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Can't Take the Heat: High Temperature Depletes Bacterial Endosymbionts of Ants

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Abstract

Members of the ant tribe Camponotini have coevolved with *Blochmannia*, an obligate intracellular bacterial mutualist. This endosymbiont lives within host bacteriocyte cells that line the ant midgut, undergoes maternal transmission from host queens to offspring, and contributes to host nutrition via nitrogen recycling and nutrient biosynthesis. While elevated temperature has been shown to disrupt obligate bacterial mutualists of some insects, its impact on the ant-*Blochmannia* partnership is less clear. Here, we test the effect of heat on the density of *Blochmannia* in two related *Camponotus* species in the lab. Transcriptionally active *Blochmannia* were quantified using RT-qPCR as the ratio of *Blochmannia* 16S rRNA to ant host elongation factor 1- α transcripts. Our results showed that 4 weeks of heat treatment depleted active *Blochmannia* by >99 % in minor workers and unmated queens. However, complete elimination of *Blochmannia* transcripts rarely occurred, even after 16 weeks of heat treatment. Possible mechanisms of observed thermal sensitivity may include extreme AT-richness and related features of *Blochmannia* genomes, as well as host stress responses. Broadly, the observed depletion of an essential microbial mutualist in heat-treated ants is analogous to the loss of zooanthellae during coral bleaching. While the ecological relevance of *Blochmannia*'s thermal sensitivity is uncertain, our results argue that symbiont dynamics should be part of models predicting how ants and other animals will respond and adapt to a warming climate.

Introduction

Bacterial endosymbionts of insects include the most specialized and tightly constrained symbioses known in the animal world (recently reviewed in [1]). In particular, long-term bacterial mutualists inhabit an estimated ~10–15 % of insect species [2], live within specialized host cells (called bacteriocytes), and typically provide nutritional functions. Often these bacteria supplement specific amino acids or vitamins that are missing in an unbalanced diet of the host (such as plant sap or vertebrate blood) or perform more general functions such as nitrogen recycling. By virtue of their nutritional supplementation, these mutualists allow insect hosts to thrive on diets and live in habitats that would be insufficient otherwise. The bacteria are transmitted maternally, typically via the egg. As expected from

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this transmission mode, they have coevolved with their respective insect group, often for tens or hundreds of millions of years.

Given their interdependent nature, these insect–bacterial mutualisms may be fragile in the face of a changing environment [3]. In fact, the evolutionary trajectory of bacteriocyte-associated mutualists leads to consistent patterns of genome degradation, which may constrain adaptation of the bacteria and insect host alike and leave these associations vulnerable to environmental stresses such as increased temperature. Evolution in these bacteria is shaped by reductive genome evolution [1], a syndrome typically characterized by extreme AT richness, reduced structural integrity of proteins, and severe genome reduction that includes the loss of cell membrane components.

Predicted heat sensitivity of insect mutualists has been borne out by lab and field experimental studies. We know that many bacteriocyte-associated mutualists are depleted or lost entirely when the insect host is exposed to high temperatures (reviewed in [4]). For example, elevated temperature depletes obligate bacterial mutualists in aphids [2, 5, 6], weevils [7] and cockroaches [8]. While results are difficult to compare across studies, due to variation in experimental conditions and potential contributions of facultative symbionts [9] that might not have been screened, results clearly point to heat sensitivity of several bacteriocyte-associated mutualists.

The goal of this study was to assess the thermal tolerance of the endosymbiont *Blochmannia*, which has coevolved with Camponotini, an ant tribe that includes *Camponotus*, the second most diverse ant genus with >1,000 species [10]. *Blochmannia* lives within bacteriocytes that line the ant midgut, as well as within ovaries, consistent with its maternal transmission from host queens to offspring. Despite severe genome reduction (to ~0.71–0.79 Mb), *Blochmannia* retains nitrogen recycling and amino acid biosynthetic functions that reflect its nutritional role in hosts [11–13]. Endosymbiont contributions to ant fitness may include more rapid and successful colony founding by mated queens, the ability of workers to rear brood more effectively, and pathogen defense [14, 15]. *Blochmannia* can be eliminated from worker ants in the lab with antibiotics, with no apparent increase in worker mortality [16]. However, the endosymbiosis is considered an obligate mutualism, since the bacteria enhance key aspects of ant colony establishment and success [14, 15], and *Blochmannia* occurs in all field-collected Camponotines sampled to date [17].

Here, we test the sensitivity of this mutualism to heat by monitoring the presence of transcriptionally active endosymbionts using real-time qPCR (RT-qPCR). Endosymbiont abundance was quantified as the ratio of *Blochmannia* 16S rRNA transcripts to *Camponotus* elongation factor 1- α (EF1 α) transcripts. We report that the ant-*Blochmannia* relationship is unstable under daily high temperatures of 37.7 °C. The observed thermal instability of this obligate mutualism may have implications for understanding potential responses of the ant host to climate change.

Materials and Methods

Ant Collection

Three *Camponotus* colonies were collected in the field and included in this study. First, a colony of *Camponotus pennsylvanicus* (colony ID cpenn1) was collected on 30 June 2009 in Falmouth, MA, USA (GPS: N41.58°34.71'42.78", W70.61°36.44'26.08"). The vast majority of ants were workers, but significant numbers of alates and last (fourth)-instar larvae were also collected. In addition, we collected two colonies of *C. chromaiodes*, a species that is very closely related to *C. pennsylvanicus*. These colonies included: cchrom1 collected on 11 April, 2012 in Raleigh, NC, USA (GPS: N35°45'44.15", W78°41'2.12") and cchrom2

collected on 13 June, 2012 in Raleigh, NC, USA (GPS: N35°53'32", W78°35'12"). Both *C. chromaiodes* colonies contained various castes distributed throughout a large, mostly rotten log. Only distinctive minor and major workers were included in containers for experimental treatments.

Ant Maintenance

Throughout the experiments, ants were reared inside plastic containers (L×W×H=33×23×7.6 cm) with side walls smeared with fluon (Insect-a-Slip, BioQuip Products, Inc., CA) to prevent ants from escaping. All ant cages were provided with Bhatkar's ant diet [18] supplemented with frozen German cockroach (*Blattella germanica*). Tubes for water (with cotton stop) and hiding were wrapped with black paper. An extra water bottle with a paper towel stop was provided in each cage to prevent dehydration. Ants clustered on wet surfaces such as the paper stop during heat treatment. Ants were fed twice per week, at which time the water bottle and old food were replaced and any dead ants removed. Ant diet was stored at 4 °C and made fresh each month.

Experimental Treatments

For all colonies, media workers were excluded for the purpose of clarity in caste determination (minors versus majors) and interpretation of results. The structures of each experiment are detailed as "Electronic Supplementary Material" (ESM) Supplementary Methods. Briefly, several minor worker ants (10 to 15) from all collected ant colonies were frozen within 36 h of field collection to assess initial endosymbiont densities ($t=0$). Remaining minor workers (and other stages such as unmated queen alates when available) were divided into replicate groups and reared at distinct temperatures: room temperature (RT), heated, and for cpenn1, cool (Table 1). The RT treatments were kept on the bench top with min/max temperatures recorded using lab thermometers. Cool and heated treatments were kept in Percival incubators (Percival Scientific Inc., IA, USA) at precise temperatures and a photoperiod of 14 h of light and 10 h of dark, with night/day temperature shifts occurring over a 3-h ramp time. Experiments were continued for 4 or 16 weeks. At several points throughout each experiment, a subset of the sample (two to nine ants) were removed from the dishes and frozen at −80 °C for later analysis. Molecular analyses below were restricted to gasters of minor workers and unmated female alates. Ant survival was high throughout the experiments. For instance, the mortality during the 16-week experiment was less than 20 % for heat-treated workers and less than 10 % for the workers kept at room temperature.

Dissection of Samples

For workers, we used whole gasters for bacterial density measurements unless otherwise described. Whole gasters were snipped from frozen ants using dissecting scissors. For some minor ants in colony cchom2, we dissected the gaster before freezing at −80 °C. These workers were euthanized by freezing on dry ice, the gaster snipped off into RNAlater (Life Technologies) to protect RNA integrity as tissues thawed, and then dissected in RNAlater into two parts: the midgut and the 'rest of the gaster' (the abdomen without the midgut). Likewise, for cpenn1 female alates, we dissected the gaster of frozen samples into three portions for separate analysis: queen midgut, ovaries, and the 'rest of the gaster' (which included other organs, storage molecules, the exoskeleton, etc.). Heads of minor workers served as negative controls in RT-qPCR below.

RNA Preparation

Total RNA was prepared from dissected tissues of each individual ant with TRIzol reagent (Invitrogen). RNA samples were treated with Turbo DNase (Ambion) to remove trace

amount of genomic DNA. The absence of DNA was confirmed using regular PCR with total RNA as template for selected samples from each batch of RNA samples. The concentration of RNA samples was quantified with NanoDrop 1000 spectrophotometer and stored at -80°C for later analysis.

Quantitative PCR

We used one-step real-time, quantitative reverse-transcription PCR to measure the copy numbers of *Blochmannia* 16S rRNA transcripts and host EF1 α within each individual sample (iScript One-Step RT-PCR kit with SYBR Green, Bio-Rad). Transcript copy numbers were quantified in absolute terms, using plasmid standards of known copy numbers in each of the RT-qPCR plates. The ratio of *Blochmannia* 16S rRNA/host EF1 α was then calculated to compare the relative endosymbiont abundances across samples. The RT-qPCR conditions, primer sequences, PCR efficiencies, and methods for plasmid curves are provided as ESM Supplementary Methods.

Statistical Analysis

Statistics were performed with JMP Pro 9 (SAS, NC). The nonparametric Wilcoxon test was used to compare the 16S rRNA:EF1 α ratio among all temperature treatments and time points, within each experiment. Significance is reported at the $p < 0.05$ level.

Results

Copy Number of *Blochmannia* 16S rRNA Varied Across Temperature Treatments

Absolute copy number of *Blochmannia* 16S rRNA per host individual varied widely among samples, notably decreasing two to three orders of magnitude under heat treatment (ESM Supplementary Fig. 1; Supplemental Table 1). By contrast, we detected a very little variation in the absolute copy number of the host EF1 α transcript (ESM Supplementary Fig. 1). This pattern confirms the utility of EF1 α as a reference gene to normalize for any variation in the amount of total RNA isolated from a given sample. In light of the consistency of EF1 α transcript abundance across samples, the observed temperature-related variation in the 16S rRNA:EF1 α ratio reflects variation in 16S rRNA transcript copies (not variation in EF1 α). We thus interpret the 16S rRNA:EF1 α ratio as reflecting the abundance of transcriptionally active *Blochmannia*.

Heat Depletes Transcriptionally Active *Blochmannia* in Ant Workers

Minor workers subjected to heat treatment (up to 37.7°C during the day) for 3 to 4 weeks showed a significant decline of endosymbiont transcripts, as represented by the ratio of 16S rRNA: EF1 α . For *C. chromaiodes* cchrom1, this ratio was significantly lower in the heated treatment than the RT treatment at $t=3$ and 4 weeks (Fig. 1a). The ratio significantly declined between the $t=3$ and $t=4$ under heat treatment. Similar results were observed in *C. pennsylvanicus* workers (Fig. 1b). When considering only the $t=3$ -week time point, the ratio under heat treatment was significantly lower than the cool treatment (but not significantly different from RT treatment). By 4 weeks, heat-treated *C. pennsylvanicus* workers showed significant endosymbiont depletion compared to either RT or cool treatments.

Heat Depleted Transcriptionally Active *Blochmannia* in Midguts and Ovaries of Unmated Ant Queens

We tested the effects of temperature on transcriptionally active *Blochmannia* in the midgut versus ovary of unmated *C. pennsylvanicus* queens collected from the nest. In both midguts and ovaries, the 16S rRNA:EF1 α ratio was consistently lower in heat-treated than RT samples (Fig. 2). The further decline of this ratio between 3 and 4 weeks suggested

continued symbiont loss (although comparisons involving the limited $t=4$ sample were not significant). At $t=4$, ratios were exceedingly low (0–1.9), falling within the range for negative controls (0.04–1.2 for worker heads) and thus consistent with complete symbiont elimination (Supplemental Table 1). The 16S rRNA:EF1 α ratio in the remainder of the queen gaster (after the midgut and ovaries were removed) was also exceedingly low (0–2.6) (Supplemental Table 1).

Four-Month Heat Treatment of Minor Workers Failed to Eliminate Bacterial Transcripts

In order to test for complete *Blochmannia* elimination from workers, minor ants from *C. chromaiodes* colony cchrom2 were subjected to a longer heat treatment of 16 weeks. Upon field collection ($t=0$), the 16S rRNA:EF1 α ratio in workers was quite high (mean >12,000; Fig. 3). Compared to these initial values, a significant decline occurred by week 4 in both RT and heat-treated workers. This decline represents a 73 % reduction in the ratio for RT-treated ants and a more pronounced, 97 % reduction in heat-treated ants. Significant *Blochmannia* depletion in heat-treated (compared to RT) samples was observed at $t=7$, 12 and 16 weeks. Ants at RT showed a significant increase in the 16S rRNA:EF1 α ratio between 12 and 16 weeks, suggesting potential recovery from earlier symbiont loss.

Our data indicate that 12–16 weeks of heat treatment significantly depleted *Blochmannia*, as heat-treated workers showed exceedingly low values for the 16S rRNA:EF1 α ratio (Fig. 3; Supplementary Fig. 2) and for absolute 16S rRNA copy number (Supplementary Fig. 1). In two worker individuals, these values fell within the range observed for negative controls (Supplementary Fig. 2). Thus, while *Blochmannia* persist at low levels in most heat-treated workers, complete elimination likely occurred in a subset of heat-treated individuals.

Blochmannia are Abundant in Worker Midguts But Not Other Abdomen Organs

In the absence of the colony queen, worker ovaries may develop and foster endosymbiont replication [16]. As noted above, most of our worker samples were whole gasters, which include the midgut and any developed ovaries. To test the potential contribution of any *Blochmannia* populations in ovaries, we included samples in which worker midguts were dissected and separated from the rest of the gaster (which contains any developed ovaries and other abdomen organs). During dissections of minor workers, we did not detect any ovary development. RT-qPCR of samples confirmed that the midgut was the major organ harboring bacterial transcripts (Fig. 4; Supplementary Fig. 2). This pattern indicates that when considering whole gaster samples, the observed decline of the 16S rRNA:EF1 α ratio under heat treatment mostly reflects a depletion of midgut-associated *Blochmannia*.

Discussion

Combined, our results indicate that heat treatment significantly reduces the abundance of *Blochmannia* 16S rRNA transcripts in ant workers and female alates. In each experiment we performed, the average ratio of *Blochmannia* 16S rRNA to EF1 α transcripts declined by >99 % after just 4 weeks of heat treatment, compared to RT controls sampled at the same time point. This result implies a loss of transcriptionally active endosymbionts due to bacterial death. Interestingly, in most ant individuals, very low levels of *Blochmannia* transcripts persisted above the range found in negative controls even after 16 weeks of heat treatment, suggesting the heat did not entirely eliminate all endosymbionts. An avenue for future work will be to investigate possible recovery of endosymbiont populations if heat-treated ants are returned to room temperature.

Previous studies have used antibiotics to deplete *Blochmannia* from *Camponotus* [14–16]. Based on qPCR of *Blochmannia* genes in gDNA samples, a 75 % endosymbiont reduction in

Camponotus fellah workers occurred after 3 months of rifampicin treatment [15]. Our qPCR results, though performed differently (using RNA rather than gDNA, and normalizing against a host transcript) suggest a more effective and rapid endosymbiont depletion using heat. That is, the >99 % reduction of endosymbionts that we observed was achieved after just 4 weeks of heat treatment. Thus, heat exposure provides an alternative approach to render aposymbiotic or nearly aposymbiotic ant hosts when studying the ant-symbiont interactions.

Endosymbiont densities are often measured by using RT-qPCR based on genomic DNA [19]. Due to the shorter half-life of RNA compared to DNA, quantifying endosymbiont transcripts (such as 16S rRNA) should offer a more sensitive approach to measure the abundance of metabolically active bacteria. That is, RNA is expected to degrade relatively quickly after bacterial death, whereas lingering gDNA from dead cells may over-estimate endosymbiont abundance. For example, RT-qPCR assays targeting 16S rRNA were ten times more sensitive than those targeting 16S rDNA in citrus pathogen, *Candidatus Liberibacter asiaticus* [20]. This said, disadvantages of targeting transcripts include greater caution required when working with degradable RNA and higher associated costs.

In contrast to apparent *Blochmannia* death at highs of 37.7 °C, the related free-living bacterium *Escherichia coli* is known to survive up to 55 °C [21]. While the mechanisms underlying the unusual heat sensitivity of *Blochmannia* remain unclear, genomic features of this and other long-term obligate mutualists could make these bacteria particularly vulnerable to thermal stress. First, the typically extreme AT richness of DNA in *Blochmannia* and most other bacteriocyte-associated mutualists (though exceptions exist [22]) could be unstable under high temperature, due to the lower thermostability of AT pairs or destabilizing effects of AT pairs on DNA stacking [23]. Coupled with AT-richness, endosymbionts typically have thermolabile structural RNAs [24] and show reduced structural integrity of proteins due to biases amino acid substitutions [25]; these structural changes may heighten heat sensitivity. However, the relatively moderate GC content of (54 % [26]) of the heat-sensitive weevil endosymbiont (*Sitophilus oryzae* primary endosymbiont or SOPE) suggests that base compositional biases alone cannot entirely explain thermal sensitivity. Contributing factors might also include minimal transcriptional responses of endosymbionts to environmental changes [27, 28] including heat stress [27], and the loss of cell membrane components.

Alternatively, thermal sensitivity of endosymbionts may also be caused by as-yet uncharacterized stress responses of the host. While heat did not increase mortality of adult ants in our experiments, heat-treated alates and workers showed signs of physiological stress. In general, alates in the heat-treated samples dealated themselves, which may be a sign of heat stress. Some evidence suggests that when queens are not able to fly to mate, they shed wings and adopt worker roles or even queen-like roles though they are infertile [29]. In addition, larvae disappeared (probably eaten) immediately in heat-treated dishes, and lasted longest in cool temperatures. Among workers, signs of heat stress included their gathering on wet surface areas within dishes, such as the wet paper towel or wet cotton ball, and remaining there for the entire hot temperature period. Preliminary work showed that without these wet surface areas available, worker mortality greatly increased under heat (Fan and Wernegreen, personal observation). Workers might drink more water under heat or use these surfaces for evaporative cooling.

Our results demonstrate heat susceptibility of *Blochmannia*, but they do not address the possibility of symbiont adaptation to changing temperatures. Previous studies in other symbiotic systems indicate that temperature adaptation in microbial partners of insects can occur, with important implications for host fitness and ecology. For example, adaptive

evolution among fungal associates of leaf-cutter ants led to increased cold-tolerance, enabling winter fungiculture and allowing the expansion of this association northward [30]. In addition, while genome reduction and lack of horizontal transfer may limit adaptive evolution in long-term endosymbionts of insects (see above), recent work offers a precedent for ongoing temperature adaptation despite these genomic constraints. In populations of *Buchnera*, the long-term mutualist of aphids, a recurrent mutation within a regulatory region of the gene *ibpA* disrupts the expression of this small heatshock protein, further reducing the (already limited) heat shock response of this endosymbiont [31]. Aphids containing *Buchnera* with this mutation have an advantage under cool temperatures, but are more vulnerable to thermal stress in lab [31] and field [32] settings. The rarity of the heat-sensitive mutation in natural populations (<3 % of aphids screened) is consistent with selection favoring IbpA production and greater heat tolerance [33]. It remains unknown whether *Blochmannia* populations harbor genetic variation for heat tolerance, and whether any such variation may be shaped by selection within or among host ant colonies. If so, it is possible that temperature adaptation of endosymbionts could influence thermal responses of the *Blochmannia*-Camponotine relationship.

In sum, while the ecological relevance of *Blochmannia* depletion in natural settings is largely unexplored, the loss of these essential mutualists under heat may affect host responses to a warming climate. Broadly speaking, microbial symbionts mediate ecologically significant traits and therefore may facilitate adaptation to environmental variation [34]. However, highly dependent mutualisms tend to be vulnerable to environmental stresses, and the breakdown of such interactions through mutualist abandonment or coextinction may accelerate biodiversity loss under global climate change [3]. Long-term, bacteriocyte-associated mutualists of insects exemplify this vulnerability. While adaptation to temperature fluctuations is possible (see precedents above), genomic constraints likely limit the evolutionary potential of such mutualists. Our results add to growing evidence that long-term endosymbionts are sensitive to increased temperature and may constrain insects' responses to heat. In this sense, essential bacterial mutualists may be the Achilles' heel of thermal responses of their insect hosts [4].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Heat treatment depleted *Blochmannia* in both **a** *Camponotus chromaiodes* and **b** *C. pennsylvanicus* workers. Minor worker ants subjected to heat treatments for 3 to 4 weeks showed a significant decline in transcriptionally active *Blochmannia*, quantified as the ratio of *Blochmannia* 16S rRNA: ant host EF1α transcripts. In both experiments, this ratio was significantly lower under heat treatment compared to the RT or cool treatments. Bars indicate the mean $\pm$ standard error. Each bar represents **a** $n=3$ workers for RT and $n=6$ for heat-treated samples, and **b** $n=6$ for all samples except the $t=4$ heat-treated sample ($n=5$). Letters above bars indicate treatment levels that were significantly different from each other based on pairwise comparisons ($p<0.05$; Wilcoxon test). The duration of the experiment is noted by weeks, where $t=0$ the starting point of the experiment soon after field collection

Fig. 2.

Heat treatment depleted *Blochmannia* from tissues of unmated ant queens. In *C. pennsylvanicus*, *Blochmannia* transcripts declined in midguts (*Qg*) and ovaries (*Qov*) of alate queens under heat treatment, compared to RT or cool treatments. The abundance of *Blochmannia* transcripts was quantified as the ratio of *Blochmannia* 16S rRNA: ant host EF1α transcripts. Bars represent the mean $\pm$ standard error. Each bar represents $n=3$ ants for all treatments, except for the heat-treated, $t=4$, *Qov* and *Qg* samples for which $n=2$ due to limited sample availability. Letters above bars indicate treatment levels that were significantly different from each other based on pairwise comparisons ($p < 0.05$; Wilcoxon test). The duration of the experiment is noted by weeks, where $t=0$ the starting point of the experiment soon after field collection

Fig. 3.

Blochmannia transcripts declined under long-term (16-week) heat treatment in *Camponotus chromaiodes*. The abundance of *Blochmannia* transcripts was quantified as the ratio of *Blochmannia* 16S rRNA: ant host EF1 α transcripts. Bars indicate the mean $\pm$ standard error. Each bar represents $n=9$ ants, except for the 4-week samples for which $n=3$. Letters above bars indicate treatment levels that were significantly different from each other based on pairwise comparisons ($p<0.05$; Wilcoxon test). The duration of the experiment is noted by weeks, where $t=0$ the starting point of the experiment soon after field collection. *Qg*, dissected queen midgut; *Qov*, dissected queen ovary

Fig. 4.

The majority of *Blochmannia* in worker gasters reside in midgut tissues. *Camponotus chromaiodes* minor workers were exposed to heated or RT treatments, and the 16S rRNA:EF1 α ratio was measured from the midgut and the rest of the gaster (including any ovaries) after 16 weeks. For both the midgut and the rest of the gaster, the ratio was significantly lower under heat treatment. The difference between the two tissue types indicates that the midgut is the major organ harboring *Blochmannia* transcripts. Bars represent the mean $\pm$ standard error ($n=3$). Letters above bars indicate treatment levels that were significantly different from each other based on pairwise comparisons ($p<0.05$; Wilcoxon test)

Table 1

Temperature regimes (degree Celcius were performed on the bench top and min/max temperatures recorded with lab thermometers. Heated and cooled treatments were performed in an incubator with programmed daytime (d) and nighttime (n) temperatures

	RT	Heated	Cool
cpenn1	24–25	25n, 37.7d	14n, 25d
cchrom1	21–22	23.3n, 37.7d	NA
cchrom2	21–22	23.3n, 37.7d	NA

NA not applicable