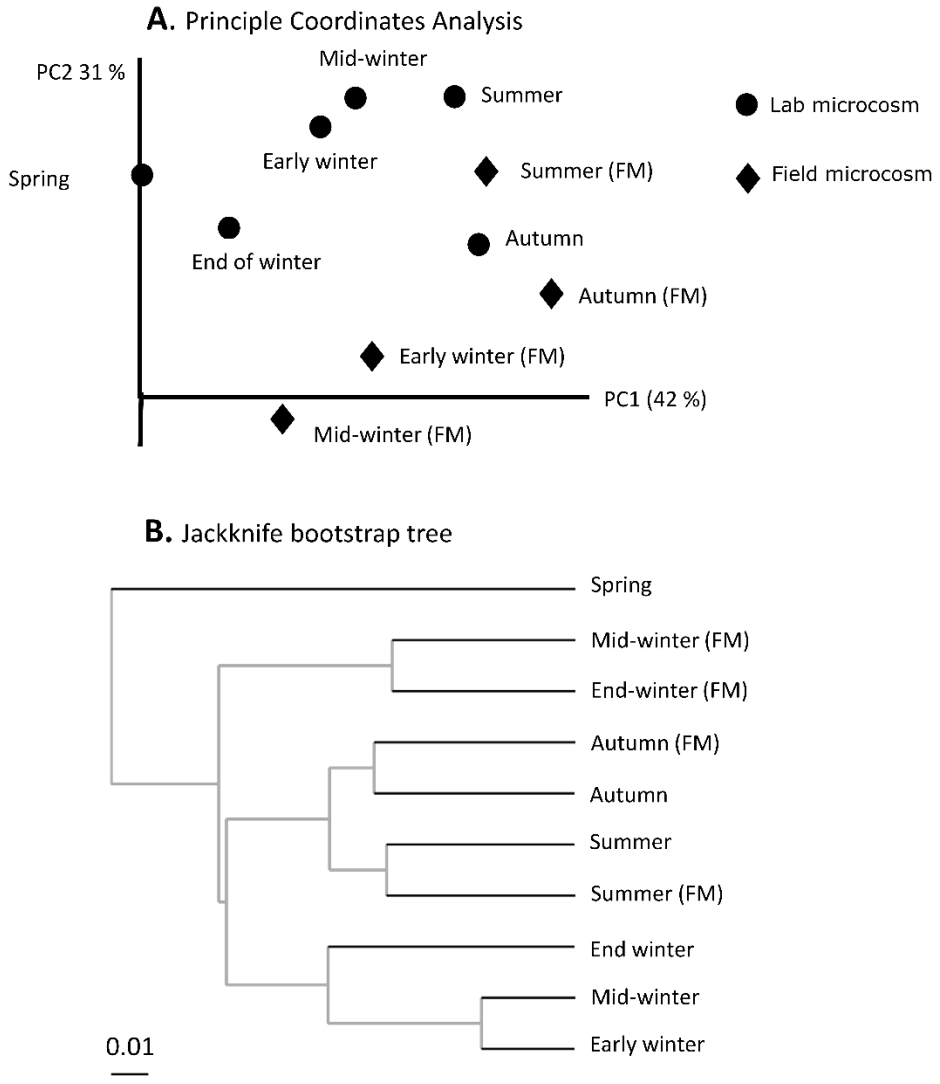
Figure 1. Temperature and photoperiod of simulated overwintering conditions of *Gryllus veletis*. Grey dashed lines line represents hours of daylight; black lines represent temperature. Asterisks indicate sampling points, in order of summer, autumn, early winter, mid-winter, late winter, and spring Alternating white and shaded regions represent the span of a season. Sampling begins in summer, under rearing conditions.



652

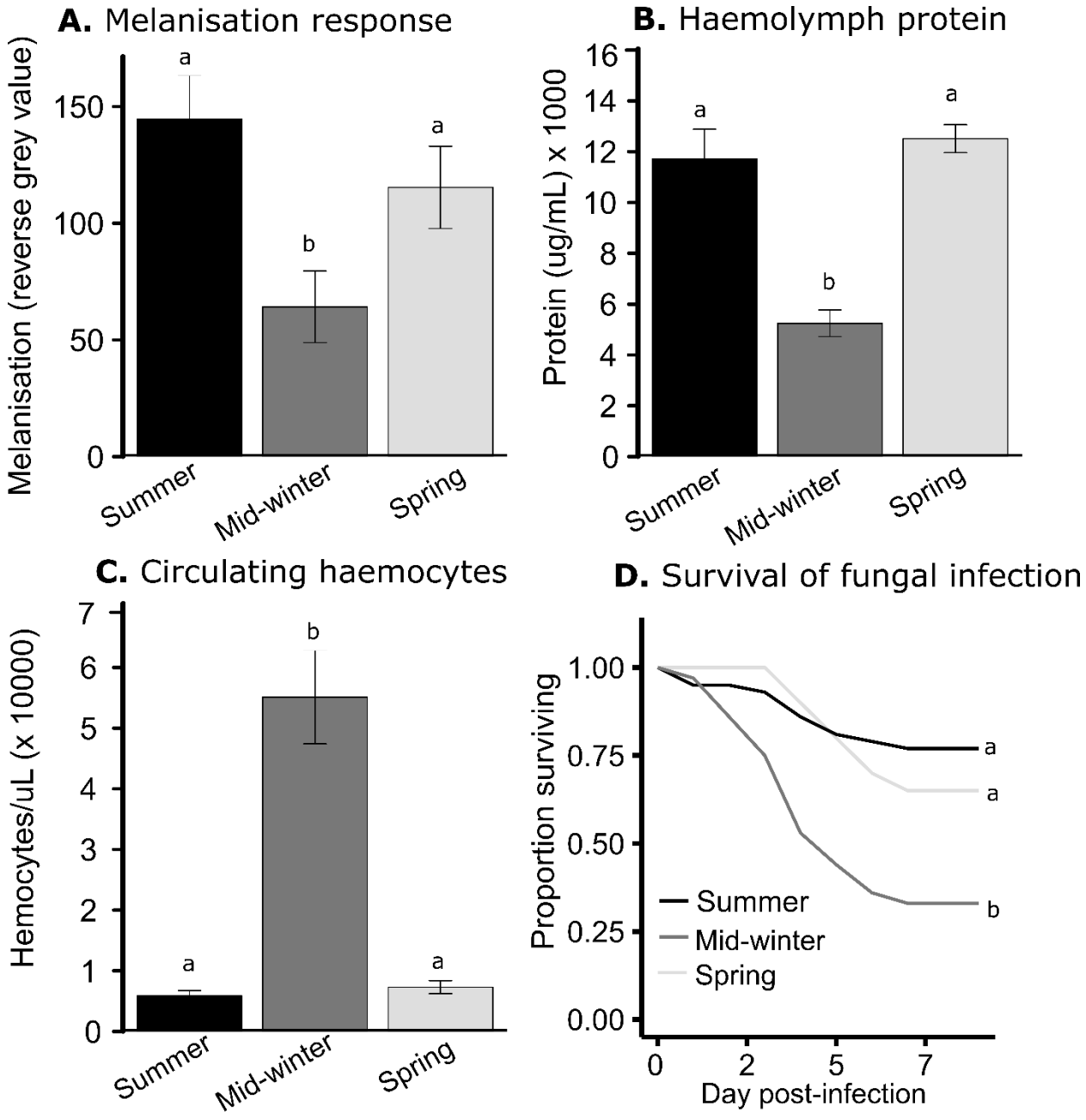
653 **Figure 2. Relative abundance of the dominant phyla and species or genera within phyla that show the most variation across**
654 **season in the microbiome of the hindgut in *Gryllus veletis***. Relative abundance data for all taxa including those poorly resolved
655 available in Figures S3 and S4. Dominant taxa represent those accounting for > 1% of the relative abundance, and/or those
656 demonstrating a >5% change in relative abundance over season. Top panels represent crickets in a lab microcosm and bottom panels
657 represent crickets in a simulated field-like microcosm. A/B. Dominant phyla; C/D. Dominant species within the Proteobacteria; E/F.
658 Dominant genera within the Firmicutes.

659



660

Figure 3. Measures of beta-diversity in the microbiome of *Gryllus veletis* across season and between two microcosms. A. Principal coordinates analysis (PCoA) of the composition of the gut microbiome. B. Jackknife bootstrap tree as a measure of validation for the PcoA. Grey lines indicate a bootstrap level of 75 – 100 %. Circles represent the lab microcosm, and diamonds represent the field-like microcosm. Output from the PCoA is relative, so we provide no scale on the axis in panel A.



661

Figure 4. Measures of immune activity and haemolymph protein in summer, mid-winter and spring in *Gryllus veletis* in a laboratory microcosm. **A**. the strength of the melanisation response against a simulated pathogen (n = 5-10 per season) **B.** Concentration of haemolymph protein as a correlate for substrate available for the melanisation response (n = 5-10 per season). **C.** Concentration of haemocytes in the haemolymph (n = 5-16 per season). **D.** Survival against the fungal entomopathogen, Metarhizium anisopliae (n = 10 per season). Different letters indicate seasons that differ significantly from each other. Error bars indicate SEM.

662