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Seasonal shifts in gut microbiota and cold tolerance metrics in a northern population of *Reticulitermes flavipes* (Blattodea: Rhinotermitidae)

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Eastern subterranean termites, *Reticulitermes flavipes* (Kollar), are widely distributed across North America where they are exposed to a broad range of environmental conditions. However, mechanisms for overwintering are not well understood. Wisconsin is a unique location to study mechanisms of cold tolerance as it represents the northern boundary for persistent *R. flavipes* populations. In this study, we evaluated seasonal shifts in cold tolerance using critical thermal minimum (CT_{min}) and supercooling point (SCP) and examined how these measurements correlate to changes in the microbial community of the termite gut. Results showed seasonal acclimatization to cold, which is consistent with the use of behavioral freeze-avoidant mechanisms. However, these insects also demonstrated an increased susceptibility to freezing later in the season, which may be tied to changes in gut microbiota. Our results found shifts in the composition of the gut microbiome in *R. flavipes* between mid- to late summer and early to late fall. These differences may be suggestive of a change in metabolism to adjust to a period of reduced feeding and increased metabolic stress during overwintering. Specifically, results showed an increased abundance of *Methanobrevibacter sp.* (Euryarchaeota) associated with cold, which may be indicative of a metabolic shift from acetogenesis to methanogenesis associated with overwintering. Further work is needed focusing on specific contributions of certain gut microbes, particularly their role in metabolic adaptability and in providing protection from oxidative stress associated with changes in environmental conditions.

Key words: cryobiology, temperature tolerance, thermal stress, microbiome

Introduction

Habitat-specific conditions such as temperature, moisture, phenology, and the availability and quality of food resources have considerable impacts on the population dynamics of all organisms on earth. Latitudinal as well as temporal temperature variability have been identified as driving the diversity, abundance, and biological activity of organisms (Chown and Nicolson 2004, Shimadzu et al. 2013). While insects have adapted to seasonal changes in temperature, predictions of global climate change suggest temperature fluctuations will increase in both frequency and magnitude (Deser et al. 2012), which will undoubtedly result in changes in species distributions associated with increased exposure to variability in environmental conditions and thermal stress. How insects respond to shifts in environmental conditions can be directly tied to underlying physiological adaptability (e.g., intracellular, biochemical, and metabolic adjustments used for maintaining internal equilibrium). In terms of thermal tolerance, physiological adaptability is likely to include mechanisms for contending with changes in internal temperature and for modulating cellular homeostasis while preserving functionality within the insect host and its microbial symbionts (Woon et al. 2019). Therefore, studies examining physiological temperature tolerance and the factors associated with thermal tolerance limits can be useful for defining the fundamental niche for a particular species or population and can be used to predict changes in distribution

and/or dispersal dynamics under varied environmental conditions (Teets and Denlinger 2013, Jorgensen et al. 2021).

Termites in the genus *Reticulitermes* (Rhinotermitidae) are widely distributed throughout the temperate regions of North America and are responsible for billions of dollars in damage to human-built structures annually (Su 2002). Although *Reticulitermes* spp. abundance is highest in warmer, southern climates, members of this genus have been found to occur farther north than any of the other subterranean termite genera (Esenther 1969, Weesner 1970, Husby 1980, Strack and Myles 1997). Churn Creek and Kamloops, British Columbia (~50 °N), are the coldest locations where *Reticulitermes sp.* have been documented (Vickery and Kevan 1985, Scheffrahn et al. 2015). However, these northern populations are not likely representative of naturally founded colonies but are probably the result of anthropogenic introduction into regions outside their normal range.

Several differences have been noted for northern *Reticulitermes* compared to more southern populations. In terms of colony growth, dispersal by alate reproductives is favored in the south, while colony growth in northern *Reticulitermes flavipes* (Kollar) populations generally occurs through secondary (neotenic) reproductives (Grace 1996a, Scaduto et al. 2012). Other differences noted for *Reticulitermes* at the northern edge of their distribution include a reduction in agonism (i.e., aggressive or defensive behavioral responses) between adjacent colonies as shown in Toronto by Grace (1996b). This is supported by studies on a related species (*Coptotermes formsanus* Shiraki), in which short-term cold exposure (i.e., 4 °C for 1 h) resulted in termites remaining non-aggressive for several days, even after conditions improved (Shelton and Grace 1997, see also Howick and Creffield 1980). A loss of genetic diversity associated with introduced populations, where colony growth arises from relatively few founding individuals, has also been suggested to affect aggression (Scaduto et al. 2012). Essentially, a lack of intercolony aggression could be considered adaptive for colony survival at the extreme northern edge of subterranean termite distribution by allowing for an open colony structure consisting of millions of individuals across several interconnected colonies (see Vargo et al. 2013).

Within Rhinotermitidae, the eastern subterranean termite *R. flavipes* is the most broadly distributed species in North America and thus has the most potential for demonstrating physiological plasticity to variations in environmental conditions. Across its northern range, the effect of climate, specifically prolonged exposure to subzero winter temperatures, has long been hypothesized to limit termite spread and restrict establishment of new termite populations (Urquhart 1954, Esenther and Gray 1968, Esenther 1969, Grace 1987). This is supported by the apparent absence of dispersal by winged reproductives (Esenther 1969, Grace 1996a), a trait that is common in other cold-adapted organisms (Wagner and Liebherr 1992). Based on published literature, the northern boundary for persistent *R. flavipes* populations is likely below 45 °N with few exceptions (Watson 1948, Watson and Thompson 1958, Kirby 1965, Esenther 1969, Harris 1970, Austin et al. 2005, McKern et al. 2006, Scaduto et al. 2012, Arango et al. 2015). However, the boundary for field colonies that do not rely on heated structures as a means for overwintering is probably closer to 43 °N based on data for termite populations from Janesville, Wisconsin, USA (42.7 °N) (Esenther 1969, Arango et al. 2015), Point Pelee, Ontario, Canada (41.9 °N) (Kirby 1965, Husby 1980, Raffoul et al. 2011), Middlesex Fells Reservation, Massachusetts, USA (42.45 °N) (Bulmer et al. 2001), and Dansville, Michigan, USA (42.6 °N) (Leadbetter and Breznak 1996) (Fig. 1).

Results from the relatively limited number of temperature tolerance studies to date suggest that these northern termite populations are freeze-avoidant, tunneling below the frost line during cold winter months (Esenther 1969, Cabrera and Kamble 2001, 2004, Hu and Song 2007, Clarke et al. 2013). During this time, termites are not suspected to enter into a state of diapause, although published research and observations of Wisconsin *R. flavipes* colonies suggest that activity during winter months is markedly decreased (Strack and Myles 1997). This is supported by laboratory experiments of *R. flavipes* from Ontario, Canada, where continuous foraging was noted as temperatures decreased from 20 to 10 °C followed by a major reduction in foraging activity below 5 °C. As temperatures fell below 5 °C, the majority of termites were observed to be toward the bottom of the containers, supporting the hypothesis that moving downward in soil is likely a major behavioral modification used to mediate exposure to unfavorable temperatures in these insects (Strack and Myles 1997). Hu and Song (2007) provide further evidence that *R. flavipes* use behavioral mechanisms to survive cold based on observations of termite distributions in the upper and lower sections of glass tubes exposed to different temperature regimens under laboratory conditions. In terms of physiological adjustments,

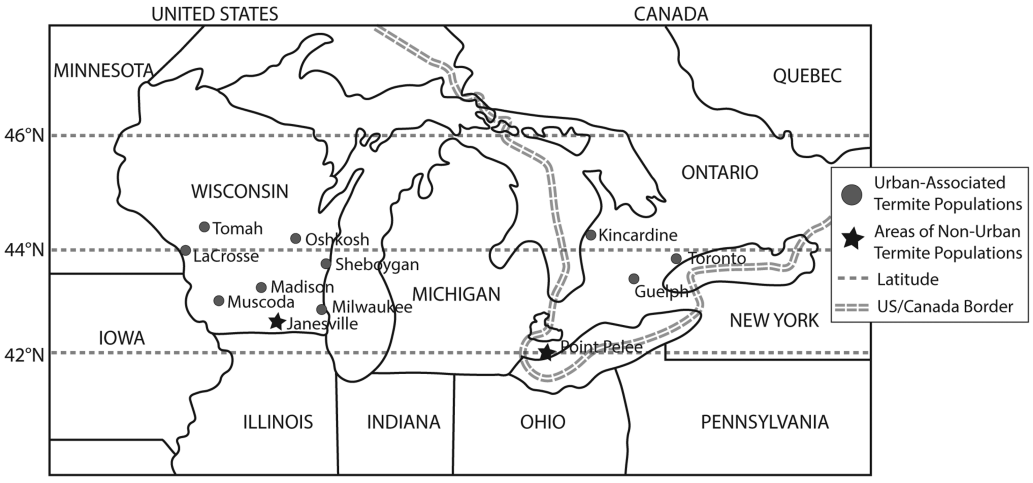

Fig. 1. Simplified map showing the northern distribution of certain *R. flavipes* populations (note: only select populations are shown to highlight the major areas of activity) (data are based on personal observations as well as locality information provided by Urquhart 1954, Kirby 1965, Esenther and Gray 1968, Esenther 1969, Husby 1980, Grace 1987, Strack and Myles 1997, Raffoul et al. 2011, Scaduto et al. 2012, Arango et al. 2015, Maynard et al. 2015.

Husby (1980) noted low osmotic pressure in the hemolymph of winter-collected *R. flavipes*, which he suggested resulted from a switch to fat metabolism caused by a cessation in protozoan activity in the hindgut, resulting in lower levels of sugars in the hemolymph. However, Cabrera and Kamble (2001) did not show an increase in fat content associated with decreasing temperature, suggesting that lipid reserves are not likely to be stored before winter. Together, these data support the hypothesis that *R. flavipes* does not enter into any specific state of extended diapause to overwinter but rather survives in a relatively inactive state where metabolism continues, although at a substantially reduced metabolic rate. This type of behaviorally active cold tolerance strategy is likely to support greater thermal tolerance plasticity by facilitating more rapid shifts from relatively inactive to active states, thereby allowing for feeding and nest maintenance behaviors when conditions are favorable.

While these behavioral and metabolic cold tolerance adjustments help to enable overwintering survival, they undoubtedly impose additional physiological constraints on these insects. For example, the behavior of tunneling below the frost line for overwintering may increase stress related to hypoxia and desiccation as there is decreased gas exchange and water availability in frozen soil (Lighton 1998). In addition, as termites are thought to reduce feeding and slow their metabolic rate rather than entering into a state of diapause (Strack and Myles 1997), normal metabolic processes would be constrained. The physiological shifts associated with this type of behaviorally active, cold tolerance strategy likely centers around conserving energy, maintaining cellular functioning and homeostasis, and lessening oxidative damage from the formation of stress-induced reactive oxygen species (ROS). In short, adaptive measures that balance metabolic demands with mechanisms to contend with various forms of metabolic stress are likely to be integral in the cold tolerance strategy utilized by subterranean termites.

Variability in the composition of the termite gut microbiome may be a key factor in contending with overwintering conditions, as these organisms would be readily able to respond to metabolic remodeling associated with alterations in respiration, nutrition, and metabolic rate (Ferguson et al. 2018). In addition, because of their rapid growth rate, microbes can evolve more quickly to adapt to changing conditions compared to their insect host (Sepulveda and Moeller 2020). Thus, we hypothesize that in termites, microbially mediated cold tolerance strategies would require enough flexibility to respond to shifting environmental conditions while also maintaining a core microbial community required for meeting baseline metabolic demands.

The overall goal of this work was to further elucidate the potential factors contributing towards cold tolerance in *R. flavipes*. Specifically, we measured seasonal changes in physiological cold tolerance using supercooling point (SCP) and critical thermal minimum (CT_{min}) as both have been used to evaluate seasonal variations in cold tolerance in other insect taxa and are thought to be highly comparable among species and populations (Sinclair et al. 2015). We also examined seasonal shifts in termite gut microbiome as well as how they relate to SCP and CT_{min} based on the hypothesis that seasonal changes in the physiological and metabolic requirements of the termite host will be reflected in the composition of the gut microbiome.

Materials and Methods

Air and Soil Temperature

Air temperature values were obtained from the UW Extension Ag gridded weather data records for Janesville, Wisconsin (latitude 42.8°N/ longitude −89.2°W) (http://agweather.cals.wisc.edu). Soil temperature data at three depths (20.3, 50.8, 101.6 cm) were taken from the UW Extension Ag NWS Co-Op Observer Reports from soil thermometers in Union Grove, Wisconsin (Station ID UGRW3), which is located approximately 55 miles west of Janesville but at a similar latitude.

Termite Collections

Specimens of *R. flavipes* (Kollar) were collected in cardboard traps (i.e., cardboard cut to 30.5 × 30.5 cm and grouped together in a 2.5-cm-deep wooden frame with a wire mesh on one side) from a forested site in Janesville, Wisconsin (N42°42.362 × W089°01.830) dominated by ash (*Fraxinus*) and hickory (*Carya*) tree species. Termites were collected between the months of July and November, which were grouped together by season for analysis. Termites were maintained in the original cardboard collection materials in the dark and under room temperature conditions prior to being evaluated in cold tolerance assays; termites were tested no more than 24 h after collection. At each collection date, a subset of workers was frozen at −80 °C for 16S rRNA amplicon sequencing. Only one site was sampled for this work as it is currently the only known population in Wisconsin occurring mainly independent of urban structures. Other known areas of termite activity were excluded because of their likely exposure to insecticidal treatments, which could impact results.

Supercooling Point (SCP)

Methods for determining supercooling points were adapted from several published studies (Cabrera and Kamble 2004, Schoville et al. 2015, Arango et al. 2021). Individual worker termites from field sampling dates ($n = 16$) were placed into 1.5-ml microcentrifuge tubes. T-type thermocouples were wrapped near the base with cotton rounds and pushed into the tube to keep the thermocouple in place, near the termite at the bottom of the microcentrifuge tube. Tubes were then inserted into a floating tube rack and carefully placed into a programmable water bath (software: LabWise, water bath: Grant Instruments R1), which was programmed for an initial 10-min acclimation at 20 °C followed by a decrease in temperature at a rate of 0.5 °C/min until reaching 5 °C, after which the rate of temperature decrease was 0.2 °C/min until SCP was reached. The supercooling point was determined by a spike in temperature as measured by the data logger (USB TC-08 PicoLog), representing the temperature at which freezing is initiated. Data were analyzed in a two-tailed Mann–Whitney test using Bonferroni corrected *P*-values and statistical significance was determined at $P \leq 0.05$. All statistical analyses were done using PAST 4.10 (Hammer et al. 2001).

Critical Thermal Minimum (CT_{min})

Critical thermal minimum (CT_{min}) was examined using a specially designed aluminum cooling block coupled to a programmable water bath (Grant Instruments R1) with insulated plastic tubes, which allowed for cycling of 50:50 propylene glycol and water between the water bath and aluminum block. The temperature of the water bath was controlled using LabWise software, which was programed for an initial 10 min at 20 °C followed by a decrease in temperature at a rate of 0.2 °C/min. Groups of termite workers were added to 5 of the 6 small Petri dishes (60 × 15 mm) attached to the center of the aluminum cooling block with thermal conducting tape. Each culture plate had a small hole drilled in the lid to prevent condensation and to allow insertion of a K-type thermocouple for temperature measurements in the sixth plate, which was monitored using a thermocouple data logger (USB TC-08 PicoLog). Measurements of CT_{min} were recorded based on the temperature at which termites

display a lack of muscle coordination as determined by an inability to right themselves after being gently flipped over with a paint brush. Once CT_{min} was reached, the termite was removed from the test dish into a 12-well plate and allowed to recover at room temperature. Evaluations of CT_{min} were performed on a total of 30 workers per sampling date. Data were analyzed in a two-tailed Mann–Whitney test using Bonferroni corrected *P*-values and statistical significance was determined at $P \leq 0.05$. All statistical analyses were done using PAST 4.10 (Hammer et al. 2001).

DNA Extraction and 16S rRNA Amplicon Sequencing

Termite workers ($n = 20$) from each of the collection time points were sterilized externally by rinsing in 70% ethanol, and their guts were extracted directly into ZR bashing bead lysis tubes (Zymo Research, Irvine, CA). A mix of 600 µl of tissue and cell lysis solution and 2 µl Proteinase K from the MasterPure Complete DNA and RNA Purification Kit (Epicentre-Lucigen, Middleton, WI) was added to each sample tube and all tubes were then vortexed vigorously for 2 min. Hereafter, DNA extraction methods followed the Epicentre-Lucigen total nucleic acids purification protocol.

Polymerase chain reaction was done using indexed MiSeq primers targeting the V4 region of the 16S rRNA gene [primers: forward—5'-GTGCCAGCMGCCGCGGTAA-3'; reverse—5'-GGACTACHVGGGTWTCTAAT-3'] (Kozich et al. 2013). Reaction mixtures for each sample (24 µl) included 12 µl KAPA HiFi HotStart ReadyMix (2X) (KAPA Biosystems, Boston, Massachusetts), 1.5 µl NanoPure water, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), and 8 µl diluted DNA (20 ng/µl). All samples were run in triplicate under the following conditions: initial denaturation at 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. A non-template control reaction was included and submitted for sequencing.

Amplicon libraries were sequenced with the Illumina MiSeq platform using paired-end sequencing at the University of Wisconsin-Madison Biotechnology Center. Reads were preprocessed, assembled, aligned, and classified using the mothur pipeline (Schloss et al. 2009). The classification was based on the Silva SEED release 132 databases. Operational taxonomic units (OTUs) were defined using a 99% similarity threshold. The richness of OTUs was estimated using the Chao-1 index (Chao and Chiu 2016), and non-metric multidimensional scaling and permutational analysis of variance (PERMANOVA) were used to quantify differences among samples and assess statistical significance, using PRIMER-e v 7.0.13.

Principal coordinate analysis (PCoA) was used to evaluate similarity in bacterial community across collection dates. A distance-based redundancy analysis, utilizing axis scores from the PCoA data, was used to determine if there was any correlation between bacterial community and either of the thermal tolerance variables (SCP or CT_{min}) (Legendre and Anderson 1999). Maaslin analysis was then used to identify specific OTUs that were correlated with SCP or CT_{min} ($P \leq 0.05$) (Mallick et al. 2021). Microbial community data were evaluated using MicrobiomeAnalyst (Dhariwal et al. 2017, Chong et al. 2020) to compare shifts in the abundance of certain taxa associated generally with seasonal changes from mid-summer to late fall.

Results and Discussion

Air and soil temperatures reported during the collection period are given in Fig. 2. These data show soil temperatures at all three depths tended to be lower than ambient air temperature during the warmer months and decreased at a slower rate than air temperature during late fall particularly at a depth of 101.6 cm, demonstrating the buffering effect of soil. By November, air temperature and soil temperature at depths of 20.3 and 50.8 cm were consistently at or below 5 °C, and by December, soil temperature was below freezing to a depth of 50.8 cm. Therefore, termites would have to tunnel to or below 50.8 cm by December to avoid freezing. This is supported by Husby (1980; Ontario, Canada) and Esenther (1969; Wisconsin, USA), who documented termites tunneling to depths at or greater than 100 cm during the coldest winter months.

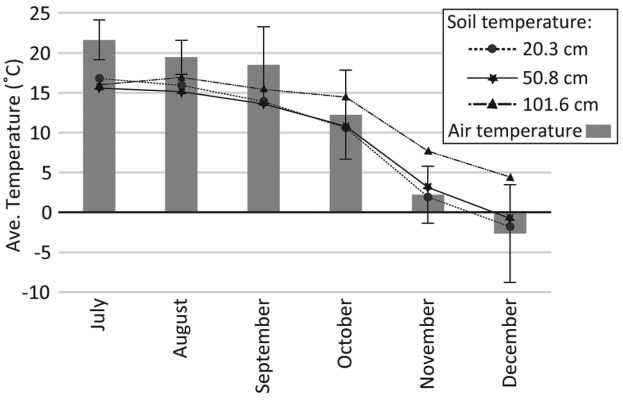

Fig. 2. Average soil temperatures at three depths ($n = 15$/depth) (lines) and monthly air temperatures (bars) ($n = 30$–31) reported during the collection period for this study (error bars represent standard deviation). Data obtained from the UW Extension Ag gridded weather data records and NWS Co-Op Observer Reports (http://agweather.cals.wisc.edu).

Although soil temperature data recorded here are low, microclimate characteristics likely have a substantial impact on actual soil temperatures at a very fine scale. Factors including rock piles (Danks 1991), snow cover (Shorthouse et al. 1980, Coulson et al. 1995), and proximity to residual heat associated with urban structures (e.g., building foundations, sewer pipes, etc. (Myles and Grace 1991)) have all been shown to influence soil microclimate conditions. Characteristics that are likely to impact microclimate conditions for termites collected in this study include a dense understory of mayapple (*Podophyllum peltatum* L.), dame's rocket (*Hesperis matronalis* L.), garlic mustard (*Alliaria petiolata* (M. Bieb.) Cavara & Grande), and Virginia creeper (*Parthenocissus quinquefolia* (L.) Planch.) as well as an abundance of leaf litter and dead fallen trees, particularly ash trees decimated by emerald ash borer (*Agrilus planipennis* Fairmaire). This site was selected for study as it is not directly adjacent to urban structures and thus, termites examined here are unlikely to be relying on heated buildings for overwintering. While specific microclimate factors were not evaluated in the present study, favorable microhabitat selection undoubtedly plays a significant role in overwintering survival in these insects, allowing termites to disperse to areas where they would be seldom exposed to temperatures below 0 °C. In northern climates, snow cover in particular has the potential to have a strong influence on subterranean termite success as even small depths of snow cover (i.e., 1.27 cm) provide a strong buffering effect on soil temperatures (Mail 1930). Various climate change scenarios have predicted decreases in the amount of snow cover, which would introduce increased variability in microclimate conditions including more frequent freeze-thaw cycles in soil (Sinclair 2001). Thus, although soil provides protection from rapid fluctuations in temperature, increased environmental variability, such as a change in snow cover, could have a substantial impact on termite survival in northern climates.

The few studies that evaluate overwintering strategy and temperature tolerance in *R. flavipes* have concluded that these insects utilize a freeze-avoidant cold tolerance strategy as they show a complete inability to survive any degree of internal ice formation (Sponsler and Appel 1991, Davis and Kamble 1994, Cabrera and Kamble 2004, Hu and Appel 2004, Clarke et al. 2013, Arango et al. 2021). However, they can develop varying levels of chill tolerance based on data showing acclimation to cold and short-term survival at subzero temperatures in both seasonal and laboratory-based studies (Davis and Kamble 1994, Arango et al. 2021). The importance of cold acclimation in developing chill tolerance was demonstrated for *R. flavipes* by Davis and Kamble (1994), where they reported freezing to be the cause of lethal damage in cold-acclimated individuals, while cold (not freezing) was the main cause of lethal damage in individuals that received little to no cold acclimation. Evidence of seasonal changes in critical thermal limits (CT_{min} and CT_{max}) associated with fluctuating seasonal habitat temperatures was examined in field colonies of two rhinotermitid species, *R. flavipes* and *C. formosanus* from Auburn, Alabama (Hu and Appel 2004). They found slight variations in CT_{max}, while CT_{min} showed wider seasonal fluctuations, decreasing in the fall and winter months and increasing in the spring and summer months. Results from the present study found critical thermal minimum decreases significantly from between 8.0 and 6.4 °C in mid-summer and early fall to between 5.9 and 5.0 °C in late fall (Fig. 3). These seasonal CT_{min} adjustments are likely necessary to facilitate protection of neuromuscular functioning at low temperatures and thereby enhance termite's ability to utilize behavioral freeze avoidance strategies during the winter months.

In addition to increasing chill tolerance at low temperatures (i.e., decreased CT_{min}), many freeze-avoidant organisms also show increased supercooling ability through the production and/or accumulation of cryoprotectants or antifreeze proteins/peptides (e.g., trehalose, glycerol, sorbitol, mannitol, glucose, fructose, inositol, ethylene glycol, alanine, and proline) (Doucet et al. 2009, Duman et al. 2010 and references within) or through the removal of potential ice nucleating agents (e.g., calcium carbonate, calcium phosphate, potassium phosphate, potassium urate, sodium urate, uric acid, certain amino acids, proteins, steroids, and ice-nucleating microorganisms) (Strong-Duman 1982, 2001, Duman and Horwath 1983, Gunderson et al. 1990, Lee et al. 1996, Mugnano et al. 1996, Kawahara 2002, Clark and Worland 2008, Duman et al. 2010, Lee 2010). Many of these compounds not only serve to lower freezing temperature (i.e., decrease SCP) but many also function as osmolytes that help prevent osmotic damage to cells and stabilize cell membranes (Lee et al. 1993a, Kawahara 2002, Teets and Denlinger 2013).

Supercooling point results from this study are atypical from other freeze-avoidant organisms, however, in that *R. flavipes* show increased susceptibility to freezing later in the season. Specifically, SCP was found to increase significantly, ranging from –4.34 to –5.04 °C during the mid to late summer months to between –4.16 and –1.46 °C in early to late fall (Fig. 3). These results agree with those of Davis and Kamble (1994) and Arango et al. (2021), which also found increased susceptibility to freezing associated with seasonal decreases in temperature or cold acclimation. Thus, supercooling through the production of cryoprotectants does not appear to be utilized as a mechanism of cold tolerance in *R. flavipes*, which was also suggested by Husby (1980).

Results from analyses of the termite gut microbiome associated with collection season are shown in Fig. 4 (for alpha diversity, see Supplementary Figure S1). These results suggest that there are no substantial changes in the relative abundance of the core phyla comprising the *R. flavipes* gut microbiome. However, dendrogram

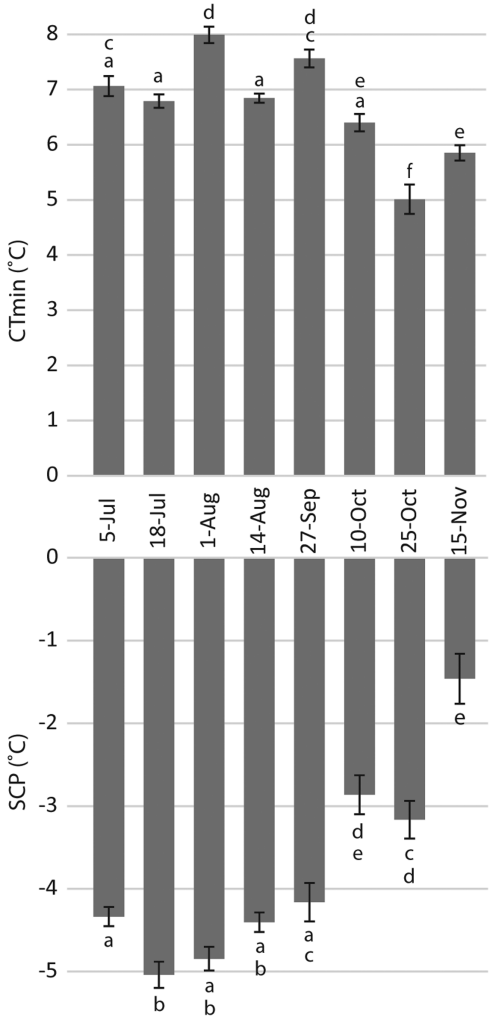

Fig. 3. Seasonal changes in CT_{min} (top) ($n = 30$) and SCP (bottom) ($n = 16$) (dates with the same letter are not significantly different, $P \leq 0.05$) (bars represent standard error).

clustering analysis does indicate a distinct seasonal component in the overall composition of the *R. flavipes* gut microbiome, which may be facilitating some degree of plasticity to changing environmental conditions or may be the result of changes in feeding/nutrient levels to meet overwintering energy requirements.

It is possible that the increase in SCP in termites collected later in the season is related to changes in the termite microbiome, particularly as various microbial organisms have been shown to be some of the most effective ice nucleators in insects (Lee et al. 1993b, Kawahara 2002, Lorv et al. 2014, Roeters et al. 2021). This is supported by the findings of Cabrera and Kamble (2004), which showed supercooling point to be the lowest in *R. flavipes* workers treated with antibiotics or defaunating using oxygen treatment. Microbial phyla containing known ice nucleation active bacteria include a diversity of Proteobacteria, particularly members of Gammaproteobacteria: Pseudomonadaceae, Enterobacteriaceae, and Xanthomonadaceae (e.g., *Stenotrophomonas*, *Erwinia*, *Xanthomonas*, *Pantoea*, *Pseudomonas*), as well as some members of Alphaproteobacteria, Firmicutes (*Lysinibacillus*, *Bacilli*), and Actinobacteria (Failor et al. 2017). The elimination or retention of ice-nucleating bacteria is a well-known component of the overwintering strategy in many freeze-avoidant insects. This may

be one factor driving seasonal shifts in lineages of Firmicutes and Proteobacteria that have been consistently documented across a broad range of insect taxa related to overwintering (e.g., *Drosophila melanogaster* Meigen (fruit fly), *Porcellio scaber* Latreille (wood louse), *Gryllus veletis* (Alexander and Bigelow) (field cricket)) (Ferguson et al. 2018, Moghadam et al. 2018, Horváthová et al. 2019, Sepulveda and Moeller 2020). Although this does not appear to hold true for termites evaluated here, the result suggests that the benefits of maintaining particular microbial species or populations for acclimating to cold temperatures may negate their potential ice nucleating capacity particularly if freezing can be avoided by other means.

The relationship between cold tolerance data and seasonal changes in gut microbiome is shown in Fig. 5. These results showed axis 2 to be strongly correlated with SCP ($P \leq 0.001$) and only weakly correlated with CT_{min} ($P = 0.022$). However, axis 1 was not significant with either SCP ($P = 0.67$) or CT_{min} ($P = 0.89$) (Supplementary Table S1), suggesting that other variables, which may not be directly tied to cold tolerance metrics, are driving much of the seasonal variability in the termite microbiome. Specific taxa that did

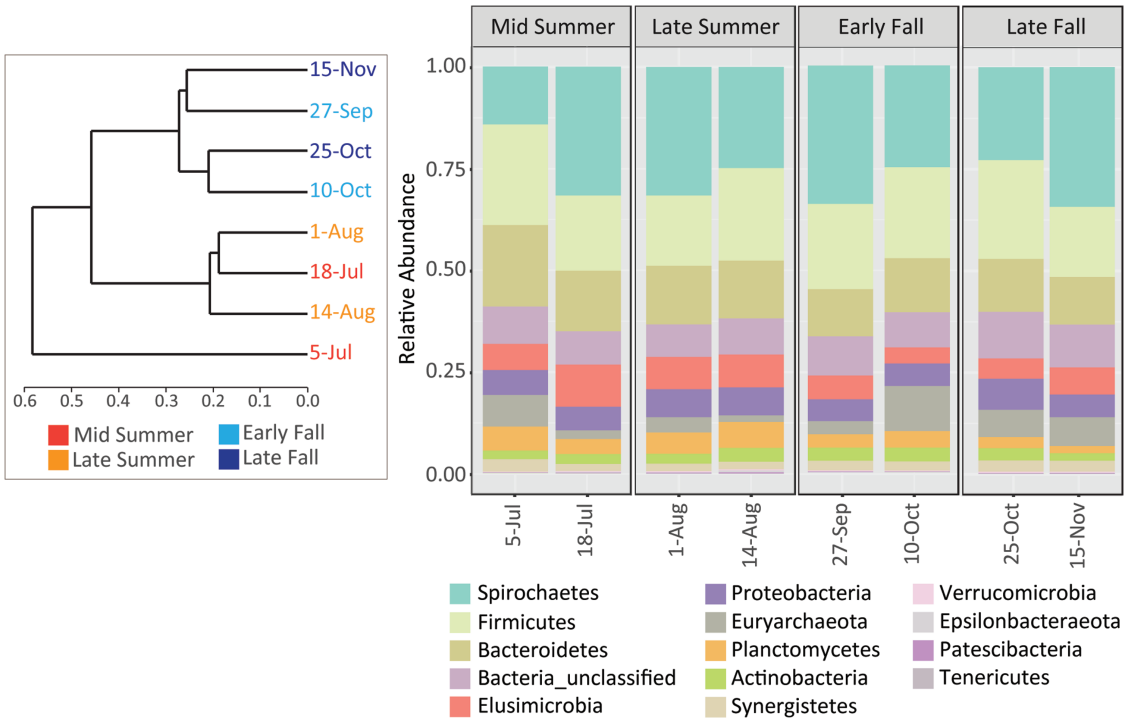

Fig. 4. Dendrogram analysis (left) and relative abundance (right) of core microbial phyla in the termite gut associated with collection date ($n = 20$ termite guts/collection date).

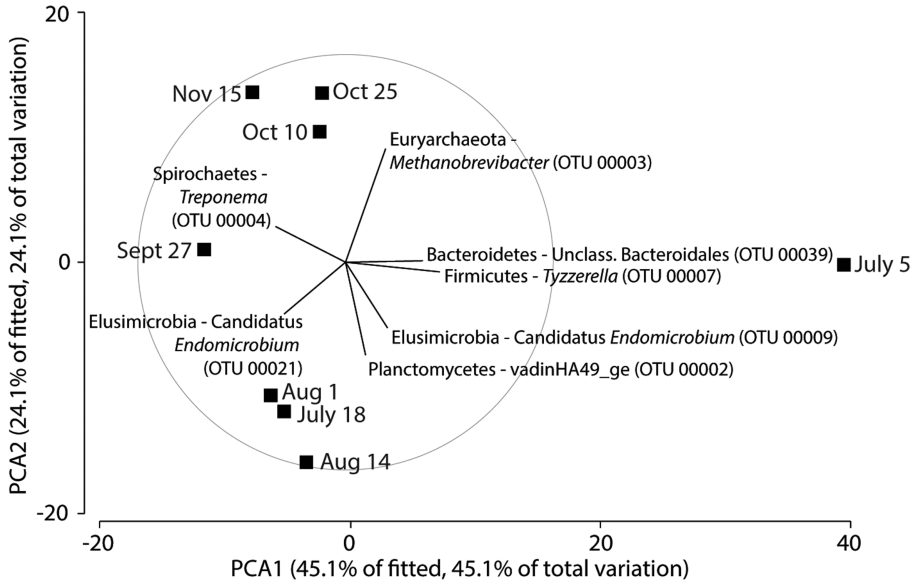

Fig. 5. Distance-based redundancy analysis showing principal coordinate analysis of bacterial community structure and OTUs correlated with SCP or CT_{min}.

show a significant correlation to either SCP or CT_{min} are provided in Supplementary Table S2.

While there was no major restructuring observed in the composition of the termite gut microbiome seasonally, there were two microbial taxa that did appear to demonstrate a seasonal shift in relative abundance; specifically, members of Elusimicrobia and Euryoarchaeota, both of which are involved in hydrogen-based metabolism in the termite gut by way of acetogenesis and methanogenesis, respectively (Breznak and Switzer 1986). In termites, acetogens and methanogens play important roles in carbon and electron flow and are responsible for reoxidation of H_2 that is formed during fermentation (Breznak and Switzer 1986, Brauman et al. 1992). Our results found Elusimicrobia to be significantly negatively correlated to Euryarchaeota ($P = 0.021$) with increased abundance of Elusimicrobia occurring during the warmer summer months and increased abundance of Euryarchaeota associated with colder months (Fig. 6). Fluctuations in the partial pressure of oxygen and hydrogen are thought to drive associations between these taxa (Leadbetter and Breznak 1996), which may be related to adaptive changes in respiration.

In *R. flavipes*, methanogens have been found primarily associated with the hindgut wall, either by direct attachment or adhering to prokaryotic filaments lining the hindgut wall (Leadbetter and Breznak 1996). Methanogens have also been identified as ecto- and endosymbionts of protozoa (Leadbetter and Breznak 1996, Fröhlich and König 1999, Tokura et al. 2000), where they have been demonstrated to compete with other hydrogenotrophic bacteria for hydrogen produced by protists (Odelson and Breznak 1983). Acetogenesis typically outcompetes methanogenesis in *R. flavipes* as the primary electron sink when H_2 concentrations are high (Brauman et al. 1992). However, methanogenesis is more likely to outcompete acetogenesis in anoxic environments in which CO_2 reduction is the primary electron sink reaction and where H_2 concentrations are limiting (Breznak 1994, Karekar et al. 2022).

Therefore, the increased abundance of *Methanobrevibacter* found in this study later in the season could suggest a shift in metabolism from acetogenesis to methanogenesis associated with changes in respiration and oxidation of the gut. Ferguson et al. (2018) suggested that insect guts may become increasingly anaerobic in the winter, tied to the physiological response of closing external cuticular spiracles when exposed to cold. This not only reduces gas exchange but is thought to be important for limiting access by potential pathogens (Hajek and Leger 1994) or ice nucleating bacteria (Olsen et al. 1998) and preventing desiccation through body water loss (Danks 2000). While it is unknown if termites close their spiracles associated with cold exposure, gas exchange would already be severely limited in subterranean termites that rely on tunneling deep in soil to avoid freezing as there would be a shift in the O_2/CO_2 concentration, which would impose constraints on normal aerobic metabolism.

An additional role for members of Euryarchaeota in cold tolerance may be to provide protection against oxidative stress as certain methanogenic lineages have evolved strong antioxidant properties (Lyu and Lu 2015, 2018). Strains of *Methanobrevibacter* isolated from termites have been shown to produce catalase-like enzymes capable of decomposing hydrogen peroxide, which is likely formed from O_2 reductions by other members of the gut microbial community, and thus may help in protecting against oxidative stress from ROS generated through this process (Leadbetter and Breznak 1996, Tholen et al. 2007). When produced in high concentrations, these ROS can be extremely detrimental to biological organisms and cells.

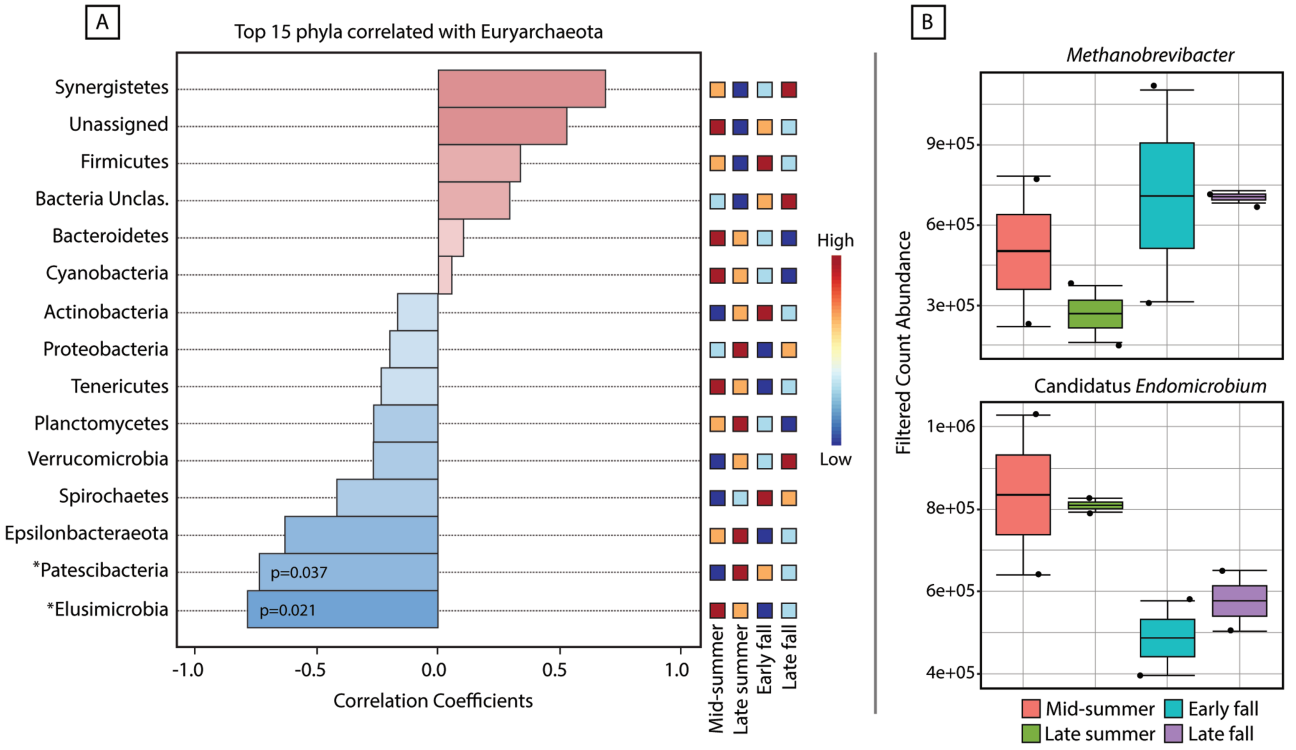

Fig. 6. A) Pattern search plot of the top 15 phyla showing a positive (bars to the right of 0.0) or negative (bars to the left of 0.0) correlation with Euryarchaeota (mini heatmap to the right indicates whether the abundance of that phylum is higher or lower across seasonal groups); B) seasonal changes in the abundance of Euryarchaeota: *Methanobrevibacter* and Elusimicrobia: Candidatus *Endomicrobium*. *Statistical analysis used Pearson *r* for distance measure correlation on rarified data.

Environmental stresses such as cold can enhance ROS production and threaten cell viability leading to issues including lipid peroxidation, protein oxidation, nucleic acid damage, enzyme inhibition, and cell death (Sharma et al. 2012). To avoid oxidative injury caused by environmentally induced oxidative stress, ROS is managed through the production of antioxidants like superoxide dismutase, catalase, and peroxidase, which are often increased associated with exposure to environmental stresses (Sharma and Dubey 2005, Mishra et al. 2011). Therefore, members of Euryarchaeota in the termite gut may be performing the dual role of methanogenesis to meet metabolic demands while also providing protection against oxidative stress. However, these hypotheses still need to be evaluated in future studies.

Summary and Conclusions

Results from this study showed *R. flavipes* undergo seasonal temperature acclimatization to cold, however, these insects were also more susceptible to freezing as external temperatures decreased. Increased susceptibility to freezing in conjunction with cold acclimatization in *R. flavipes* suggests that the benefits of physiological changes associated with cold must outweigh the risk of freezing or that the risk of freezing is relatively low and likely well-avoided by other means (e.g., tunneling below the soil frost line, microhabitat selection). Changes in the gut microbiome observed in this study may be the result of favoring those organisms that are involved in mediating adaptive responses to thermal stress (e.g., preventing oxidative damage from ROS and/or maintaining cellular homeostasis by regulating ion balance) or those that are necessary for meeting metabolic energy demands. Examining the relationship between gut microbiome and expression of stress response genes (e.g., catalase, superoxide dismutase, peroxidase, heat shock proteins (HSPs 70, 40, 110, 60)) during temperature acclimatization would help in elucidating the underlying mechanisms of cold tolerance in these insects including the role of specific microorganisms in adaptability to changing environmental conditions.

A major question that remains is in understanding the relationship between overwintering mechanisms and how they affect termite phenotype, fitness, reproduction, and dispersal. Along their northern range, metabolic energy usage in these insects is likely directed at low-temperature survival rather than in energy expensive pathways such as formation of alate reproductives. However, formation of alates may increase if changes in global climate result in milder winter conditions, allowing for a longer period of active feeding annually. The link between the gut microbiome and its contribution to metabolic adaptability represents an exciting area for future research. Further studies should focus on examining the physiological and metabolic constrains on northern termite populations and the underlying mechanisms contributing toward their establishment and persistence in colder climates.

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Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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