**Implications of Ambient Heat for Immunity and the Gut Microbiome of a Wild Primate**

by

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## Dedication

This dissertation is dedicated to the people who have supported my dreams unconditionally: my family, my fiancé, and my steadfast friends.

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## Abstract

Temperature is a ubiquitous environmental stressor that drives the evolution of tolerance to distinct thermal environments. High temperatures, in particular, place strong selective pressure on individuals by disrupting cellular stability and oxygen availability. Understanding responses to ambient heat is therefore essential for predicting how thermal environments influence health and evolutionary fitness. Research on thermal tolerance has largely emphasized thermoregulatory adaptations that help maintain stable body temperatures. However, heat tolerance is also limited by the ability of essential biological processes to proceed at elevated temperatures. Thermal tolerance, therefore, reflects not only how effectively organisms regulate body temperature but also the fundamental constraints that heat imposes on physiology. Investigating how these constraints contribute to heat tolerance is critical for predicting the consequences of heat exposure and understanding how thermal tolerance evolves.

In this dissertation, I examine how naturally occurring variation in ambient temperature influences two systems expected to be highly sensitive to heat: the immune system and the gastrointestinal microbiome. However, much of what we know about the effects of heat on these systems comes from experimental research, and it remains unclear whether temperature is a strong predictor of their function in natural populations. Therefore, this work evaluates the importance of ambient temperature in shaping these systems in real-world contexts, while also assessing the specific consequences of high temperatures for health and survival.

I conducted this work in wild white-faced capuchin monkeys (*Cebus imitator*) living in a tropical dry forest at the Taboga Forest Reserve in Guanacaste, Costa Rica. High temperatures likely represent an important environmental stressor for this species, and the study site experiences pronounced seasonal temperature variation, including distinct periods of extreme heat. Together, these conditions provide a valuable natural system for evaluating the physiological consequences of heat in a species predicted to be especially sensitive to high temperatures. I measured immune activity using urinary neopterin, a noninvasive biomarker of

cell-mediated immune activation. I also characterized gut microbiome taxonomic composition using 16S rRNA sequencing and functional potential using metagenomic sequencing.

Ambient temperature was a strong predictor of immune activity and gut microbiome composition compared to commonly considered demographic and ecological factors (e.g., age, sex, social group, diet, and rainfall). Because these data were collected from a wild population, these patterns could reflect responses to confounding ecological variables that co-occur with hot periods rather than direct responses to temperature. However, the observed changes in immune activity and the gut microbiome closely resemble responses documented in experimental studies of heat stress. This suggests that the patterns observed here reflect the effects of ambient heat and underscore the importance of temperature in influencing the function of these systems.

My results suggest that the sensitivity of the immune system and gut microbiome to high temperatures may reduce host resilience to heat. Elevated temperatures were associated with reduced immune activity and microbiome shifts consistent with a loss of functions beneficial to host health. These effects could increase the risk of infectious disease or inflammatory bowel disorders during exposure to high temperatures. While experimental work has suggested that heat-induced changes in these systems may constrain heat tolerance through disease risk, these findings provide some of the first evidence of how these processes operate in natural populations. These findings can also help predict the health and fitness consequences of rising global temperatures from climate change.

# Chapter 1 Introduction

## 1.1 Background

Biological processes are inherently sensitive to ambient temperature because thermal conditions directly influence metabolic rate. As temperatures rise, enzymatic reactions proceed more quickly, increasing metabolic activity and enhancing the efficiency and productivity of processes such as locomotion and reproduction (Arnoldi et al., 2025; Somero, 2004). However, these benefits of heat occur within a limited thermal range. Exposure to high temperatures can also compromise protein structural stability (Fields, 2001) and cause oxygen demand to exceed supply (Pörtner, 2002), reducing the energy available to maintain typical metabolic function. As a result, heat exposure can impair growth and reproduction (Pörtner & Knust, 2007) and, in extreme cases, lead to tissue damage and death (Leon & Helwig, 2010). Consequently, understanding how organisms respond to ambient heat is essential for predicting how thermal environments shape individual health and fitness outcomes.

Species have evolved thermal tolerances that reflect the temperature ranges of their historical environments. For example, thermal tolerance often follows a latitudinal gradient, with broader tolerance ranges observed in species at high latitudes where there is greater seasonal variation in temperature (Addo-Bediako et al., 2000; Sunday et al., 2010). These patterns indicate that different thermal environments impose distinct selective pressures on individuals (Buckley & Huey, 2016). Importantly, intraspecific variation and heritability of thermal tolerance fulfill the conditions under which natural selection can shape these traits (Hangartner & Hoffmann, 2016; Nati et al., 2021).

Research on the evolution of thermal tolerance has largely focused on thermoregulatory adaptations that help maintain stable body temperatures despite changes in ambient conditions (Angilletta et al., 2002, 2010). Heat tolerance, specifically, depends on coordinated physiological and behavioral mechanisms that reduce heat load, such as cellular stress responses (Lindquist & Craig, 1988), evaporative cooling (Campos & Fedigan, 2009), microclimate selection (Barrows, 1981), and changes in activity patterns (Iii et al., 2006). However, heat tolerance is also

constrained by the ability to maintain sufficient metabolic function at high temperatures. Sufficient heat exposure causes metabolic processes to deteriorate due to the destabilizing effects of heat on cells (Fields, 2001) and the mismatch between oxygen supply and demand (Pörtner, 2002). Therefore, heat tolerance isn't solely determined by thermoregulatory adaptations, but also by the fundamental constraints of heat on metabolism (Sinclair et al., 2016). Investigating how these constraints affect diverse biological processes is essential for predicting the consequences of high temperatures and understanding how thermal tolerance evolves.

### 1.2 Study systems

Evaluating which biological processes are most affected by heat can provide insight into specific systems that constrain thermal tolerance. In this dissertation, I focus on two systems expected to be particularly vulnerable to elevated temperatures: the immune system and the gut microbiome (Evans et al., 2015; Sepulveda & Moeller, 2020). Both systems are sensitive to heat stress and play essential roles in supporting health, disease resistance, and energy balance.

Research on heat exposure and the immune system spans over 50 years, originating from early efforts to understand the immunostimulatory effects of fever. Acute heat exposure can enhance immune activity and promote inflammatory responses that, in the context of fever, improve pathogen defense and health outcomes (Hasday, 2000). However, chronic heat exposure can suppress immune function, reducing the immune system's ability to respond effectively to challenges (Chauhan et al., 2021; Inbaraj et al., 2016). Consistent with these findings, much empirical work has linked heat-induced immunosuppression to increased infection risk and poorer health outcomes (Hu et al., 2007; Meng et al., 2013). Therefore, disease vulnerability from exposure to high temperature may be a key mechanism linking heat exposure to reproductive success.

Although this idea has been widely proposed, there is limited evidence showing how this process operates in natural populations. To date, research on the negative effects of heat on the immune system has been conducted almost entirely in laboratory and experimental settings (Maloney & Duffy, 2024). Although this work is essential for isolating the effects of heat, it may not fully capture how the immune system responds to high temperatures under natural conditions. Quantifying the heat experienced by individuals in real life is challenging because high temperatures are rarely static or uniform. Instead, variation in weather (M. R. Kearney et al., 2012), microclimate exposure (M. Kearney et al., 2009), and thermoregulatory behavior

(Campos & Fedigan, 2009) shape thermal exposure in ways that are difficult to replicate experimentally. Consequently, there is limited evidence for how this mechanism functions in wild populations. Field studies of wild animals provide a powerful complementary approach for examining how immunity responds to natural variation in ambient temperature under ecologically realistic conditions.

Our understanding of the consequences of heat for the gut microbiome is far more limited compared to the immune system. However, increasing recognition of the microbiome's importance for host health has driven growing interest in evaluating the effects of heat for microbiome composition and function (Sepulveda & Moeller, 2020). The gut microbiome supports essential physiological processes for the host, including energy balance, digestion (Flint et el., 2012), immune function (Corrêa-Oliveira et al., 2016), and even aspects of brain function (DeCasien et al., 2026). Experimental studies suggest that heat stress can alter both the composition and functional capacity of the gut microbiome in ways that may compromise these beneficial roles. For example, across diverse host species, temperature has been shown to drive consistent shifts in microbial composition and metabolic function (Huus & Ley, 2021; Sepulveda & Moeller, 2020). These changes have been linked to altered digestion (Fontaine et al., 2018), disrupted immune regulation (Chen et al., 2018), and, in some cases, reduced reproduction and survivorship (Zhang et al., 2019). Together, these findings have led to the hypothesis that heat-driven changes in the gut microbiome may constrain host heat tolerance.

However, similar to the immune system, this evidence comes almost entirely from experimental and laboratory study systems (Huus & Ley, 2021; Sepulveda & Moeller, 2020). As a result, we have little understanding of how these processes occur in a natural population, or even if temperature is an important predictor of the gut microbiome in real-world environments. Research on the gut microbiome of wild hosts has historically focused on ecological drivers of composition, such as changes in diet, rather than abiotic factors like temperature (Amato, 2016). Further, because the microbiome is sensitive to diverse environmental stimuli, it remains unclear whether the effects of heat stress under experimental conditions occur in natural environments.

In this dissertation, I attempt to fill these knowledge gaps by examining how ambient heat influences the immune system and gut microbiome of a wild population of white-faced capuchin monkeys (*Cebus imitator*). Specifically, I ask: (1) Does ambient temperature predict immune activity? (2) Does ambient temperature predict gut microbiome composition and

functional potential? (3) Do high ambient temperatures predict changes to these systems with health and survival implications? I show that ambient temperature is an important predictor of both of these systems in a natural environment. Specifically, I provide some of the first evidence from a wild population that heat-driven immunosuppression may increase disease risk, supporting its role as a mechanism linking heat exposure to fitness outcomes. I also show that exposure to high ambient temperatures recapitulates the effects of experimental heat stress on the microbiome. These findings provide some of the first evidence from a natural population that microbiome sensitivity to heat may constrain host tolerance to high temperatures by compromising beneficial functions and increasing gastrointestinal disease risk. Importantly, I interpret these findings in the context of climate change to help predict the consequences of the growing mismatch between evolved thermal tolerance and present-day heat exposure.

### 1.3 Study species and field site

White-faced capuchins are a useful species for studying the effects of temperature because they may be more sensitive to heat than other sympatric primates. Ecological niche models indicate that suitable habitat for capuchins is associated with a narrower temperature range compared to *Alouatta* spp. and *Ateles geoffroyi* (Johnson & Kerry, 2018). This pattern suggests that thermoregulatory stress plays an important role in determining capuchin distributions. This sensitivity may be reinforced by their daily reliance on drinking water, which likely increases vulnerability to dehydration during periods of high temperatures. (Campos & Fedigan, 2009). Consistent with this constraint, capuchins living in seasonal environments are restricted to areas with permanent water sources during the hot, dry season (Fedigan, 1996; Fedigan & Jack, 2001). Capuchins living in seasonal environments also employ a range of behavioral strategies to reduce heat load and show evidence of selection on genes related to water balance and kidney function (Campos & Fedigan, 2009; Orkin et al., 2021). These patterns suggest that high temperatures represent a significant environmental challenge for capuchins and are likely to have important physiological consequences for this species.

My study population lives in the Taboga Forest Reserve, a 516-ha fragment of tropical dry forest in northwestern Costa Rica. Tropical dry forests are highly seasonal, characterized by a hot dry season from late November through early April and a cooler wet season during the remainder of the year (Melin et al., 2014). Average daily maximum temperatures during the dry season are approximately 35 °C and regularly exceed 37 °C, compared to approximately 32 °C in

the wet season (Tinsley Johnson et al., 2020). Daily temperature variability is also greater in the dry season, where daytime temperatures increase by nearly 4 °C more than in the wet season. (Campos & Fedigan, 2009). This pronounced seasonal variation provides a wide range of ambient temperatures, including distinct periods of high temperatures, for evaluating physiological responses to heat.

Importantly, tropical dry forests undergo pronounced ecological changes between seasons. Minimal rainfall during the dry season drives widespread leaf loss in deciduous trees, reduces fruit availability, and causes many water sources to dry up, resulting in several months of resource scarcity (Melin et al., 2014). These conditions impose energetic and physiological challenges for capuchins (Bergstrom et al., 2017; Carnegie et al., 2011), and make it difficult to distinguish the effects of heat exposure from those of resource limitation (Orkin et al., 2019; Webb et al., 2023).

However, the Taboga capuchins do not appear to experience the degree of resource scarcity typically observed during the dry season in tropical dry forests. They have year-round access to water from rivers and irrigation systems and can exploit adjacent agricultural areas and non-deciduous forest (e.g., riparian and alluvial palm forest) for food during the dry season (Tinsley Johnson et al., 2020). Consistent with this, biomarkers of physiological stress do not differ between seasons at Taboga, unlike patterns reported at other sites (Beehner et al., 2022). Taboga also supports capuchin densities two to six times higher than most sites, likely reflecting greater year-round resource availability (Tinsley Johnson et al., 2020). Together, these features make the Taboga population a unique system for examining the physiological consequences of high temperatures independent of the resource scarcity that typically accompanies the dry season.

Capuchins are also a valuable species for predicting the consequences of rising temperatures due to climate change. Because tropical environments experience relatively little annual temperature variation, species in these regions typically evolve narrower thermal tolerances compared to temperate species (Addo-Bediako et al., 2000; Sunday et al., 2010). As a result, even modest temperature increases may exceed their evolved thermal tolerance. This makes tropical species particularly vulnerable to climate warming because they already live much closer to their thermal limits than temperate species (Tewksbury et al., 2008). Studying capuchins in an environment with distinct cool and hot periods, therefore, provides an

opportunity to examine physiological responses to high temperatures and anticipate the consequences of climate change.

## 1.4 Chapter overview

### *1.4.1 Chapter 2: Considering evolved thermal tolerance helps predict the immunological consequences of climate change*

Here, I review the conceptual framework for my dissertation by expanding on how the temperature sensitivity of biological processes shapes heat tolerance. I start with a summary of the evidence that thermal tolerance is an evolved trait, and use this framework to predict the immunological consequences of rising ambient temperatures due to climate change. I argue that growing mismatches between historical thermal environments and contemporary temperatures make the immune system vulnerable to dysfunction through two pathways: (1) the strong influence of temperature on metabolism, and (2) sensitivity to signals of stress that are beneficial during short-term challenges but become detrimental under chronic heat exposure. I then review how heat-driven immune dysfunction may increase disease risk and contribute to the health impacts of climate change. This review establishes the theoretical foundation for the empirical chapters that follow and highlights the importance of evaluating the effects of heat under ecologically relevant conditions.

### *1.4.2 Chapter 3 High temperatures predict decreased immune function in a wild primate*

Here, I test whether naturally occurring variation in ambient temperature predicts immune activity in wild white-faced capuchins. I use urinary neopterin as a non-invasive biomarker of cell-mediated immunity and show that neopterin concentrations increase with temperature up to a thermal optimum, beyond which they sharply decline. This nonlinear relationship is consistent with the expected temperature relationship of many biological processes. It also indicates that the predicted relationship reflects responses to ambient temperature rather than confounding factors such as resource scarcity. I also show that neopterin appears to be more sensitive to temperature in younger individuals, suggesting that the immune system in early life may be particularly vulnerable to heat. I conclude by showing that temperatures in northwestern Costa Rica have steadily increased over the past forty-five years, resulting in more days exceeding the thermal optimum observed in this species. This trend

indicates that the immunological consequences of heat exposure are likely to become increasingly important as temperatures continue to rise.

### *1.4.3 Chapter 4 High temperatures predict altered microbiome composition, metabolic capacity, and immune interactions in a wild primate*

I extend my approach from Chapter 3 to test whether ambient temperature predicts gut microbiome composition and functional potential. Consistent with patterns observed for neopterin, temperature is an important predictor of microbiome variation compared to more commonly utilized ecological and demographic variables (e.g., diet, rainfall, social group, sex, age). Temperature predicts taxonomic and functional shifts that resemble responses observed under experimental heat stress, suggesting that these changes reflect direct effects of temperature rather than confounds such as resource scarcity. I show that high temperatures may compromise essential microbiome functions that are important for host health. High temperatures also predict changes in taxonomic composition that are associated with gastrointestinal disease. These patterns suggest that microbiome sensitivity to heat may influence the thermal tolerance of capuchins. In addition, I show that microbiome responses to temperature depend on neopterin concentration, indicating that microbiome sensitivity to heat is shaped by host immunity. This likely reflects the close functional relationship between the gut microbiome and immune system.

### *1.4.4 Chapter 5 Ambient heat predicts conserved changes in gut microbiome composition and metabolic capacity independent of immune activation in a wild primate*

Finally, I investigate the mechanisms underlying microbiome sensitivity to high temperatures. Across diverse host species, microbiomes show similar responses to heat exposure, suggesting that elevated temperatures alter the gastrointestinal environment in ways that consistently favor some microbes over others. I show that ambient heat predicts similar changes in wild capuchins. Specifically, oxygen-tolerant bacteria increase relative to obligate anaerobes at high temperatures, consistent with a more oxidative intestinal environment typical of heat stress. Because inflammation can contribute to oxidative stress, I also test whether heat-induced immune activation (measured via neopterin) contributes to these changes. However, temperature predicts taxonomic and functional shifts in the microbiome independent of its effects on immune activation, suggesting that heat exerts strong, largely independent effects on the gut environment and immune system. I also show that potentially pathogenic taxa and microbial groups

commonly associated with chronic inflammatory conditions expand with rising temperatures. These changes may have important health consequences and could influence host resilience to heat exposure.

### 1.5 Conclusion

This dissertation examines how natural variation in ambient temperature influences the immune system and gut microbiome of wild white-faced capuchins. Across analyses, ambient temperature consistently emerged as a stronger predictor of immune activity and microbiome composition compared to other ecological or demographic factors. Although high temperatures at my study site mostly occur during the dry season, patterns across both systems indicate that heat itself, rather than correlated factors such as resource scarcity, is the primary driver of the observed changes. Elevated temperatures were associated with reduced immune activity and with microbiome shifts that may disrupt beneficial microbial functions and increase susceptibility to gastrointestinal disease. These findings suggest that the sensitivity of the immune system and gut microbiome to high temperatures may reduce host resilience to high temperatures and represent important physiological constraints on heat tolerance. These findings also inform predictions about the consequences of rising ambient temperatures due to climate change.

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# Chapter 2 Considering Evolved Heat Tolerance Helps Predict the Immunological Consequences of Climate Change

## 2.1 Abstract

Among the most damaging anticipated consequences of climate change are rising global temperatures that are expected to exceed the heat tolerance of many species worldwide. Species' heat tolerance reflects adaptation to their historical environments, and rapid increases in ambient temperatures create a mismatch between evolved thermal tolerance and present-day heat exposures. This mismatch has severe physiological implications because high temperatures can affect the function of molecular and organismal-level processes. The immune system, specifically, may be particularly susceptible to dysregulation under climate warming because it is highly sensitive to temperature. Although a rich body of empirical and clinical research has characterized the effects of heat on immunity, these findings are rarely interpreted in the context of evolved heat tolerance. Here, we provide a framework for predicting the immunological consequences of climate change by integrating results derived from five decades of immune research with evolutionary principles of heat tolerance. This framework offers a strong foundation for predicting the health and disease consequences of immune vulnerability to heat in a rapidly warming world.

## 2.2 Introduction

Anthropogenic climate change is causing rapid increases in ambient temperatures in many parts of the globe (Cook et al., 2013; IPCC, 2023). Exposure to high temperatures is predicted to be a particularly salient consequence of climate change because species have evolved thermal tolerances that were advantageous under historical environmental conditions (Bennett et al., 2021; Sunday et al., 2010). As a result, rapid warming exposes species to heat beyond their evolved upper thermal tolerance, generating a mismatch between historical thermal adaptations and present-day environmental exposures. Exposure to ambient heat beyond an organism's evolved heat tolerance can have pervasive physiological consequences. When heat

load exceeds the capacity for heat dissipation, it triggers coordinated stress responses (Horowitz, 2002), disrupts molecular and system-level processes (Fields, 2001; Pörtner, 2002), and, under extreme conditions, leads to tissue damage, organ failure, and death (Yan et al., 2006). Consequently, rising temperatures due to climate change pose substantial risks to the fitness and survival of organisms. Understanding how evolved thermal tolerance shapes vulnerability to warming is therefore critical for predicting responses to climate change.

The immune system may be particularly vulnerable to thermal mismatches under climate change because many immune processes are highly sensitive to temperature (Evans et al., 2015; Maloney & Duffy, 2024). Extensive experimental and clinical studies consistently demonstrate that immune responses to heat vary substantially with the intensity and duration of thermal stress (Beachy & Repasky, 2011), suggesting that immune function may be particularly susceptible to dysregulation under climate warming. Despite extensive documentation of immune responses to heat, however, these findings are rarely interpreted through an evolutionary lens that considers how immune sensitivity to heat is shaped by evolved thermal tolerance.

Empirical studies of immune responses to heat span a wide range of taxa, experimental designs, and thermal contexts, resulting in substantial heterogeneity in reported effects. Transient elevations in body temperature can enhance immune function by promoting inflammatory responses and improving pathogen response and are often considered beneficial, particularly in response to infection (Hasday et al., 2000). In contrast, sustained or extreme heat exposure frequently impairs immunity. Chronic heat exposure is associated with immunosuppression and increased disease risk (Chauhan et al., 2021; Inbaraj et al., 2016), and extreme heat can trigger excessive, non-specific inflammation that may be damaging (Iba et al., 2025). Thus, mechanisms of immune sensitivity to heat can be beneficial within evolved thermal limits but increase vulnerability to immune dysregulation when temperatures exceed those limits.

Here, we explore the immunological consequences of rising ambient temperatures driven by climate change and their implications for health and disease risk. We first review the evolutionary basis of and the physiological constraints on heat tolerance. We then propose that immune sensitivity to heat arises from two interdependent mechanisms: (1) the passive effects of temperature on metabolism and its implications for immune efficacy, and (2) active changes in immune regulation via stress signaling. We show how these mechanisms are beneficial within evolved thermal ranges but may cause immune dysregulation when temperatures exceed them.

We argue that immune dysregulation from heat stress has two distinct consequences –recurrent sterile inflammation and chronic immunosuppression– and examine their implications for health and disease risk. Finally, we highlight the utility of wild animal systems as models for quantifying the impacts of rising ambient temperatures on immunity. Our approach integrates more than five decades of empirical work on immune sensitivity to temperature with evolutionary principles of heat tolerance and provides a framework for predicting the immunological consequences of climate change.

### 2.3 Heat tolerance has evolved

Species vary widely in their thermal tolerances. For example, thermal tolerance often follows a latitudinal gradient, with broader tolerance ranges observed in species at high latitudes where there is greater seasonal variation in temperature (Addo-Bediako et al., 2000; Sunday et al., 2010). Such patterns indicate that different thermal environments impose distinct selective pressures (Buckley & Huey, 2016), leading to the evolution of tolerance to temperatures most relevant to a specific environment. Importantly, intraspecific variation in thermal tolerance (Nati et al., 2021; Sasaki et al., 2022), combined with heritability of these traits (Benfey et al., 2024; Hangartner & Hoffmann, 2016), fulfills the conditions under which natural selection can shape thermal tolerance. While both ends of the thermal tolerance range can evolve, the physiological consequences of heat exposure differ fundamentally from cold exposure. Cold exposure primarily slows metabolic processes (Fields, 2001), while heat more directly threatens cellular structure and function. Exposure to high temperatures destabilizes cellular membranes (Vigh et al., 2007), disrupts mitochondrial function (Belhadj Slimen et al., 2014), and denatures proteins, disrupting proteostasis (Ahmed et al., 2020). Disruption of proteostasis is a particularly salient consequence of heat exposure because proper protein folding underlies nearly all cellular processes and directly influences survival, longevity, and reproductive success. Consequently, exposure to high temperatures is expected to impose especially strong selective pressures on mechanisms that confer tolerance to heat.

The cellular heat shock response helps preserve proteostasis during heat exposure. The accumulation of denatured and aggregated proteins activates a group of molecular chaperones, heat shock proteins (HSPs), that refold proteins or degrade irreversibly damage ones (Lindquist & Craig, 1988; Richter et al., 2010). Exposure to high temperature rapidly (within minutes) activates heat shock transcription factors (HSFs) that promote HSP gene expression (Lindquist,

1986). The resultant HSPs are classified into families based on their molecular weights; large heat shock proteins (e.g., HSP90, HSP70) actively refold or target damaged proteins, and small HSPs (typically <30 kDa) stabilize unfolded proteins and prevent aggregation (M. E. Feder & Hofmann, 1999; Lindquist & Craig, 1988). This response is conserved across evolutionary lineages, with genes regulating HSP expression present across all taxa and cell types studied to date. Its presence in eukaryotes, archaea, and eubacteria indicates that the heat-shock response originated in single-celled organisms more than 2.5 billion years ago (Singh & Hasday, 2013). This extreme conservation reflects a universal cellular vulnerability to protein misfolding, with heat likely acting as a major selective pressure due to its destabilizing effects on protein structure.

However, the heat shock response cannot indefinitely extend heat tolerance because it is costly for cells (M. E. Feder & Hofmann, 1999; Sørensen et al., 2003). HSPs suppress general protein synthesis (Duncan & Hershey, 1984), and the chaperone functions of large HSPs are also energetically costly because they require ATP (Richter et al., 2010). These costs have been demonstrated in *Drosophila* larvae, where more severe heat stress, eliciting higher HSP70 expression than milder heat stress, reduced both cell growth rates (J. H. Feder et al., 1992) and larval survival (Krebs & Feder, 1997). The tradeoff between the immediate benefits and downstream costs of the heat shock response limits it to conferring only transient heat tolerance, providing time for behavioral adjustments and physiological mechanisms to reduce thermal load. However, the extreme evolutionary conservation of the heat shock response suggests that its benefits outweigh its costs, likely because it confers an immediate survival advantage during periods of cellular stress (Krebs & Feder, 1997). Together, these patterns reflect strong selection on cellular mechanisms that confer heat tolerance and emphasize the negative consequences of exposure to heat, especially at temperatures that exceed evolved thermal limits.

### *2.3.1 Physiology constrains heat tolerance*

Despite the severity of heat exposure for cells, the evolution of heat tolerance appears to tightly be constrained. Across species, there is far greater variation in cold tolerance than in heat tolerance (Addo-Bediako et al., 2000; Araújo et al., 2013), with most eukaryotes unable to tolerate temperatures above ~47 °C (Pörtner, 2002). Two non-mutually exclusive explanations have been proposed to explain the convergence of heat tolerance: that upper thermal limits converge on a shared optimum due to similar selective pressures, or that their evolution is

fundamentally constrained by physiology (Araújo et al., 2013; Bennett et al., 2021). There is some support for the first explanation, as the cellular consequences of heat are so severe that even infrequent exposures to high temperatures may impose strong selective pressure, particularly when such exposure results in death (Buckley & Huey, 2016). However, growing evidence also supports the latter explanation, as fundamental processes at both systems and molecular levels operate optimally within a specific thermal range. Extending tolerance at high temperatures often compromises efficiency at lower temperatures that are more likely to be experienced.

At the organismal level, heat tolerance is shaped by the capacity of oxygen delivery to meet increasing metabolic demand. Rising temperatures accelerate metabolic rate and increase oxygen demand. However, the capacity for oxygen delivery through the respiratory and circulatory systems is finite, leading to a mismatch at high temperatures where oxygen demand exceeds supply (Pörtner, 2002). As a result, aerobic metabolism becomes constrained, forcing increased reliance on anaerobic pathways that yield less energy and cannot be sustained for prolonged periods. The shift to anaerobic pathways should impose a physiological constraint on heat tolerance because multicellular animals, particularly metazoans, depend on oxygen-based metabolism to support growth, reproduction, and maintenance (Brown et al., 2004). Consistent with this prediction, empirical work shows that reductions in cardiac rate and respiratory capacity at high temperatures coincide with declines in growth, reproduction, and population abundance in fish (Pörtner & Knust, 2007). Limits on oxygen delivery are argued to represent the primary constraint on heat tolerance in metazoans, because oxygen depletion is expected to occur before the onset of direct cellular damage from heat exposure (Pörtner, 2002).

At the molecular level, heat tolerance is shaped by an inherent tradeoff between enzyme structural stability and flexibility (Somero, 2004). As temperature increases, molecular motion accelerates, and bonds become more flexible, enhancing enzymatic reaction rates. In contrast, at low temperatures, enzymes become too rigid to efficiently catalyze reactions. The positive relationship between heat and reaction rate is finite: beyond a critical threshold, heat destabilizes enzyme structure, leading to denaturation and loss of function (Fields, 2001). As a result, enzyme function is constrained to a relatively narrow thermal range. Selection for greater heat tolerance may extend function at high temperatures (Dong et al., 2018), but this typically comes at the cost of reduced efficiency at cooler temperatures that organisms are more likely to experience (Fields,

2001). These inherent thermodynamic properties of enzymes impose a fundamental limit on the evolution of heat tolerance, as enzymes must maintain sufficient reaction rates to sustain metabolic function across the range of temperatures most frequently experienced. Although the heat-shock response can transiently extend cellular tolerance to heat, this protection comes at a cost (Sørensen et al., 2003) and cannot be sustained indefinitely.

### *2.3.2 Heat constrains biological performance*

The universal thermodynamic properties of enzymes give rise to a predictable relationship between temperature and the rate or efficacy (i.e., performance) of a biological process. This relationship, known as the thermal performance curve (TPC), describes how the performance of a biological process changes across a temperature range (Arnoldi et al., 2025). On the cooler side of the curve, biological performance increases gradually to a peak, followed by a steep decline at higher temperatures, producing an asymmetric curve (Fig 2.1). The temperature at which performance is maximized is the thermal optimum, while the thermal breath is the range between the minimum and maximum temperature where performance approaches zero (Sinclair et al., 2016). TPCs are ubiquitous across different levels of biological organization and have been documented for diverse processes such as rates of photosynthesis, running speed, and population growth (Arnoldi et al., 2025). The recurrence of this curve shape across disparate processes reflects the fundamental tradeoff between enzyme flexibility and stability with temperature (Arroyo et al., 2022). The rise in performance at low to moderate temperatures is driven by increased molecular motion and reaction rates that accelerate the rate of energy-dependent processes, whereas the rapid decline beyond the thermal optimum reflects systemic failure caused by molecular instability. The abrupt collapse on the warm side of the curve emphasizes the shared molecular constraints on heat tolerance and the inherent sensitivity of biological processes to temperature (Pörtner, 2002).

Variation in thermal optima and breadth among species reflects differences in evolved heat tolerance, shaped in part by their unique thermal environments. For example, tropical species tend to exhibit higher thermal optima but narrower thermal breadths than temperate species, consistent with adaptation to a narrower range of high temperatures characteristic of the tropics (Bennett et al., 2021; Tewksbury et al., 2008). Species-specific behavioral or physiological adaptations can also influence the parameters of their thermal performance curves. For example, physiological thermoregulation broadens thermal breadth by buffering internal

body temperature from ambient conditions, increasing the range of environmental temperatures a species can tolerate (Buckley et al., 2012). Conversely, constraints on oxygen delivery in multicellular metazoans could shift performance curves relative to those of unicellular organisms (Pörtner, 2002). Together, these lines of evidence demonstrate that while mechanisms conferring heat tolerance have evolved, upper thermal limits are also fundamentally constrained by molecular and physiological trade-offs. Considering the evolutionary basis of heat tolerance provides a mechanistic framework for predicting biological responses to and physiological ramifications of climate change, allowing us to identify which species, physiological systems, and ecological contexts are most vulnerable to rising temperatures.

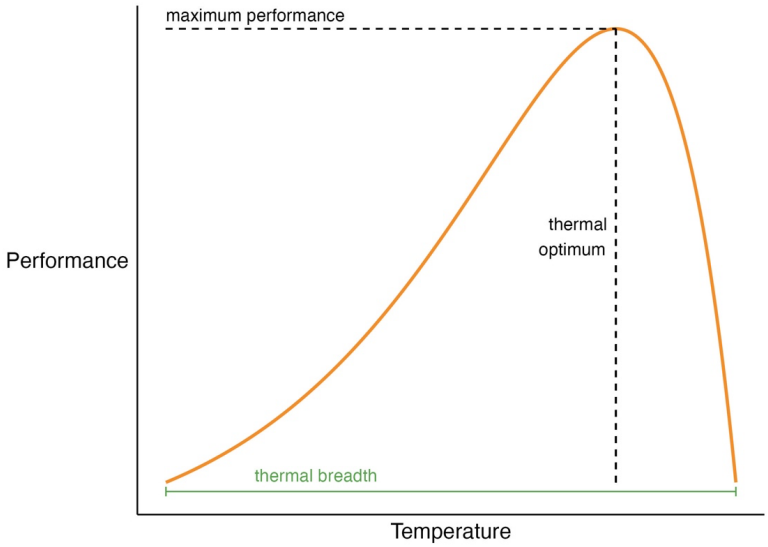



**Figure 2.1 Schematic of a thermal performance curve (TPC)**. Temperature is shown on the x-axis and the performance (i.e., rate or efficacy) of a biological process on the y-axis. The dashed horizontal line denotes the maximum attainable performance, and the dashed vertical line indicates the temperature at which maximum performance is achieved (the thermal optimum). The green horizontal line marks the range of temperatures over which performance is greater than zero, referred to as the thermal breadth. Performance increases at low to moderate temperatures as metabolic reaction rates rise, whereas the steep decline beyond the thermal optimum reflects the loss of molecular stability at high temperatures.

### *2.3.3 Climate change challenges evolved heat tolerance*

Since thermal tolerance evolves to match the range of temperatures characteristic of an organism's environment (Addo-Bediako et al., 2000; Bennett et al., 2021; Sunday et al., 2010), rapid climate warming creates an evolutionary mismatch that exposes species to temperatures that may approach or exceed their evolved heat tolerance. As described above, such exposures can lead to physiological dysfunction through whole-body constraints on oxygen delivery (Pörtner, 2002) and compromised enzyme function (Fields, 2001) that affects the performance of

biological processes (Arroyo et al., 2022). Consistent with these expectations, climate warming has driven widespread geographic range shifts, with many species moving toward higher latitudes or elevations to track suitable thermal environments (I.-C. Chen et al., 2011). Empirical evidence for range shifts spans diverse taxa from insects (Parmesan et al., 1999) to mammals (Moritz et al., 2008). However, the capacity to track shifting climates is finite, and in many species, dispersal ability is constrained by geographic barriers, ecological suitability, and habitat disturbance (Lenoir et al., 2020). This increases the likelihood of chronic or repeated exposure to high temperatures that species did not evolve to tolerate.

A typical consequence of exposure to high temperatures is heat stress, when core body temperature rises above the typical range for a species. Heat stress occurs when environmental heat load exceeds the organism's capacity for heat dissipation, triggering behavioral and physiological responses aimed at restoring a normal body temperature (Bernabucci et al., 2010). Heat stress often activates coordinated neurological and hormonal responses to reallocate energetic resources to cope with the thermal exposure (Alvarez & Johnson, 1973; Horowitz, 2002). Therefore, throughout this paper, heat stress refers specifically to elevations in body temperature that elicit compensatory responses to dissipate thermal load, arising from exposure to high ambient temperatures. Species exposed to heat at the upper limits of their thermal tolerance are more vulnerable to heat stress, highlighting why range shifts can be an effective strategy to avoid these thermal conditions. Although other environmental factors, such as humidity and air flow, can affect heat load (Renaudeau et al., 2012), here we focus on ambient temperature as the primary driver of heat stress.

In the remainder of this article, we expand on how a growing evolutionary mismatch between evolved heat tolerance and rising ambient temperatures will impact the immune system. We consider two primary mechanisms that affect immune sensitivity to heat: 1) thermal performance effects on immunity, and 2) sensitivity of immune regulation to signals of physiological stress. We discuss contexts in which these mechanisms are beneficial within evolved thermal ranges but may lead to immune dysregulation from heat stress under climate change. Specifically, we illustrate that heat-driven immune dysregulation may cause recurrent sterile inflammation and chronic immunosuppression and expand on the health consequences of each. In this way, we consider immune sensitivity to heat within the context of evolved thermal tolerance and use this framework to predict immune vulnerability to climate change.

### 2.4 Thermal performance properties affect immune sensitivity to heat

Immune responses depend on a variety of cellular mechanisms that in turn rely on enzyme-mediated reactions, making immunity subject to the same thermal constraints as other biological processes. As a result, immunity is expected to follow a thermal performance relationship, increasing with temperature to an optimum and declining rapidly thereafter (Fig. 2.1). We define immune performance as the capacity of the immune system to mount an effective response to a challenge (Sandmeier, 2024), which is commonly assessed using functional assays such as bacterial killing ability or lytic capacity (Demas et al., 2011). Like other biological processes, moderate increases in temperature should enhance immune performance by increasing metabolic rates, resulting in more available energy for processes such as phagocytosis, cytotoxic activity, and the production of effector molecules. This relationship will persist until reaching a thermal optimum, beyond which elevated temperatures disrupt protein stability, membrane integrity, and cellular metabolism, causing a rapid decline in immune performance. Importantly, because this relationship arises from the thermodynamic properties of enzymes rather than active immune regulation, thermal performance represents a passive mechanism of immune sensitivity to temperature. It enhances immunity under some conditions but can also render the immune system vulnerable to dysregulation when heat exposures exceed evolved thermal tolerance.

In line with these predictions, immune thermal performance curves have been documented across a wide range of taxa. Such relationships are well characterized in ectothermic species, including reptiles (Baker & Merchant, 2018), amphibians (Bakewell et al., 2021; Moretti et al., 2019), and insects (Ferguson et al., 2016), where body temperature closely reflects ambient conditions. However, immune thermal performance curves have also been observed in endotherms, including hamsters (Xu & Wang, 2023), voles (Xu et al., 2019), and primates (Lucore et al., 2024), demonstrating that high ambient temperatures can also affect the immunity of species with physiological thermoregulation. Differences in immune thermal performance curves among species likely reflect variation in evolved heat tolerance. For example, immune performance in ectotherms often peaks at relatively low temperatures (approximately 18–25 °C) (Bakewell et al., 2021; Ferguson et al., 2016; Moretti et al., 2019), whereas studies in endotherms suggest that immune performance peaks at higher temperatures, around 30 °C (Lucore et al., 2024; Xu et al., 2019; Xu & Wang, 2023). Higher thermal optima in endotherms

likely reflect the ability of physiological thermoregulation to increase tolerance to ambient conditions. Variation in immune thermal optima among species may also reflect adaptation to distinct thermal environments. For instance, immune performance declines above 25 °C in several species of Rhinella toads (Moretti et al., 2019), yet continues to increase through 34 °C in the desert-dwelling toad Anaxyrus punctatus (Bakewell et al., 2021), consistent with its adaptation to hotter desert climates. Together, these patterns suggest that immune performance is constrained by evolved heat tolerance from adaptation to distinct thermal environments.

Some of the strongest empirical evidence for the thermal performance properties of immunity comes from in vitro experiments with human immune cells. These studies show that short-term exposure to temperatures within the human febrile range (approximately 38–41 °C) can enhance the performance of innate immune cells, including increased phagocytic activity and bacterial killing in macrophages and neutrophils, over durations of roughly 2–6 hours (Leijh et al., 1981; Roberts, Norbert & Steigbigel, 1977). In contrast, exposure to temperatures above the febrile range (approximately 42–45 °C) rapidly impairs immune performance (Peterson et al., 1976; Sebag et al., 1977) and can be lethal to cells even over short periods. For instance, exposure to temperatures between 42–43 °C has been shown to cause rapid cell death in dendritic cells within as little as 15 minutes (Hatzfeld-Charbonnier et al., 2007). Together, these findings illustrate a clear thermal performance relationship for human immune cells and suggest that the benefits of fever do not solely arise from direct pathogen inhibition but rather an increased immune advantage at warmer temperatures (Hasday & Singh, 2000).

Importantly, the same thermodynamic principles apply whenever immune cells experience elevated temperatures, regardless of whether warming coincides with infection. For example, empirical studies demonstrate that heat stress within the febrile range induced by exercise (Jimenez et al., 2007; Peake et al., 2008) or passive heating (Pilch et al., 2023) is associated with increased immune activity, evidenced by enhanced mobilization and count of circulating leukocytes. Growing mismatches between heat tolerance and rising ambient temperatures will expose immune systems to thermal conditions they did not evolve to tolerate, increasing the risk of immune dysregulation from thermal performance effects. Repeated exposure to moderate heat stress may increasingly elevate immune activity in the absence of infection, promoting recurrent sterile inflammation (i.e., inflammation in the absence of pathogens). Even though the heat shock response can transiently support increased immune

performance during moderate heat exposure, it cannot indefinitely preserve cellular integrity under sustained or extreme thermal stress. As a result, prolonged to moderate heat or extreme heat exposure that exceeds immune thermal optima may lead to periods of immunosuppression.

## 2.5 Immune sensitivity to signals of stress affects immune sensitivity to heat

During heat stress, the effects of thermal performance on immunity do not act in isolation. In addition to the passive effects of heat, immune function is highly sensitive to signals of physiological stress, which convey information about internal challenges and actively coordinate an appropriate immune response (Matzinger, 2002; Webster et al., 2002). Heat stress can engage this system, providing an additional route through which elevated temperatures influence immunity. Therefore, while thermal performance reflects the passive consequences of temperature on immunity, stress signaling represents an active regulatory mechanism that can reshape immune function in response to heat. Immune sensitivity to physiological stress also constitutes a second mechanism by which mismatches between rising ambient temperatures and evolved thermal tolerance may increase the risk of immune dysregulation under climate change.

The immune system is responsive to a variety of molecules that signal physiological stress, but here we focus on two classes of signals that are reliably elicited by heat stress. These are (1) damage-associated molecular patterns (DAMPs), which are endogenous indicators of cellular damage (R. Chen et al., 2025), and (2) neuroendocrine signals that reflect systemic physiological stress (Webster et al., 2002). Sensitivity to these stress signals is beneficial because they coordinate immune responses to match the nature and severity of a challenge. However, climate change is expected to alter the intensity, duration, and frequency of heat-induced stress signaling, potentially increasing the risk of immune dysregulation. We expect the nature of this dysregulation to depend on the duration and severity of the heat stress because it will elicit different types of stress signals. Acute and severe heat stress promotes sterile inflammation through DAMPs (R. Chen et al., 2025), whereas chronic heat stress causes immunosuppression through neuroendocrine signaling (Cain & Cidlowski, 2017). In the remainder of this section, we examine how these stress signaling pathways shape immune processes during heat stress, and the contexts in which their effects may contribute to immune dysregulation under climate change.

### *2.5.1 Heat stress generates damage-associated molecular pathways*

DAMP signaling promotes rapid, localized immune responses to tissue damage and is particularly important for initiating inflammation under sterile conditions (Matzinger, 2002). In this context, immune sensitivity to DAMPs is beneficial because it drives inflammation required for effective tissue repair. DAMPs comprise a diverse set of endogenous molecules, including peptides, lipids, and nucleic acids, that are actively excreted by stressed cells or passively released during cell death. Extracellular DAMPs stimulate innate immune receptors, increasing proinflammatory cytokine and chemokine expression, while intracellular DAMPs activate inflammasome pathways that similarly promote inflammation (R. Chen et al., 2025; Ma et al., 2024). Together, these pathways rapidly recruit leukocytes to sites of cellular damage.

Heat stress elicits predictable cellular responses that generate DAMPs and promote sterile inflammation. For example, HSPs can act as DAMPs when released extracellularly following sterile damage (Zininga et al., 2018). At high concentrations, extracellular HSP (e.g., HSP60, HSP70) bind to Toll-like receptors 2 and 4 on innate effector cells and stimulate expression of proinflammatory cytokines (Asea et al., 2002; Quintana & Cohen, 2011). Heat stress also promotes oxidative damage that generates DAMPs. High temperatures accelerate metabolic rate and mitochondrial respiration, increasing the production and leakage of reactive oxygen and nitrogen species (RONS) from the electron transport chain (Belhadj Slimen et al., 2014). While RONS in small quantities are necessary for effective immune defense (Andrés et al., 2022), RONS production that exceeds antioxidant capacity can damage cells (Finkel & Holbrook, 2000). Because RONS are generated primarily within the mitochondrial matrix, mitochondria are disproportionately vulnerable to oxidative damage, particularly their lipid membranes and DNA (mtDNA), which has reduced repair capacity compared to nuclear DNA (Zanini et al., 2023). Oxidative damage to mitochondrial membranes allows oxidized mtDNA to escape into the cytosol (Green & Kroemer, 2004), where it can activate innate immune receptors and promote cell death (R. Chen et al., 2025). Extracellular mtDNA binds to Toll-like receptor 9 on innate immune cells, increasing proinflammatory cytokine expression (Zanini et al., 2023). Cytosolic mtDNA similarly promotes inflammatory signaling by activating inflammasomes and cytosolic DNA–sensing pathways (West & Shadel, 2017). Because oxidized mtDNA is resistant to degradation, it can accumulate in the cytosol and prolong immune activation (Gehrke et al., 2013; Zanini et al., 2023). Importantly, mtDNA damage further impairs mitochondrial respiration, exacerbating RONS production and reinforcing this cycle (Kirkinezos & Moraes,

2001). Therefore, by promoting mtDNA oxidation and HSP expression, heat stress may increase DAMP signaling and contribute to sterile inflammation.

#### *2.5.1.1 Heat-driven DAMPs cause sterile inflammation*

Studies of heat stroke demonstrate that heat stress can induce sterile inflammation through DAMP signaling. Heat stroke is a severe form of heat stress typically defined by prolonged elevations in core body temperature, often exceeding ~40°C (Yan et al., 2006). It elicits a massive systemic inflammatory response, the magnitude of which closely resembles that observed in sepsis. However, rather than being driven by pathogen-associated molecular patterns, this response is largely initiated by DAMPs released during heat-induced cellular damage (Iba et al., 2025). Excessive inflammatory activation during heat stroke can result in widespread tissue injury and contribute to multi-organ dysfunction (Yan et al., 2006). Even moderate heat exposure may generate DAMPs sufficient to promote sterile inflammation. Elevated core temperature from intensive exercise increases extracellular mtDNA levels and inflammatory markers, such as circulating leukocytes (Ohlsson et al., 2020; Trumpff et al., 2021). Exercise can also increase extracellular heat shock proteins that are associated with higher white blood cell counts (Gibson et al., 2014; Horn et al., 2007).

Climate change may increase the risk of immune dysregulation by increasing the frequency and intensity of DAMP signaling. As rising ambient temperatures exceed evolved thermal tolerance, heat stress is expected to become more frequent and severe, elevating the likelihood of DAMP generation and recurrent sterile inflammation. This risk may be particularly pronounced for mitochondrial DAMPs. Repeated heat exposure can promote cumulative mitochondrial dysfunction, increasing mtDNA oxidation and the probability of mtDNA release into the cytosol (Belhadj Slimen et al., 2014; Green & Kroemer, 2004). Therefore, even if individual episodes of moderate heat stress are insufficient to elicit strong DAMP signaling, repeated heat exposure may progressively increase cytosolic mtDNA and the likelihood of engagement with intracellular innate immune sensors. In this way, thermal mismatches under climate change may convert an otherwise beneficial damage-sensing adaptation into a driver of recurrent sterile inflammation.

### *2.5.2 Neuroendocrine signals of stress affect immune regulation*

The immune system is also responsive to signals of stress from the central nervous system (CNS). The CNS is sensitive to diverse stimuli, including adverse ecological or social environments and psychological distress, and uses the stress response to reallocate energy to cope with these challenges (Sapolsky et al., 2000). However, the effects of stress on immunity depend strongly on the duration of the stressor. Acute stress, lasting minutes to hours, transiently enhances immune activity by mobilizing leukocytes to the skin and is considered beneficial for responding to potential infection associated with injury (Dhabhar, 2009). In contrast, chronic stress, sustained over hours, days, weeks, or months, is consistently associated with immunosuppression from reduced inflammatory signaling (Glaser & Kiecolt-Glaser, 2005). Here, we focus on the immunosuppressive consequences of chronic stressors to assess how prolonged exposure to temperatures above the upper limits of thermal tolerance affects immune regulation.

The CNS primarily interacts with the immune system via glucocorticoids produced by the hypothalamic pituitary (HPA) axis and catecholamines (e.g., epinephrine and norepinephrine) produced by the sympathetic-adrenal-medullary (SAM) axis. The SAM and HPA axes function as an integrated stress response in which detection of a stressor by the sympathetic nervous system induces the rapid release of catecholamines, followed by subsequent glucocorticoid secretion from the adrenal cortex (Sapolsky et al., 2000). Chronic secretion of neuroendocrine hormones shifts immune investment away from Th1-mediated cellular immunity and toward Th2-biased, humoral responses (Padgett & Glaser, 2003; Webster et al., 2002). Elevated circulating glucocorticoids preferentially engage the glucocorticoid receptor (GR), which is broadly expressed across immune cell types (Cain & Cidlowski, 2017). GR activation reduces the expression of proinflammatory cytokines and promotes the expression of anti-inflammatory cytokines (Elenkov & Chrousos, 2002). Catecholamines reinforce this shift by activating β-adrenergic receptors expressed on Th1 cells. This suppresses Th1-associated cytokine signaling, permitting increased Th2 differentiation (Elenkov, 2007; Padgett & Glaser, 2003). Therefore, sustained glucocorticoid and catecholamine signaling suppresses cell-mediated immunity by limiting proinflammatory effector activity, while promoting humoral immune processes.

While suppression of non-specific inflammation during sustained stress may reduce the risk of self-inflicted tissue damage (Elenkov, 2007; Elenkov & Chrousos, 2002), extensive experimental evidence shows that prolonged neuroendocrine stress signaling compromises the

immune system's ability to fight infection. For example, mice subjected to restraint stress and infected with a virus exhibit reduced virus-specific cytokine production and impaired T-cell responses (Glaser & Kiecolt-Glaser, 2005; Sheridan et al., 1998). Importantly, blocking glucocorticoid receptors restores cytokine production and T-cell function, demonstrating that the immunosuppressive effect of glucocorticoids reduces the immune system's ability to respond to infectious challenges (Sheridan et al., 1998). In humans, chronically stressed populations show reduced immune responses to vaccination, a useful proxy for increased infection risk because it reflects a diminished ability to mount effective responses to immune challenges (Glaser & Kiecolt-Glaser, 2005). Additional work demonstrates that individuals who report sustained psychological distress are more likely to develop illness following inoculation with a rhinovirus (Stone et al., 1992).

#### *2.5.2.1 Chronic heat stress causes immunosuppression*

Importantly, immunosuppression from chronic heat stress reduces the ability to respond to infection. For example, mice exposed to repeated heat stress (e.g., daily exposure to 38 °C for several hours) show reduced immune responsiveness to vaccination (Hu et al., 2007; Meng et al., 2013). An extensive body of work, largely focused on livestock, further demonstrates the immunosuppressive effects of chronic heat exposure (Bagath et al., 2019; Inbaraj et al., 2016). In these studies, animals are exposed to elevated ambient temperatures for extended periods, typically 2–7 weeks at approximately 30–40 °C, depending on species. Prolonged heat exposure consistently results in broad suppression of immune function, including reductions in leukocyte counts and inflammatory signaling (Safa et al., 2019; Wojtas et al., 2014). Evidence from humans subject to chronic heat exposure from their occupations similarly shows reduced innate immune activity and lower white blood cell counts (Jafari et al., 2020; Yadav et al., 2024).

Although relatively few studies have directly linked immunosuppression from heat stress to neuroendocrine signaling, emerging evidence supports this connection. For example, pigs exposed to prolonged heat stress (21 days at ~35 °C) show elevated circulating glucocorticoids in conjunction with reduced white blood cell counts and decreased expression of IL-2 and IFN-γ, consistent with suppression of cell-mediated immunity (Ju et al., 2014). These findings suggest that sustained neuroendocrine activation during prolonged heat exposure contributes to immunosuppression. Therefore, while immune sensitivity to neuroendocrine signals of stress is

beneficial within evolved thermal limits, it leaves the immune system vulnerable to dysregulation when ambient temperatures exceed those limits.

### 2.6 Heat-driven immune dysregulation has important implications for health and disease

In the preceding sections, we outlined two mechanisms by which heat affects immunity: the passive effects of temperature on immune performance and the active regulation of immune function to signals of stress (Fig. 2.2A). We show that these mechanisms affect immunity in distinct ways that could cause immune dysregulation when ambient temperatures exceed evolved heat tolerance (Fig. 2.2B). We propose that heat-driven immune dysregulation under climate change will cause recurrent sterile inflammation and chronic immunosuppression (Fig. 2.2C). Recurrent sterile inflammation could occur when moderate heat stress transiently elevates immune activity or causes repeated generation of DAMPs that engage inflammatory pathways. Immunosuppression may arise due to thermal constraints on immune performance or when chronic heat stress engages neuroendocrine pathways that actively suppress inflammatory responses. Importantly, this framework helps reconcile opposing immune responses to heat by showing how these mechanisms produce different effects on immunity depending on the intensity and duration of the heat exposure.

Although we have discussed the distinct effects of thermal performance and sensitivity to stress signals, their impacts on immune function are not mutually exclusive. For example, sterile inflammation observed during moderate heat stress from exercise or passive heating likely reflects the combined effects of increased immune performance and activation of DAMP signaling pathways. Further, immunosuppression during chronic heat stress likely reflects the combined effects of neuroendocrine suppression of immune activity and the constraints on the heat-shock response to sustain cellular heat tolerance over extended periods. Disentangling the relative contributions of these mechanisms in immune responses to heat is likely to remain challenging because they are difficult to isolate experimentally. Consequently, predicted immune dysregulation with climate change should be viewed as the product of both passive thermal performance constraints and changes in active immune regulation via stress signaling. Next, we consider the distinct health implications of sterile inflammation and immunosuppression in the context of climate change (Fig. 2.2D).

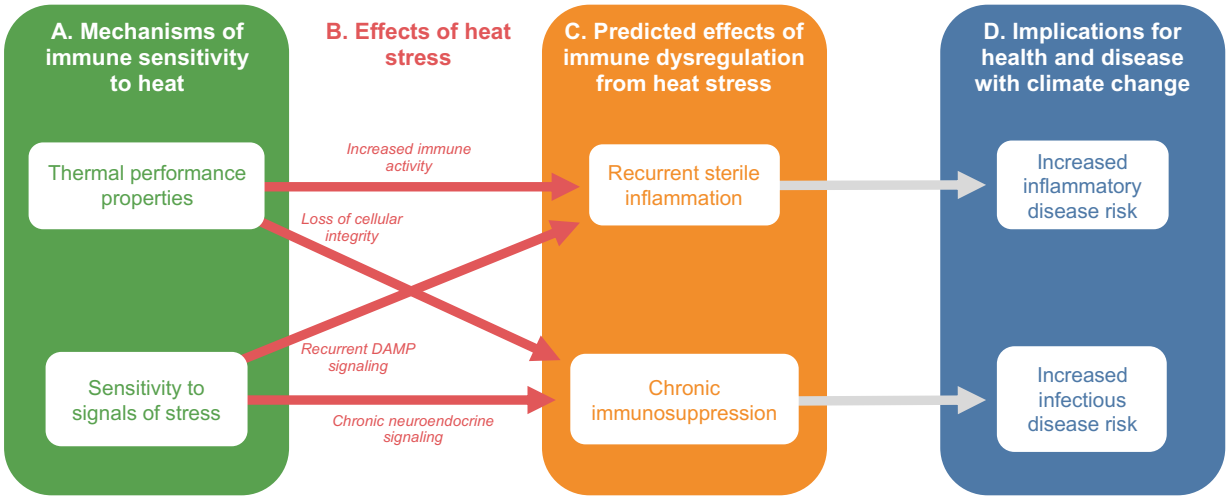



**Figure 2.2 An evolutionary framework for predicting the immunological consequences of climate change.** Panel A illustrates two core mechanisms that affect immune sensitivity to heat. Section B shows how these mechanisms render the immune system vulnerable to dysregulation during heat stress. Panel C depicts the predicted consequences of immune dysregulation from heat stress, and Panel D highlights the downstream health implications.

### *2.6.1 Sterile inflammation increases inflammatory disease risk*

Heat-driven sterile inflammation is expected to increase under climate change as rising temperatures both elevate immune activity through thermal performance effects and promote inflammation via DAMPs (Fig 2.2). Although DAMPs are beneficial for tissue repair, recurrent or unresolved signaling contributes to chronic inflammation implicated in a wide range of diseases [e.g., cardiovascular, neurodegenerative, and autoimmune disorders (Ma et al., 2024; Roh & Sohn, 2018)]. The DAMPs we predicted to be most relevant to heat stress, HSPs, and mtDNA, have been directly linked to inflammatory pathology. HSP70 and HSP96 are elevated in rheumatoid arthritis and promote inflammation through TLR activation of innate immune cells (Huang et al., 2009; Martin et al., 2003), while oxidized mtDNA drives excessive type I interferon production in systemic lupus erythematosus (Caielli et al., 2016). This suggests that DAMPs from recurrent heat stress may exacerbate the development or severity of inflammatory diseases.

Heat-driven DAMPs may also contribute to age-associated inflammatory pathology. Aging is characterized by chronic, low-grade inflammation [i.e., inflammaging (Franceschi et al., 2000)], which increases the risks of non-communicable diseases such as cancer, cardiovascular disease, diabetes, and neurodegenerative disorders (Fulop et al., 2018). Endogenous danger signals are thought to play a key role in driving inflammaging, and mtDNA is likely central to this process. mtDNA mutations accumulate with age, leading to impaired respiratory function,

increased RONS production, and cellular senescence (López-Otín et al., 2023; Zanini et al., 2023). Consistent with this, circulating levels of extracellular mtDNA increase with age and are associated with elevated proinflammatory cytokines (Pinti et al., 2014). Heat stress may accelerate these processes because high temperatures similarly increase RONS production and oxidative mitochondrial damage (Belhadj Slimen et al., 2014). Exposure to high ambient temperatures from heat waves has already been associated with accelerated biological aging in humans (S. Chen et al., 2025). Together, these findings suggest that rising temperatures could promote inflammatory processes implicated in aging via heat-driven DAMP signaling, with consequences for the risk of developing age-related diseases.

Heat-driven sterile inflammation could also cause dysbiosis of the gut microbiome, defined as alterations in microbial composition and metabolic function that disrupt community homeostasis. Inflammation alters the intestinal environment by increasing epithelial oxygenation and RONS production. These conditions are poorly tolerated by obligate anaerobes but favor the expansion of aerobic and facultatively anaerobic taxa with more metabolic flexibility (Zeng et al., 2017). This shift away from obligate anaerobes toward oxygen-tolerant groups, particularly Proteobacteria, is a hallmark of dysbiosis and reflects the ability of these taxa to exploit inflamed environments (Hughes et al., 2017). Consistent with this mechanism, elevated temperatures are associated with declines in anaerobe-dominated phyla such as Firmicutes and increases in Proteobacteria across diverse host species (Sepulveda & Moeller, 2020). These shifts can disrupt beneficial host–microbiome interactions. For example, obligate anaerobes ferment dietary fiber and produce short-chain fatty acids, which serve as important energy sources for the host and are key immunomodulators (Corrêa-Oliveira et al., 2016; Flint et al., 2012). In contrast, expansion of Proteobacteria is often associated with inflammatory bowel disease and includes many known gut pathogens, such as *Escherichia coli*, *Klebsiella* spp., and *Proteus* spp (Rizzatti et al., 2017). Together, these findings suggest that heat-driven sterile inflammation may indirectly increase disease risk by promoting dysbiosis of the gut microbiome.

As outlined here, dysregulated sterile inflammation has clear implications for inflammatory disease risk and may also adversely affect health by accelerating aging processes and disrupting the gut microbiome. Further research is needed to quantify the extent to which heat-driven sterile inflammation contributes to these outcomes, including how the magnitude and

frequency of heat stress shape inflammatory pathology. This will be critical for understanding how climate change will influence disease risk through immune vulnerability to heat.

### *2.6.2 Heat-induced immunosuppression will increase infectious disease risk*

We also predict that chronic heat stress will elevate infectious disease risk by suppressing immune function through sustained neuroendocrine stress signaling and thermal performance constraints on immunity (Fig. 2.2). Chronic stress across diverse contexts is consistently associated with impaired immune defense, including reduced vaccine responsiveness and increased infection risk (Glaser & Kiecolt-Glaser, 2005). Consistent with this pattern, prolonged heat exposure suppresses both innate and adaptive immune responses (Bagath et al., 2019), indicating that temperatures exceeding evolved thermal tolerance may compromise host resistance to infection. As climate warming increases, growing mismatches between evolved heat tolerance and ambient temperatures will likely increase the prevalence and severity of infectious disease from chronic immunosuppression.

Climate change is also predicted to increase infectious disease risk by altering host–pathogen interactions. Warming temperatures are expected to shift the geographic ranges of infectious disease vectors. Empirical evidence already shows marked expansion of vector species and increased prevalence of vector-borne diseases, including tick-borne encephalitis (Gray et al., 2009) and mosquito-borne diseases such as West Nile virus, Chikungunya, and dengue (El-Sayed & Kamel, 2020). Range expansion is particularly concerning because it exposes immunologically naïve populations to novel pathogens, a risk exacerbated by immunosuppression. At the same time, rising temperatures will facilitate vector and pathogen survival during historically cold seasons, removing an important constraint on population growth (Harvell et al., 2002). Consistent with this prediction, warming has been associated with increased frequency and severity of mosquito-borne illness (Mora et al., 2022). We expect these changes, combined with immunosuppression from chronic heat exposure, to substantially increase infectious disease burden under climate change (Fig. 2.3).

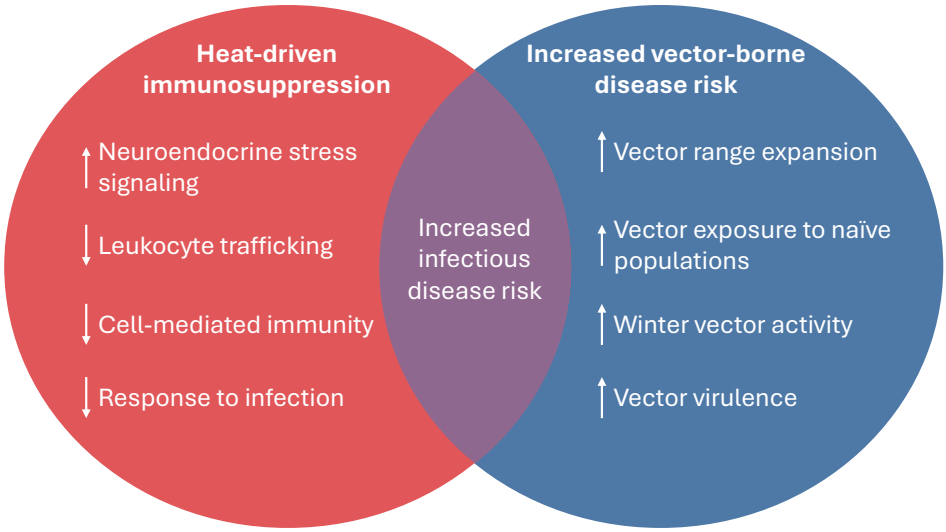



**Figure 2.3 Overlapping drivers of infectious disease risk under climate warming.** The red circle summarizes the consequences of heat-driven immunosuppression, while the blue circle shows climate-driven changes in vector activity. Their overlap illustrates that rising infectious disease risk results from both reduced host resistance to infection and increased exposure to more virulent disease vectors.

Heat-driven immunosuppression may disproportionately increase vulnerability to infectious disease in younger individuals. Early-life immunity relies more heavily on innate defenses, while adaptive, antigen-specific responses develop gradually with cumulative pathogen exposure (Simon et al., 2015). Sustained glucocorticoid signaling is therefore particularly problematic because it suppresses the nonspecific inflammatory responses that are critical in early life. This has led to speculation that the immune-related effects of climate change will disproportionately affect young individuals relative to other populations (Lucore et al., 2024; Swaminathan et al., 2014).

Beyond increasing susceptibility to novel infections, immunosuppression may also impair the control of existing infections. Many viruses can remain in a latent state for extended periods during which hosts remain asymptomatic. Stress has been shown to increase the likelihood of reactivation of these infections and has been studied extensively in the context of herpes simplex virus (HSV). For example, experimentally stressed mice exhibit greater reactivation of HSV (Padgett, Sheridan, et al., 1998) caused by neuroendocrine suppression of HSV-specific cytotoxic T-cell responses (Wonnacott & Bonneau, 2002). Consistent with these findings, humans who report chronic psychological stress have more frequent recurrences of genital herpes (Cohen et al., 1999). Together, this evidence suggests that immunosuppression from chronic heat stress may increase the reactivation of latent infections under climate change.

Similarly, stress-induced immunosuppression also impairs wound healing. Mice subjected to restraint stress exhibit significantly delayed wound closure, but this effect is reversed by blocking glucocorticoid receptors (Padgett, Marucha, et al., 1998). Congruent patterns have been observed in humans, where individuals experiencing chronic psychological stress show slower wound healing (Kiecolt-Glaser et al., 1995). Chronic heat exposure produces similar effects, where mice subjected to chronic heat stress (i.e., 43 °C for two weeks) showed delayed wound healing (dos Santos-Silva et al., 2017). Slower wound healing may, in turn, increase the risk of secondary infections with climate change (Coates et al., 2020).

As outlined here, immunosuppression has direct consequences for infection risk, particularly for young individuals, and may also indirectly exacerbate disease burden by slowing wound healing. When combined with the increasing virulence of vector species, immunosuppression could further increase infectious disease risk under climate change (Fig. 2.3). Future research is needed to quantify how heat-induced immunosuppression influences infection rates and disease severity. This type of research is critical for improving predictions of public health risks posed by climate warming.

## 2.7 Studies of wild animals can help evaluate the immunological consequences of climate change

Most research on the relationship between heat and immunity has been conducted under controlled experimental conditions. This work is essential for quantifying the effects of temperature on immune function and for identifying the mechanisms through which heat influences immunity (Evans et al., 2015; Maloney & Duffy, 2024). However, such studies are limited in their ability to capture the complex effects of climate change on the immune system and its implications for health and disease. This limitation arises because experimental research gains control at the expense of ecological realism (Zipple et al., 2023). By design, experimental studies simplify the conditions under which heat is experienced to isolate its effects. But heat is not encountered as a static or uniform stressor in real life. Changes to weather conditions combined with variation in microclimate exposures make it difficult to quantify the heat experienced by individuals in natural environments (Kearney et al., 2012). Further, many species possess complex behavioral adaptations for dissipating heat load that can be difficult to recreate under experimental conditions (Campos & Fedigan, 2009; Iii et al., 2006). Consequently, controlled experiments alone cannot capture immune vulnerability to climate warming in real-

world populations. Wild animal models, therefore, provide a uniquely powerful system for evaluating the immunological consequences of climate change. Studying immunity in this context allows direct observation of how immune systems respond to naturally occurring variation in ambient temperature. Wild systems also inherently include key sources of variation that shape heat tolerance, such as life-history stage, nutritional state, prior thermal exposure, and thermoregulatory behavior (Campos & Fedigan, 2009; Sinclair et al., 2016). This context allows more accurate identification of the temperature conditions that produce immune dysregulation with meaningful health consequences.

Unfortunately, studying immunity in wild animals presents substantial logistical and methodological challenges. Many commonly used immune biomarkers require serum or plasma samples (Demas et al., 2011) that can be difficult to collect from wild animals. Recently, non-invasive biomarkers such as urinary neopterin and soluble urokinase plasminogen activator receptor (suPAR) have expanded opportunities to study immune activation in wild animals. Neopterin reflects IFN-γ–mediated macrophage activation and has been used to assess temperature-driven variation in cell-mediated immunity (Lucore et al., 2022, 2024). In contrast, suPAR indicates broad inflammatory activation and is elevated during sterile tissue damage (Higham et al., 2020), making it useful to measure inflammation from heat exposure. Importantly, non-invasive sampling allows more frequent measurement of immune biomarkers, making it possible to identify the temperature ranges associated with meaningful changes in immunity under natural conditions (Lucore et al., 2024). Longitudinal studies that consistently monitor individuals over years or decades (Alberts & Altmann, 2012; Lonsdorf et al., 2022) are particularly well-positioned to use these biomarkers to track immune responses to climate change in real-time. Therefore, incorporating perspectives from wild systems is critical for generating accurate predictions of the immunological consequences and associated health risks of our warming climate.

### 2.8 Conclusion

In this review, we develop an evolutionary framework for predicting the immunological consequences of climate change by interpreting extensive evidence for immune sensitivity to heat in the context of evolved thermal tolerance. Although temperature effects on immune processes are well documented, they have rarely been considered from this perspective. By bridging these literatures, we show that immune responses to heat arise through two broad

mechanisms: passive thermal effects on immune performance and active regulation of immune function via signals of stress. While both mechanisms can be beneficial within evolved thermal tolerance, they also create vulnerability to immune dysregulation when environmental temperatures exceed upper thermal limits. Our framework also helps reconcile opposing immune responses to heat by showing how these mechanisms produce different effects on immunity depending on the intensity and duration of heat exposure. However, predicting immune dysregulation under climate change is challenging because real-world heat exposures are shaped by ecological, physiological, and behavioral contexts that are difficult to replicate experimentally. As a result, studies of wild populations, combined with an evolutionary perspective on thermal tolerance, are essential for predicting the immunological consequences of an increasingly warming climate.

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# Chapter 3 High Temperatures Are Associated With Decreased Immune System Performance in a Wild Primate

## 3.1 Abstract

Rising temperatures due to climate change are predicted to threaten the persistence of wild animals, but there is little evidence that climate change has pushed species beyond their thermal tolerance. The immune system is an ideal avenue to assess the effects of climate change because immune performance is sensitive to changes in temperature and immune competency can affect reproductive success. We investigate the effect of rising temperatures on a biomarker of non-specific immune performance in a wild population of capuchin monkeys and provide compelling evidence that immune performance is associated with ambient temperature. Critically, we found that immune performance in young individuals is more sensitive to high temperatures compared to other age groups. Coupled with evidence of rising temperatures in the region, our results offer insight into how climate change will affect the immune system of wild mammals.

## 3.2 Introduction

Human-induced climate change is affecting the health of our planet in many devastating ways. Increasing temperatures are predicted to have major effects on wild animal populations and are frequently cited as an important threat to their persistence (Bellard et al., 2012; Bennett et al., 2021). There is evidence across a diversity of taxa that climate change is already affecting species distributions (Helmuth et al., 2006; C. D. Thomas & Lennon, 1999), behavior (Telemeco et al., 2009), and physiology (Lovegrove et al., 2014; Pörtner & Knust, 2007), yet there has been little evidence that temperature changes have direct effects on individual fitness. The immune system, one of the body's main defense mechanisms against infection, is an ideal avenue in which to assess the effects of rising temperatures because it is sensitive to changes in ambient temperature (Dittmar et al., 2014) and an important determinant of individual fitness outcomes. Many hypotheses posit the negative effects of climate change on immune function (Skevaki et

al., 2024; Swaminathan et al., 2014), however, a relationship between the immune system and temperature has yet to be demonstrated in any wild mammal. Here, we provide compelling evidence that immune performance is associated with temperature in a natural population of tropical mammals and discuss the implications for population health in the face of climate change. Specifically, we examine how a marker of immune performance is affected by extended periods of high temperatures in a population of wild capuchin monkeys in Costa Rica.

The consequences of rising temperatures have primarily been observed in ectotherms because they lack thermoregulatory mechanisms to buffer the negative effects of thermal load on physiological performance (Pörtner & Farrell, 2008; Sinclair et al., 2016) and, by extension, growth and reproduction (Pörtner & Knust, 2007). Thermal performance curves are often used to illustrate how physiological performance changes across a temperature range. Performance steadily increases with temperature until reaching a thermal optimum, beyond which performance quickly decreases (Dowd et al., 2015). System performance can become heat-compromised at the far end of the temperature range due to anaerobic conditions created at high temperatures (Pörtner, 2002; Pörtner & Knust, 2007). Less is known about the relationship between physiological performance and temperature in endotherms because the effects of ambient temperature are buffered by thermoregulatory mechanisms. However, we know that temperature is an important predictor of immune performance because decreases in immune performance at high temperatures are well documented in livestock under experimental conditions [e.g. cows (Bagath et al., 2019), chickens (Quinteiro-Filho et al., 2010)]. No comparative work exists on wild endotherms, particularly tropical species which are predicted to be vulnerable to shifts in temperature because they are adapted to a narrower temperature range than species at higher latitudes (Stillman, 2003; Tewksbury et al., 2008)

The immune system consists of two main branches, nonspecific immunity (i.e. innate resistance to infection) and adaptive immunity (i.e. acquired resistance to specific pathogens). Here, we focus on nonspecific immunity because it is the first line of defense against infection and deploys an immediate, nonspecific defense while adaptive immunity builds a pathogen-specific response (Ellis, 2001; Turvey & Broide, 2010). Nonspecific immunity is the primary line of defense for infants and juveniles while the rest of their immune system develops (Levy, 2007). Therefore, non-specific immune competency in early life has implications for long-term health outcomes (McCusker & Warrington, 2011). Neopterin is an excellent way to measure the

nonspecific immune system performance because it reflects the activation of nonspecific immune cells (Murr et al., 2002) and can be collected non-invasively from urine (Higham et al., 2015; Lucore et al., 2022). Specifically, neopterin is excreted by macrophages and monocytes stimulated by interferon-gamma (IFN-γ) produced by the Th-1 cellular immune response during the initial stages of infection (Eisenhut, 2013; Murr et al., 2002). From here on, we define immune performance as Th-1 cell activation of macrophages and monocytes.

Urinary neopterin has been used to monitor disease status (Behringer et al., 2017) and immune ontogeny (Lucore et al., 2022; Schneider-Crease et al., 2022). However, no current studies have used neopterin to measure the effects of abiotic conditions on immune performance. In this study, we examine changes in urinary neopterin (n=670 samples) in 54 wild white-faced capuchin monkeys (*Cebus imitator*) to evaluate the relationship between temperature and immune performance. Air maximum and minimum temperatures were collected every 15 minutes and averaged 15 days before sample collection to evaluate how long-term temperature trends are associated with neopterin concentration. We also evaluated age-specific trends of temperature and neopterin using two years of longitudinal sampling of individuals ranging from one month to 36 years of age. To situate our results within the larger context of climate change, we show evidence of rising temperatures in Northwestern Costa Rica using a global atmospheric model (Global Modeling and Assimilation Office (GMAO), 2015).

### 3.3 Results

We found a relationship between maximum temperature and neopterin in capuchin monkeys that resembles a thermal performance curve (Dowd et al., 2015; Pörtner & Farrell, 2008; Sinclair et al., 2016) where neopterin concentration increases with temperature until reaching a thermal optimum, at 30˚C, beyond which neopterin concentration quickly decreases (Fig. 3.1). Modeling the relationship with minimum temperature as a predictor yielded the same results (Fig. S3.1), therefore, we will report the results for maximum air temperature only. The steep decline in neopterin between 30-31 ˚C is typical of a thermal performance curve where performance drops quickly after passing the thermal optimum. The practically linear relationship between neopterin and temperature between 24˚ and 30˚C corroborates in vitro studies where rising temperatures increase immune performance (Baronzio et al., 2006; Knippertz et al., 2011).

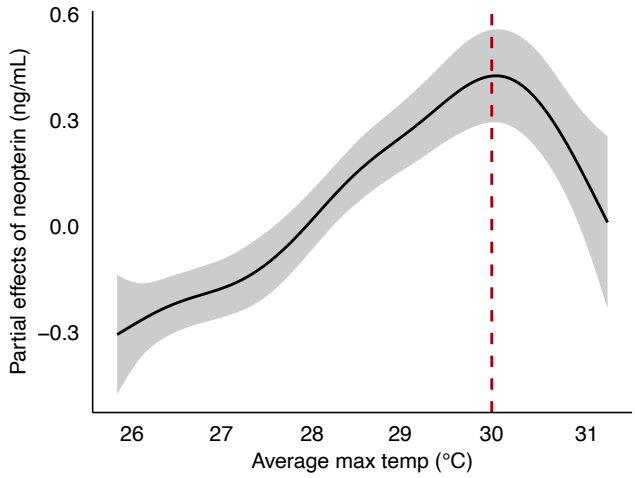



**Figure 3.1 Relationship between neopterin and temperature.** Partial effects plot of the generalized additive model that evaluates the non-linear relationship between neopterin concentration and average maximum temperature in the 15 days before sample collection. The relationship resembles a thermal performance curve where the dotted red line indicates the thermal optimum for immune performance after which we see a steep decline in performance.

Neopterin shows a U-shaped relationship with age ($ß_{age2}$ = 4.81, Fig. 3.2) where increased levels in early life indicate a reliance on non-specific immunity (Levy, 2007), and increased levels in late life are characteristic of immune system senescence (Franceschi & Campisi, 2014). Importantly, we found that the relationship between temperature and neopterin is also associated with age; in our best-fitting model temperature shows a reliable negative interaction effect with age ($ß_{temp*age2}$ = -4.20, Fig. 3.2). To interpret the interaction effect between a polynomial and continuous predictor, we plotted the U-shaped relationship between age and neopterin across discrete temperature bins (Fig. 3.3). Similar to the thermal performance curve, neopterin concentration increases across all ages up to 30 ˚C (Fig. 3.3A-C). Above 30˚C, there is a flattening of the curve, particularly on the left side depicting young individuals (Fig. 3.3D), which is emphasized when the curves are superimposed on each other (Fig. 3.3E). Importantly, we found no reliable effect of sex, reproductive state, or season (as measured by average rainfall; $ß_{rain}$=0.02) on neopterin (Fig. S3.2, Table S3.5) indicating that the relationship between neopterin and temperature is not dependent on seasonal temperature changes.

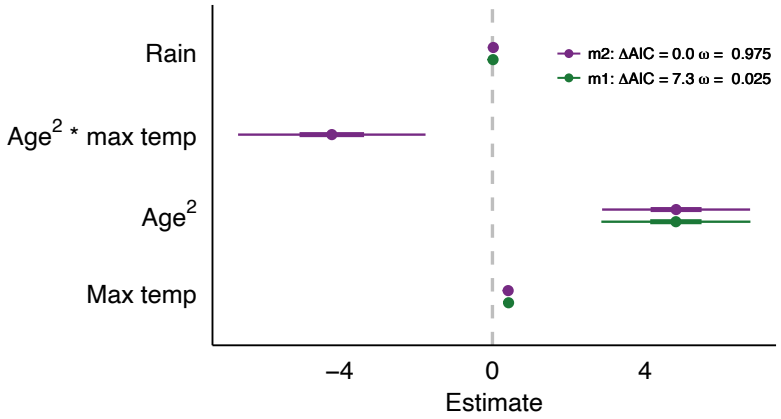



**Figure 3.2 Model results for neopterin.** Comparison of generalized linear mixed models for models with greater than >0.001 % of the model weight. Similar results are seen across models. Thick bars represent 50% CI, and thin bars represent 95% CI. Both models support the U-shaped relationship between neopterin and age. Model 2 holds the most model weight and includes the reliable interaction effect between age$^2$ and max temperature. Rain shows no reliable effect.

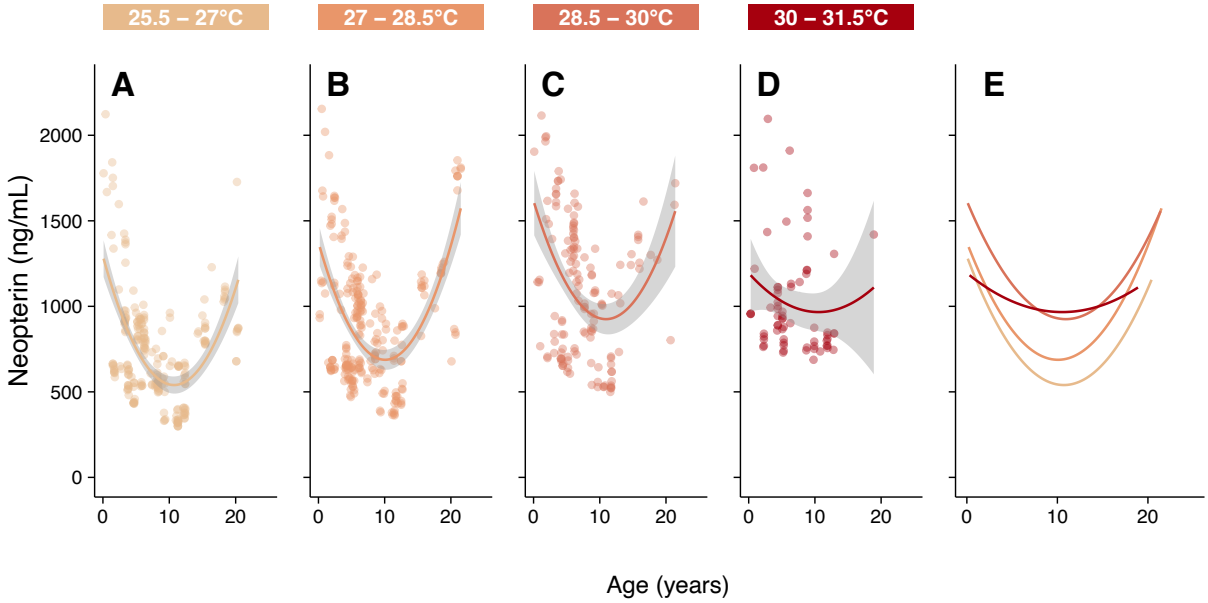



**Figure 3.3 Visualization of the interaction effect between age and temperature.** The relationship between age and neopterin concentration is plotted across four, discrete temperature ranges. (A-D) The top panel shows the discrete temperature bins where each bar lists the temperature cut-offs for its corresponding plot. The bars become increasingly dark to indicate rising temperatures. The predictions from the best-fitting generalized linear model are plotted in the color of the corresponding bar that lists the temperature range for each plot. Grey shading represents 95% confidence intervals. Plot (E) shows the same curves from plots (A-D) overlapping to emphasize the flattening of the U-shaped neopterin-age relationship at temperatures above 30˚C, particularly in young individuals.

Temperatures are rising in Northwestern Costa Rica (Fig. 3.4), showing an average increase of 0.18˚C each decade since 1980 ($ß_{date}$ = 5.05-5, SE = 1.66-6). Rising temperatures have resulted in more days and longer periods where temperatures are above 30˚C, the thermal

optimum for Th-1 cellular immune performance in our study population. For example, between 1980 and 2014, only 18% of 20 days recorded average temperatures above 30˚C, compared to the last decade when the number increased to 34%. This equates to an 89% increase in the number of 30˚C-plus days in the last decade.

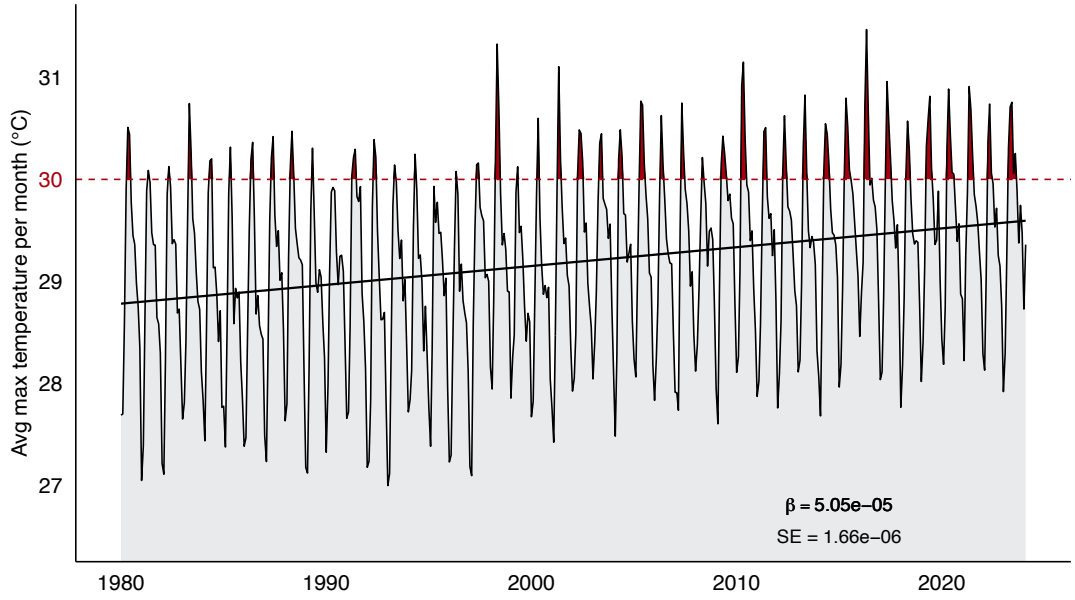



**Figure 3.4 Climate change in Northwestern Costa Rica.** Longitudinal change in average monthly maximum temperature over the last forty-three years visualized using data from the MERRA-2 global atmospheric dataset. The black line represents a linear model that shows an increase in daily maximum temperature over time. The horizontal red line at 30˚C represents the thermal optimum for immune performance after which we see a steep decline in performance. The shaded red highlights the increasing number of days and longer periods above 30˚C.

## 3.4 Discussion

Here, we provide compelling evidence that immune performance is associated with temperature in a natural mammalian population and show that immune performance in young individuals is more sensitive to high temperatures than other age groups. Average temperatures above 30˚C for an extended period, at least 15 days, are associated with a steep decline in neopterin concentration (Fig. 3.1). The decline in neopterin concentration after 30˚C is most pronounced in young individuals indicating that immune performance in early life is especially affected by high temperatures (Fig.3.3). Given that temperature was modeled as a 15-day average, these results represent the association between neopterin and extended periods of elevated temperature during which behavioral (Campos & Fedigan, 2009) and physiological (Orkin et al., 2021) adaptations to cope with short-term heat-stress are likely less effective at buffering thermal-load. Coupled with evidence of rising temperatures in the region (Fig. 3.4), our

results offer some insight into how climate change could affect the immune system of wild mammals.

Thermal performance curves are rarely observed outside of laboratory conditions where ecological variables can influence the temperature-performance relationship (Childress & Letcher, 2017). Despite this, the relationship between neopterin and temperature resembles a thermal performance curve, indicating the importance of temperature in determining immune performance in wild populations. Importantly, the thermal optimum at 30 ˚C is not necessarily physiologically optimal because it appears to be driven by temperature as opposed to infection or other challenges that require an immune response. Further, since system performance can become heat-compromised at the far end of the temperature range, the observed drop in performance after 30 ˚C could indicate that this population may soon, or already experience periods where Th-1 cellular immune activation is heat-compromised (Sinclair et al., 2016). White blood activation is a critical defense mechanism in response to initial infection; impaired activation could reduce immune competency, increase instances of infection, and affect long-term health outcomes. This trend is specifically concerning in our study population because white-faced capuchins in Northwestern Costa Rica are endemic to tropical dry forests that are highly seasonal (Portillo-Quintero & Sánchez-Azofeifa, 2010); therefore, they are likely adapted to a wider temperature range compared to many tropical specialists (Campos & Fedigan, 2009; Orkin et al., 2021). Given the evidence that our study population could be nearing the limit of their thermal range, it is likely other tropical species are experiencing more severe effects of heat-compromised performance.

High neopterin in young individuals indicates reliance on non-specific immunity in early life while the adaptive immune system develops (Levy, 2007). The decline in neopterin over the first five years of life is typical of other non-human primate species (Schneider-Crease et al., 2022) (Fig. 3.3A-C), and likely represents the period of development for the adaptive immune system. Here, we show that the neopterin-temperature relationship is associated with age (Fig. 3.2) where young individuals have lower neopterin than expected at high temperatures compared to other age groups (Fig. 3.3). Young individuals may reach their thermal optimum at lower temperatures or may exhibit a steeper decrease in performance after 30 ˚C compared to other age groups. Under both scenarios, young individuals likely approach heat-compromised Th-1 cell activation faster than older individuals indicating that immune performance in early life is

especially affected by high temperatures. Heat-compromised white blood cell activation in early life could leave individuals vulnerable to infection because the other branches of their immune system remain undeveloped. Lack of immune competency in early life is associated with higher risks of infection (McCusker & Warrington, 2011) and stunting (D. Thomas & Way, 2023) which can negatively affect reproductive success (Dewey & Begum, 2011) or result in death before reproductive age (Fleischmann et al., 2021). Therefore, the susceptibility of young individuals to heat-compromised immune performance may be an important mechanism by which rising temperatures due to climate change could increase extinction risk through direct effects on individual fitness.

The temperature-neopterin relationship does not appear to be a product of seasonal shifts in temperature or confounding factors associated with seasonality. Seasonal changes in tropical dry forests are associated with shifts in other factors (e.g. parasite load, resource scarcity) that can affect immune performance either directly or indirectly by stressing other physiological processes. However, neopterin is not associated with rainfall (Fig. 3.2) which is a reliable measurement of season in tropical dry forests (Murphy & Lugo, 1986). We acknowledge that the lack of intracellular parasite measurements is a limitation of this study as parasites can show seasonal patterns of infection (Raharivololona & Ganzhorn, 2010; Rondón et al., 2017) and have been associated with neopterin concentrations (Schneider-Crease et al., 2022). However, the relationship between parasitic infection and neopterin is not straightforward (Müller et al., 2017; Schneider-Crease et al., 2022) and requires further research. Given that climate change is affecting the emergence and distribution of infectious diseases (Alan Pounds et al., 2006; Epstein, 2001), understanding the link between temperature, parasites, and immune performance will likely help predict long-term health outcomes in the coming decades.

Temperatures are rising and will result in longer periods where temperatures are above the thermal optimum for immune performance in our study population. In the last decade, the number of days above 30˚C in Northwestern Costa Rica showed an 89% increase compared to the previous three decades (Fig. 3.4). This trend is expected to continue, resulting in an overall temperature increase between 4˚ to 6˚C by 2080 (Stan & Sanchez-Azofeifa, 2019). Given the steep decline in immune performance between 30˚C and 31.5˚C, a 4˚ to 6˚C increase promises periods where immune performance is heat-compromised. It is important to acknowledge that the immune system deploys multifaceted and redundant mechanisms (Turvey & Broide, 2010) of

which we have only measured one and it is, therefore, difficult to predict the effects of heat-compromised Th-1 immune activation on long-term health outcomes with much certainty. However, given that diverse immune mechanisms have been shown to respond to temperature (Bagath et al., 2019; Baronzio et al., 2006; Dittmar et al., 2014), it is likely that rising temperatures due to climate change could affect other branches of immunity and likely compound negative health outcomes. Future research would benefit from the collection of additional immune biomarkers from naturally occurring populations to gain a broader understanding of how temperature affects overall immune performance in wild mammals. Long-term research is also necessary to identify and quantify the effects of heat-compromised immune performance on individual fitness outcomes, as this could be a mechanism by which climate could increase extinction risk in diverse taxa. As temperatures continue to increase at faster rates, we expect species will continue to experience more diverse and severe physiological effects due to our changing – and increasingly challenging – climate.

## 3.5 Materials and Methods

### *3.5.1 Site information and sample collection*

Between Sep 2021 and Aug 2023 we collected 676 urine samples from three habituated groups of wild *C. imitator* associated with the Capuchinos de Taboga research project in the Taboga Forest Reserve. The Taboga Forest Reserve is a 516-ha tropical dry forest in the Guanacaste region of Northwestern Costa Rica that is owned by the Universidad Técnica Nacional. It is an important piece of a fragmented biological corridor connecting the Tempisque River Basin to the Guanacaste Mountains (Tinsley Johnson et al., 2020). We collected samples from 54 individually identified individuals ranging from one month to 35 years of age. For individuals <7, age was calculated by birth date; for individuals >7 years age, was estimated based on body condition by one of us (JO) with more than two decades of experience observing wild white-faced capuchins. We collected samples opportunistically, from individuals who appeared healthy upon visual inspection, using a clean catch method (Bergstrom et al., 2017) to avoid rainfall and fecal contamination between the hours of 5:00AM to 6:00PM. We collected samples from leaves or the ground only when they did not appear contaminated with soil or feces. We then transferred the sample to 1.8 mL tubes using disposable pipets and immediately

stored samples in a portable cooler on ice for up to 7 hours. We stored the samples in a -20˚C freezer at the end of the field day.

### *3.5.2 Sample analysis*

We measured the neopterin concentration of all samples using a commercially available competitive exclusion neopterin ELISA (Neopterin ELISA, Ref. RE59321, IBL International GMBH, Hamburg, Germany) in the Capuchinos de Taboga field laboratory. The kit was developed to detect neopterin concentration in human serum, plasma, and urine, but has been validated for use in the wild *C. imitator* population associated with the Capuchinos de Taboga research project (Lucore et al., 2022). We thawed, centrifuged, and aliquoted all samples to remove precipitate and measured sample water volume using a handheld refractometer *(Aichose, Ref. SR0021-ATC)* to control for sample concentration. We then diluted samples to 1:64 with manufacturer assay buffer and ran the assay using the manufacturer protocol. We reran samples with a CV higher than 15.5%. We re-diluted and reran samples with low binding (>85 % binding) or high binding (<15 % binding) on the standard curve. The inter-assay variation for 26 plates was 9.48%.

### *3.5.3 Temperature observations*

To evaluate the relationship between neopterin concentration and temperature, we used observations from the Palo Verde meteorological station located at the Organization for Tropical Studies (OTS) Palo Verde research station, 10.345° N, 85°.339 E, approximately 20.81 km southwest of the Taboga Forest Reserve. Palo Verde is one of the last remaining fragments of tropical dry forest in Central America similar to the Taboga Forest Reserve (Stern et al., 2002). We used the OTS Palo Verde climate data due to habitat similarity and close geographical location to our study site. The OTS Palo Verde meteorological station records maximum and minimum air temperature every 15 min.

Since the OTS Palo Verde climate data are only available from 2007 onward, we used the Modern-Era Retrospective analysis for Research and Applications, Version 2 [MERRA-2(Global Modeling and Assimilation Office (GMAO), 2015)] developed by the NASA Goddard Space Flight Center (GSFC) and Global Modeling and Assimilation Office (GMAO) to evaluate long-term temperature trends in the Guanacaste region. MERRA-2 is a global atmospheric dataset that provides numerous meteorological variables on a horizontal grid with a spatial resolution of

0.625° × 0.5° longitude–latitude. We extracted the average daily maximum air temperature at 2m above surface level from the years 1980 through 2023 at 10.5° N, 85.625° E, the closest available point on land to the OTS Palo Verde meteorological station. To ensure the two data sets were comparable, we standardized the MERRA temperatures by calculating the deviation from the mean temperature of the Palo Verde data and adding the deviation to the MERRA data.

### *3.5.4 Statistical analysis*

For our neopterin data, we removed three samples that were 4 SD above the mean before conducting our analysis. We also removed three samples associated with the oldest individual in our population (34 years of age at the beginning of the study) to standardize the age spread across social groups. Excluding this individual did not change our results (Tables S3.1-S3.3). To determine the best-fitting temperature predictor for neopterin concentration, we averaged maximum temperature (measured every 15 minutes) at different time intervals before sample collection. We then built a group of generalized additive models (GAMs) in which each model included a different temperature variable as a predictor. Neopterin concentration was the outcome variable for all models. We also included predictors for age, and social group, which are known predictors of neopterin, and a random intercept for individual to control for repeated sampling. We scaled age and max temperature in all models for ease of coefficient interpretation. We fit age using a spline because of the known, non-linear relationship between neopterin and age. We also fit maximum temperature using a spline because we are interested in finding the best-fitting, non-linear, relationship between maximum temperature and neopterin concentration. We fit social group as a linear variable because it was a discrete variable with three levels. We used a Gamma distribution with a log link function to fit the model since the outcome variable was continuous, positive and left-skewed with a long right tail. The best-fitting model included the predictor for the average maximum temperature of the 15 days before sample collection (Table S3.4).

To compare maximum versus minimum temperature predictors, we reran this analysis using minimum temperature (measured every 15 minutes) and found the same result – the best-fitting model included the predictor for the average minimum temperature of the 15 days before sample collection. Model results were also congruent across all other time intervals (Table S3.4). Further, the non-linear relationship between maximum and minimum temperature and neopterin concentration was the same (Fig. S3.1). Therefore, since there was no difference between

temperature predictors, we chose to use the model including the average maximum temperature 15 days before sample collection to evaluate the non-linear relationship between temperature and neopterin concentration using a GAM (n = 670 samples).

We evaluated the relationship between age, temperature, and immune function using multiple generalized linear mixed models (GLMMs) and compared them using the AICc (n = 670 samples). Similarly, neopterin concentration was the outcome variable for all models, and we fit the models using a Gamma distribution with a log link function. All models included age, $age^2$, social group, average rainfall, sex, and female reproductive status as predictors. Two models included maximum temperature as a predictor and only one model included an interaction effect between $age^2$ and temperature (Table S3.5 & Fig. S3.2). To control for repeated sampling, we included a random intercept for individual. We scaled age, rain and temperature in all models for ease of coefficient interpretation.

We used the MERRA-2 (Global modeling and assimilation office, 2015) data to assess long-term patterns in maximum temperature by fitting two linear mixed effect models (LMMs) with daily max temperature as the outcome variable (n = 16103) and compared them using AIC. We fit the models using a Gaussian distribution given the normal distribution of the outcome variable. We built the first model as an intercept model and compared it to the second model which included date as the predictor. We conducted all analyses in R version 4.3.1 (R Core Team., 2021).

## 3.6 Supplementary Materials

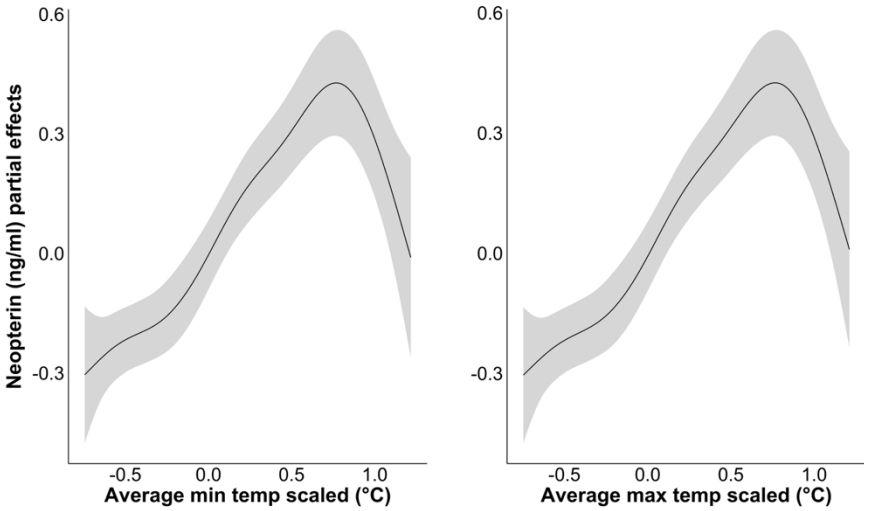



**Figure S3.1** Partial effects plot of the generalized additive models that evaluate the non-linear relationship between neopterin concentration and the average temperature in the 15 days before sample collection. The left plot visualizes the relationship between average minimum temperature compared to the right plot which illustrates the relationship between maximum temperature. Both temperature measurements yield the same results.

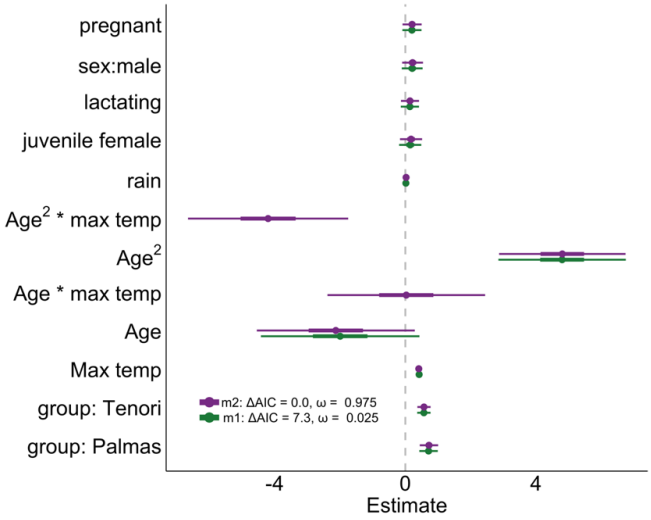



**Figure S3.2** Comparison of generalized linear mixed models for models with greater than >0.001 % of the model weight. Includes all predictor variables used in each model. Similar results are seen across models and no reliable effect is seen for reproductive status, sex, or rain. Thick bars represent 50% CI, and thin bars represent 95% CI.

**Table S3.1** Comparison of generalized additive model (GAM) results including and excluding the oldest individual in our population (34 years of age at the beginning of the study). The gam_filtered values exclude the oldest individual and are the results presented in the manuscript.

| model | variables | edf | ref.df | statistic | R-sq |
|---|---|---|---|---|---|
| gam_filtered | spline: age | 3.67 | 4.15 | 7.13 | 0.364 |
| | spline: max temp | 4.79 | 5.84 | 17.41 | |
| gam_full | spline: age | 3.99 | 4.44 | 7.54 | 0.395 |
| | spline: max temp | 4.79 | 5.84 | 17.59 | |

**Table S3.2** Comparison of generalized linear mixed model results including and excluding the oldest individual in our population. The models listed as 'full' include the oldest individual. The models listed as 'filtered' exclude the oldest individual and are the results presented in the manuscript. Here we compared both groups of models using AICc. For both groups of models, glmm_2 holds the majority of the model weight while glmm_1 holds the least of the model weight. The results do not change appreciably depending on the inclusion of the oldest individual.

| model | dAICc | df | weight |
|---|---|---|---|
| glmm_2_full | 0.00 | 11 | 60.67 |
| glmm_1_full | 0.87 | 9 | 39.33 |
| glmm_2_filtered | 0.00 | 15 | 97.50 |
| glmm_1_filtered | 7.33 | 13 | 2.50 |

**Table S3.3** Comparison of generalized linear mixed model results for models with greater than >0.001 % of the model weight including and excluding the oldest individual in our population. The models listed as 'full' include in oldest individual. The models listed as 'filtered' exclude the oldest individual and are the results presented in the manuscript. The beta coefficients and std. errors do not change appreciably depending on the inclusion of the oldest individual.

| model | predictor | estimate | std.error |
|---|---|---|---|
| glmm_2_full | Age squared | 4.54 | 0.92 |
| glmm_2_full | max temp | 0.42 | 0.05 |
| glmm_2_full | Rain | 0.02 | 0.04 |
| glmm_2_full | Age squared * max temp | -2.60 | 1.29 |
| glmm_2_filered | Age squared | 4.81 | 0.99 |
| glmm_2_filered | max temp | 0.41 | 0.05 |
| glmm_2_filered | Rain | 0.02 | 0.04 |
| glmm_2_filered | Age squared * max temp | -4.20 | 1.25 |

**Table S3.4** Comparison of the generalized additive model with temperature averages at different time intervals before sample collection to identify best fitting temperature predictor. Maximum and minimum temperature predictors show congruent results. For both temperature measurements, temperature averages 15 days before sample collection is the best fitting time period given the AICc and model weight.

| min_t_model | AICc | weight | max_t_model | AICc | weight |
|---|---|---|---|---|---|
| days15 | 0.00 | 0.32 | days15 | 0.00 | 0.35 |
| days16 | 0.71 | 0.23 | days16 | 0.91 | 0.22 |
| days14 | 1.38 | 0.16 | days14 | 1.47 | 0.17 |
| days17 | 2.10 | 0.11 | days17 | 2.50 | 0.10 |
| days18 | 2.78 | 0.08 | days18 | 3.26 | 0.07 |
| days19 | 4.32 | 0.04 | days19 | 4.85 | 0.03 |
| days13 | 4.94 | 0.03 | days13 | 5.15 | 0.03 |
| days20 | 5.80 | 0.02 | days20 | 6.29 | 0.02 |
| days21 | 7.93 | 0.01 | days21 | 8.33 | 0.01 |
| days28 | 9.62 | 0.00 | days28 | 9.84 | 0.00 |
| days12 | 14.18 | 0.00 | days12 | 13.80 | 0.00 |
| days35 | 17.81 | 0.00 | days35 | 18.00 | 0.00 |
| days11 | 20.02 | 0.00 | days11 | 19.32 | 0.00 |
| days10 | 21.64 | 0.00 | days10 | 20.98 | 0.00 |
| days9 | 22.81 | 0.00 | days9 | 22.04 | 0.00 |
| days5 | 23.12 | 0.00 | days5 | 22.56 | 0.00 |
| days7 | 26.86 | 0.00 | days7 | 25.50 | 0.00 |
| days2 | 27.13 | 0.00 | days2 | 26.74 | 0.00 |
| days3 | 27.95 | 0.00 | days3 | 27.31 | 0.00 |
| days1 | 28.05 | 0.00 | days1 | 28.17 | 0.00 |

**Table S3.5** Summary of results from the fitted generalized linear mixed models results for models with greater than >0.001 % of model weight. Results include the beta coefficient (estimate) and standard error for each variable included in each model. AICc scores and model weights are also provided.

| model | predictor | estimate | std.error | dAICc | df | weight |
|---|---|---|---|---|---|---|
| glmm_1 | group: Palmas | 0.71 | 0.14 | 7.33 | 13 | 2.5 |
| | group: Tenori | 0.57 | 0.10 | | | |
| | Age | -1.99 | 1.24 | | | |
| | Age squared | 4.80 | 0.99 | | | |
| | Rain | 0.02 | 0.04 | | | |
| | Juvenile female | 0.15 | 0.17 | | | |
| | Lactating | 0.14 | 0.14 | | | |
| | Sex:Male | 0.21 | 0.16 | | | |
| | Pregnant | 0.20 | 0.15 | | | |
| glmm_2 | max temp | 0.42 | 0.05 | 0.00 | 15 | 97.5 |
| | group: Palmas | 0.72 | 0.14 | | | |
| | group: Tenori | 0.57 | 0.10 | | | |
| | Age | -2.13 | 1.23 | | | |
| | Age squared | 4.81 | 0.99 | | | |
| | max temp | 0.41 | 0.05 | | | |
| | Rain | 0.02 | 0.04 | | | |
| | Juvenile female | 0.17 | 0.17 | | | |
| | Lactating | 0.14 | 0.14 | | | |
| | Sex:Male | 0.22 | 0.16 | | | |
| | Pregnant | 0.21 | 0.15 | | | |
| | Age * max temp | 0.03 | 1.23 | | | |
| | Age squared * max temp | -4.20 | 1.25 | | | |

### 3.7 References


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# Chapter 4 High Temperatures Predict Altered Gut Microbiome Composition, Metabolic Capacity, and Immune Interactions in a Wild Primate Species

## 4.1 Abstract

Rising temperatures due to climate change are impacting the function of critical physiological processes in wild animals. Heat stress may compromise the gut microbiome's ability to support essential host functions, highlighting an important pathway by which climate change could negatively affect numerous species. However, the relationship between the microbiome and heat stress has been studied primarily in experimental contexts and remains poorly understood in ecologically relevant environments. Here, we examine how natural thermal variation influences gut microbiome taxonomic composition and functional capacity in a wild population of white-face capuchin monkeys. We find that high temperatures are associated with altered microbiome community-level diversity, taxonomic composition, and metabolic capacity consistent with physiological responses to heat stress. We also show that the microbiome's response to high temperatures is linked to host immunity and may affect microbiome-immune interactions. These results suggest that rising temperatures may compromise essential microbiome functions and highlight a potential mechanism by which climate change could negatively impact species through their microbiomes.

## 4.2 Introduction

Rising temperatures due to climate change are predicted to push species beyond their evolved thermal tolerances and challenge them to adapt to hotter climates (Bellard et al., 2012; Bennett et al., 2021). Efforts to evaluate the physiological consequences of heat stress in wild animals have shown that high temperatures can impair aerobic capacity, reducing oxygen availability to essential systems and compromising function (Dowd et al., 2015; Pörtner & Knust, 2007). Much of this research has focused on ectotherms, whose lack of physiological thermoregulation makes them particularly vulnerable to high temperatures (Pörtner & Knust, 2007; Sinclair et al., 2016; Telemeco et al., 2009). However, mounting evidence suggests that

endotherms are also physiologically susceptible to heat (Lovegrove et al., 2014; Lucore et al., 2024), and evolved thermoregulatory mechanisms may be increasingly insufficient to cope with prolonged periods of extreme heat. Recently, research has turned to assessing the role of host-associated microbiomes in shaping how species respond to heat stress. Animals have evolved complex relationships with the microbial communities (i.e., microbiomes) that live on and inside them to the extent that hosts rely on their microbiomes to support diverse aspects of their physiology. Accordingly, microbiomes may play a key role in determining the thermal tolerances of their host species (Baldassarre et al., 2022; Fontaine & Kohl, 2023; Moghadam et al., 2018; Ziegler et al., 2017) because heat stress can deplete microbiome communities, and is associated with negative phenotypic, survivorship, and fitness outcomes (Jaramillo & Castañeda, 2021; B. Zhang et al., 2019). While this research highlights that heat is an important determinant of host-microbiome relationships, it has largely been conducted in laboratory settings and offers limited insight into how these dynamics function in ecologically relevant contexts.

The gastrointestinal tract is home to a particularly dense microbial community, the gut microbiome, that plays a crucial role in regulating host responses to environmental change (Amato, 2016), making it an ideal system to explore how species may respond to rising temperatures. High temperatures have been shown to alter community composition (Sepulveda & Moeller, 2020) and are linked to changes in host digestion, metabolism, and immune function (Chen et al., 2018; Fontaine et al., 2018; Tajima et al., 2007; Zhu et al., 2019), but these relationships have yet to be demonstrated in wild populations. A common consequence of heat stress is redox imbalance— the accumulation of reactive oxygen species (ROS) that can damage cells (Belhadj Slimen et al., 2014; Dodson et al., 2019), compromise the epithelial barrier (Lambert et al., 2002), and trigger immune activation (Koch et al., 2019). Gut microbes typically help maintain redox balance (Kunst et al., 2023; Sun et al., 2024; Yardeni et al., 2019), but heat-induced changes in community composition have been linked to reduced antioxidant metabolism (Fang et al., 2023; Liu, Ma, et al., 2023) and increased epithelial damage (Xia et al., 2022). These redox shifts may disrupt oxygen gradients, favoring facultative anaerobes over obligate anaerobes. This is supported across a variety of vertebrate species where high temperatures are consistently associated with declines in Firmicutes and Bacteroidota (Bai et al., 2022; Bestion et al., 2017; Zhu et al., 2019), phyla composed primarily of obligate anaerobes lacking robust ROS defenses (Z. Lu et al., 2018; McCord et al., 1971), and increases in Proteobacteria (Sepulveda &

Moeller, 2020), a phylum dominated by facultative anaerobes that thrive in higher-oxygen conditions (Albenberg et al., 2014; Shin et al., 2015).

The loss of obligate anaerobes during heat stress could affect the relationship between the gut microbiome and the immune system. Many obligate anaerobes ferment dietary fiber and produce short-chain fatty acids (SCFAs)(Flint et al., 2012; Hooper et al., 2002; Maier et al., 2014), which support anti-inflammatory mechanisms that promote immune tolerance of the microbiome (Corrêa-Oliveira et al., 2016; Furusawa et al., 2013; Klampfer et al., 2003; Liu, Shao, et al., 2023). A loss of these obligate anaerobes could result in reduced SCFAs and impact the immune system. Experimental studies evaluating the effects of acute heat stress in livestock show that exposure to high temperatures is associated with reduced SCFA concentrations (Tajima et al., 2007), changes in the levels of circulating cytokines, and altered gut microbiome composition (Chen et al., 2018; Yu et al., 2024), suggesting that heat-driven compositional shifts may disrupt microbiome-immune interactions and increase the risk of gut inflammation. These dynamics are likely to play an essential role in shaping animal responses to changing climates. However, interactions between heat stress and the microbiome have been studied almost exclusively in experimental contexts that examine the effect of acute periods of moderate heat stress [e.g., ~8-10 hours of exposure to temperatures ~32-34 °C, (He et al., 2019; Seo et al., 2024)] and have focused either on ectotherms [e.g., amphibians (Emerson et al., 2023; Fontaine et al., 2018), fish (Fang et al., 2023), reptiles (Bestion et al., 2017; Moeller et al., 2020)] or on domestic livestock [e.g., pigs (Xia et al., 2022), cows (Chen et al., 2018; Tajima et al., 2007; Yu et al., 2024), poultry (Liu, Ma, et al., 2023; G. Wang et al., 2021)]. Therefore, we have little understanding of how this system functions in wild populations in response to natural thermal variation.

To address this knowledge gap, here, we assess how temperature predicts gut microbiome composition and metabolic function in wild capuchin monkeys, Cebus imitator, in Costa Rica. We focus on evaluating the response to cumulative thermal load by averaging ambient temperature two weeks before sample collection, rather than attempting to measure short-term responses that are difficult to isolate in wild populations. Importantly, this predictor is also associated with thermal responses in immune performance in this population, supporting its relevance for studying the consequences of high temperatures on physiology (Lucore et al., 2024). We also evaluate the interplay between temperature, the gut microbiome, and host

immunity. Specifically, we measure host immunity using urinary neopterin, a byproduct of macrophage activity during the cellular immune response (Murr et al., 2002). Macrophages play a central role in host containment of the gut microbiome (Kelsall, 2008), and microbial metabolites, including SCFAs and saturated fatty acids, regulate macrophage activity to maintain host tolerance and prevent chronic inflammatory disorders (Corrêa-Oliveira et al., 2016; J. Wang et al., 2020). Therefore, neopterin is a valuable tool for examining microbiome-immune homeostasis.

We analyzed 11 months of paired fecal and urine samples from 36 individually identified white-faced capuchin monkeys living in tropical dry forest fragments in northwestern Costa Rica at the Taboga Forest Reserve. Tropical dry forests are highly seasonal and defined by marked changes in ecology between the dry and wet seasons (Portillo-Quintero & Sánchez-Azofeifa, 2010). During the dry season, lack of rainfall leads to food and water scarcity that has physiological consequences for capuchins (Bergstrom et al., 2017) and can influence gut microbiome composition (Orkin, Campos, et al., 2019). Because the dry season is also characterized by high temperatures, this can make it difficult to disentangle the effects of heat from those of resource scarcity. However, our study population differs from most others in that we find no evidence of seasonal differences in physiological stress responses (Beehner et al., 2022) or immunity (Lucore et al., 2024), suggesting that these monkeys are not exposed to the same seasonal stressors. This is likely because our study site is highly disturbed and bordered by secondary forest and agriculture, where capuchins can exploit anthropogenic food and water sources even during the dry season (Tinsley Johnson et al., 2020). Consequently, studying this population provides a rare opportunity to isolate temperature-driven changes in the microbiome from those driven by broader changes in seasonal ecology. We characterized microbiome composition using 16S rRNA gene amplicon sequencing (n = 277), and characterized microbiome functional capacity using shotgun metagenomic sequencing (n = 71). Our results highlight the crucial role of temperature in shaping essential gut microbiome functions in a wild endotherm species and demonstrate how temperature is related to microbiome-immune interactions.

## 4.3 Results

### *4.3.1 Temperature predicts gut microbiome taxonomic composition and functional capacity*

#### *4.3.1.1 Community-level results*

We found a strong relationship between temperature and both taxonomic and functional community-level diversity in the gut microbiome. To assess this relationship, we compared a baseline model that accounted for known predictors of microbiome composition (sex, social group, diet, and season) to a model that included both the baseline predictors and temperature (Fig. 4.1S). We measured alpha diversity using Shannon diversity index and observed richness (the total number of Amplicon Sequence Variants, ASVs, or pathways in a sample), and characterized beta diversity using the first three principal components (PC1–PC3) from a principal component analysis (PCA) to capture major axes of variation in community composition. For taxonomic community composition, the model including temperature provided the best fit (controlling for model complexity) across all community-level indices, accounting for 100% of the model weight for every principal component and alpha diversity metric (Fig. 4.1B). The strong relationship between temperature and principal components 1–3 suggests that temperature drives shifts in taxonomic composition that are substantial enough to affect community-level beta diversity. Both alpha diversity indices showed a reliable negative relationship with temperature (Shannon $\beta_{temp} = -0.49$, Observed $\beta_{temp} = -0.49$)(Fig. 4.1A). Even though the effect sizes were small compared to the principal components, alpha diversity reliably declined with increasing temperatures (Fig. 4.2S). This suggests that higher temperatures are associated with reductions in both taxonomic richness and evenness.

For functional capacity, the temperature model provided the best fit for every principal component and the total number of observed metabolic pathways, accounting for at least 96% of the model weight. In contrast, the baseline model provided the best fit for Shannon diversity, accounting for 77% of the model weight (Fig. 4.1D). Therefore, temperature was associated with shifts in individual metabolic pathways substantial enough to affect community-level beta diversity, but the relationship between temperature and functional alpha diversity was less clear. Temperature was negatively associated with the total number of unique pathways (Observed: $\beta_{temp} = -35.05$), but the magnitude and direction of this effect were uncertain due to the wide confidence intervals that include zero. The effect size for Shannon diversity ($\beta_{temp} = -0.27$) was much smaller, and the confidence intervals also included zero (Fig. 4.1C). Together, these results suggest that higher temperatures may reduce the number of unique metabolic pathways but are not associated with changes in pathway evenness.

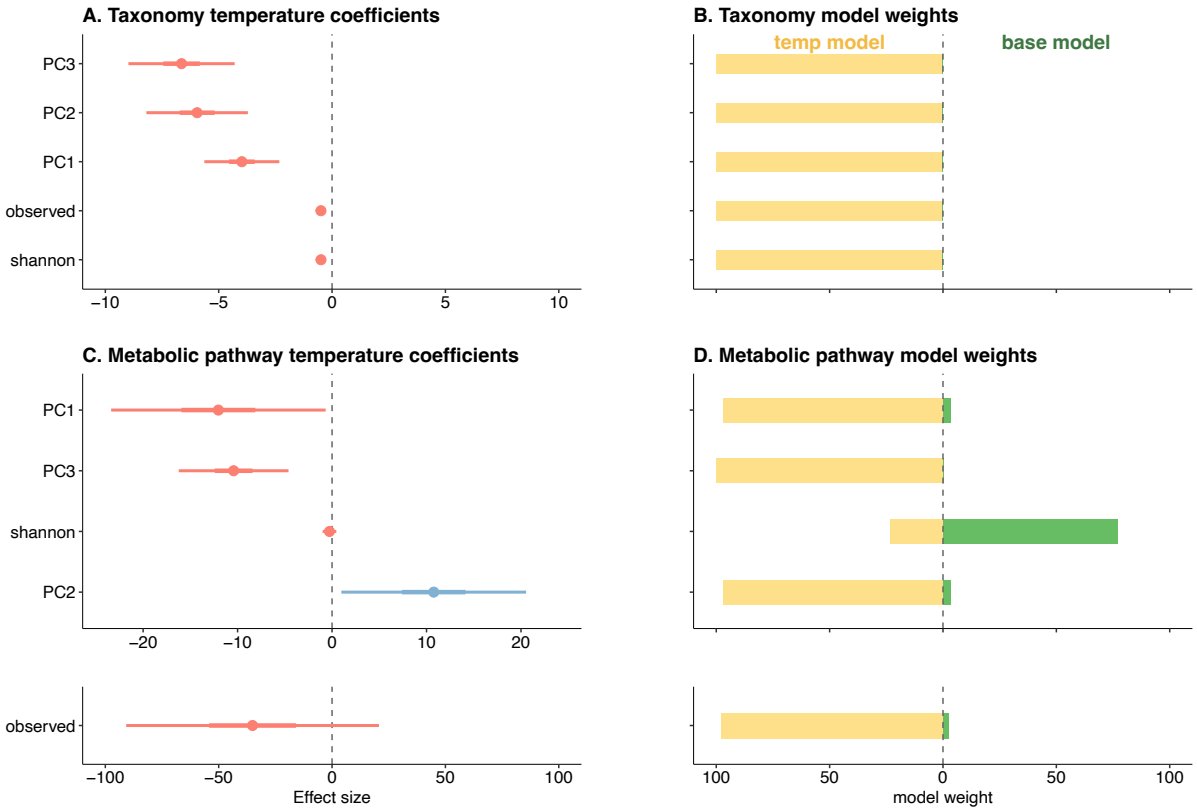



**Figure 4.1** Model comparison results assessing whether adding temperature to a base model of known microbiome predictors improves the explanation of community-level taxonomic and metabolic diversity. Panels A and C show the coefficients of the temperature predictor for community diversity metrics, including beta diversity (measured by principal components 1-3) and alpha diversity (measured by the Shannon Index and the observed number of ASVs or metabolic pathways). The thick bars represent 50% confidence intervals (CI), while the thin bars represent 95% CI. Panels B and D compare the model weights of the base model (in green) to the temperature model (in yellow). The weights of the base model plus the temperature model equal 100%. The top panels A and B show the results for community-level taxonomic diversity, while the bottom panels C and D show the results for metabolic pathway diversity. Results show a strong association between temperature and beta diversity for both taxonomic composition and metabolic capacity. In contrast, the association between temperature and alpha diversity differs by data type: taxonomic alpha diversity declines with rising temperature, whereas metabolic alpha diversity indicates a possible reduction in the number of unique pathways at higher temperatures but no relationship with pathway evenness.

### *4.3.1.2 Individual taxon and metabolic pathway results*

Temperature was also related to the abundance of individual taxa at both the family and genus levels. The temperature model provided the best fit (controlling for complexity) for 93 out of 163 taxa, accounting for at least 50% of the model weight. The majority of taxa, 82 out of 93, declined in abundance with rising temperatures. Ancestral state reconstruction performed using the temperature model weights suggests that this is a conserved microbial trait for C. imitator, particularly in key phyla such as Actinobacteria, Bacteroidota, and Firmicutes. In contrast, phyla

such as Campylobacterota and Proteobacteria appear less sensitive to temperature over evolutionary time (Fig 4.2).

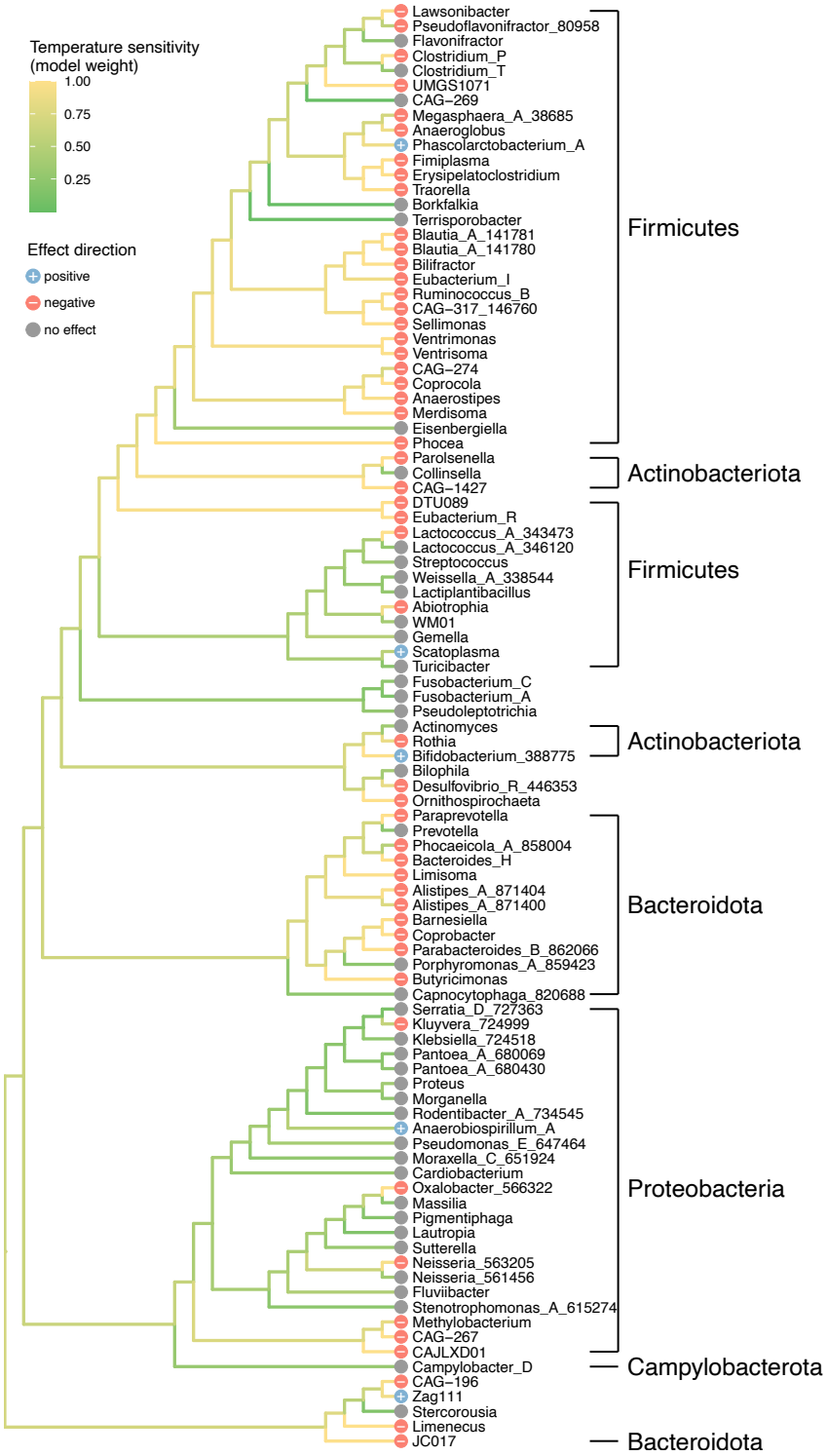



**Figure 4.2** Cladogram showing ancestral state reconstruction of temperature sensitivity for 97 genera with ≥ 0.2% average relative abundance. Branch color indicates model weight for the temperature model (includes base predictors plus temperature), with yellow representing high model weight (greater temperature sensitivity) and green representing low model weight. Genera where the temperature model accounts for ≥ 50% of the model weight are

classified as temperature sensitive and are depicted with red (negative association) or blue (positive association) circles at the branch tips. Genera with < 50% model weight are considered not temperature sensitive and are shown with grey circles. Most temperature-sensitive taxa decline in abundance with rising temperatures, especially within the Firmicutes and Bacteroidota phyla. In contrast, Proteobacteria and Campylobacterota appear less temperature sensitive compared to the other phyla.

Temperature was also related to the abundance of microbial metabolic pathways. The temperature model provided the best fit for 169 out of 383 total pathways, accounting for at least 50% of the model weight. To facilitate interpretation, we grouped 103 of these pathways into five functional categories based on shared biological roles and their apparent relationship with temperature: lipid metabolism, oxygen-driven metabolism and fermentation, redox maintenance, nucleotide mobilization, and immune regulation (Fig 4.3). Patterns in these categories suggest a metabolic restructuring that may help microbes respond to rising temperatures. For example, we found an increase in fatty acid synthesis pathways like oleate, cis-vaccenate, palmitoleate, stearate, petroselinate, and gondoate, and lipid remodeling pathways such as the mevalonate pathway and downstream isoprenoid lipid synthesis (Fig 4.3A), that may help maintain cytoplasmic membrane integrity at high temperatures (Mansilla et al., 2004; Y.-M. Zhang & Rock, 2008). However, we also found a decrease in pathways involved in lipid catabolism and recycling, including fatty acid β-oxidation, fatty acid salvage, and phospholipid turnover, which likely impairs membrane repair mechanisms (Dennis, 1994; Y.-M. Zhang & Rock, 2008) and may compromise integrity during extended periods of high temperatures. Further, we observed an increase in anaerobic pathways, including the reductive TCA cycle and several involved in glycolysis, alongside a decrease in pathways that support aerobic metabolism, including the pyruvate dehydrogenase complex, the TCA cycle, and glyoxylate cycle (Fig 4.3B). This may reflect a response to changing oxygen gradients with rising temperatures to maintain energy production (Hall et al., 1999). The anaerobic pathways primarily metabolized simple carbohydrates and coincided with a decline in complex polysaccharide fermentation, likely reflecting taxonomic shifts with rising temperatures (Flint et al., 2012).

We also observed declines in pathways that support redox maintenance and cycling (Fig 4.3C), including the synthesis of cofactors such as B vitamins (Hellmann & Mooney, 2010; Powers, 2003), coenzymes (Gout, 2018), antioxidant precursors to glutathione (Lu, 2013), NADPH production, quinone synthesis, siderophore-mediated iron transport (Dhungana & and Crumbliss, 2005; Ying, 2008), and detoxification pathways for aromatic compounds and aldehydes (Kalapos, 1999). In conjunction, we observed an increase in nucleotide and

translation-associated metabolism pathways (Fig 4.3D), such as purine and pyrimidine de novo synthesis, nucleotide salvage and recycling (Kilstrup et al., 2005; Lane & Fan, 2015), queuosine synthesis, and S-adenosylmethionine salvage (Vinayak & Pathak, 2009). This coordinated increase in nucleotide and translation-associated metabolism may reflect the consequences of redox imbalance at high temperatures because the accumulation of ROS can lead to DNA damage (Imlay, 2013). Some pathways that support redox maintenance increased with rising temperatures (Fig 4.3D), including the synthesis of antioxidant precursors and vitamin B12 (Martens et al., 2002), coenzyme metabolism (Belenky et al., 2007), and degradation of vitamin-like compounds (Linster & Van Schaftingen, 2007), which may reflect a compensatory effort to restore redox balance.

Rising temperatures were also associated with a decline in pathways that produce metabolites known to support anti-inflammatory signaling (Fig 4.3E), including synthesis of the SCFAs butyrate and propanoate (Arpaia et al., 2013; Klampfer et al., 2003), catabolism of monounsaturated fatty acids like oleate (Hung et al., 2023), and polyamine production from arginine degradation and ornithine synthesis (Nakamura & Matsumoto, 2025). However, these shifts were accompanied by an increase in select pathways that may help compensate for the loss of microbial immune regulation, including the synthesis of the saturated fatty acids (5Z)-dodecenoate and L-tryptophan biosynthesis, which leads to the production of indole derivatives that suppress pro-inflammatory cytokine signaling (Konopelski & Mogilnicka, 2022).




**Figure 4.3** Model results for the microbial metabolic pathways associated with temperature. Pathways are grouped into five functional categories (Panels A-E) based on shared biological roles and their associations with temperature. The thick bars represent 50% confidence intervals (CI), while the thin bars represent 95% CI. Summary annotations to the right of each panel describe the broad functional roles of the pathways within each category. Panel F visualizes the effects consistent with experimentally induced heat stress and how they may reinforce one another during extended periods of high temperatures.

### *4.3.2 Neopterin is related to the gut microbiome's response to temperature*

#### *4.3.2.1 Community-level results*

To assess if neopterin concentration is associated with the gut microbiome's relationship with temperature, we compared the temperature model that included known predictors of microbiome composition plus temperature to a model that included the baseline predictors and an interaction effect between temperature and neopterin concentration (Fig 4.1S). For taxonomic composition, the model including the interaction effect between temperature and neopterin concentration provided the best fit (controlling for complexity) for every principal component, accounting for at least 60% of the model weight (Fig 4.3S). Principal component 1 was the only component with confidence intervals that did not include zero. This result, along with the high model weight for every principal component, suggests that the relationship between temperature and beta diversity is associated with neopterin. In contrast, the temperature model provided the best fit for both alpha diversity metrics, accounting for at least 86% of the model weight. These results, combined with the small effect sizes (Shannon $\beta_{temp*neopterin} = -0.10$, Observed $\beta_{temp*neopterin} = -0.13$) and confidence intervals that include zero (Fig 3S), suggest that the relationship between taxonomic alpha diversity and temperature is independent of neopterin concentration.

For metabolic functional composition, the interaction model provided the best fit for every principal component and observed pathways, accounting for at least 98% of the model weight, while the temperature model provided the best fit for Shannon diversity, accounting for 63% of the model weight (Fig 4.3S). Even though principal component 3 was the only component where the confidence intervals did not include zero, the large effect sizes for all three components (PC1 $\beta_{temp*neopterin} = 17.60$, PC2 $\beta_{temp*neopterin} = -7.66$, PC3 $\beta_{temp*neopterin} = -11.44$) plus the model weights suggest that the interaction between neopterin and temperature has a meaningful influence on metabolic functional composition. The effect size and model weight for observed pathways (Observed $\beta_{temp*neopterin} = 72.78$) suggest that neopterin may be associated with the relationship between the total number of distinct metabolic pathways and temperature. However, it is unlikely that neopterin is associated with the relationship between temperature and pathway evenness due to the small effect size (Shannon $\beta_{temp*neopterin} = 0.46$), confidence intervals that include zero, and model weight for Shannon diversity.

#### *4.3.2.2 Individual taxon and metabolic pathway results*

Neopterin concentration was also associated with the relationship between temperature and the abundance of individual taxa. The interaction model provided the best fit for 20 of the 93 temperature-associated taxa, accounting for at least 50% of the model weight in each case. To interpret the interaction effect, we plotted the relationship between temperature and abundance across three discrete ranges of neopterin values (Fig 4.4). These results indicated two contrasting effects: (1) the relationship between temperature and abundance became more negative (i.e., the slopes decrease) at higher neopterin concentrations (Fig 4.4A), and (2) the relationship between temperature and abundance became more positive (i.e., the slopes increase) at higher neopterin concentrations (Fig 4.4B & C). The majority of taxa (12 out of 20) exhibited decreasing slopes at higher neopterin concentrations. These taxa were almost exclusively from the phyla Bacteroidota (5 taxa) and Firmicutes (6 taxa), and included two known SCFA producers, Parabacteroides and Alistipes (Hiippala et al., 2020; Parker et al., 2020). The taxa that exhibited increasing slopes (8 out of 20) showed two distinct patterns: one where a positive slope became steeper when neopterin was high, and another where a negative slope changed to positive when neopterin was high. These taxa included two SCFA producers, *Phascolarctobacterium* and *Eubacterium* (Louis & Flint, 2017; Wu et al., 2017), and the family *Helicobacteraceae*, which commonly includes facultative anaerobes (Alfarouk et al., 2019).

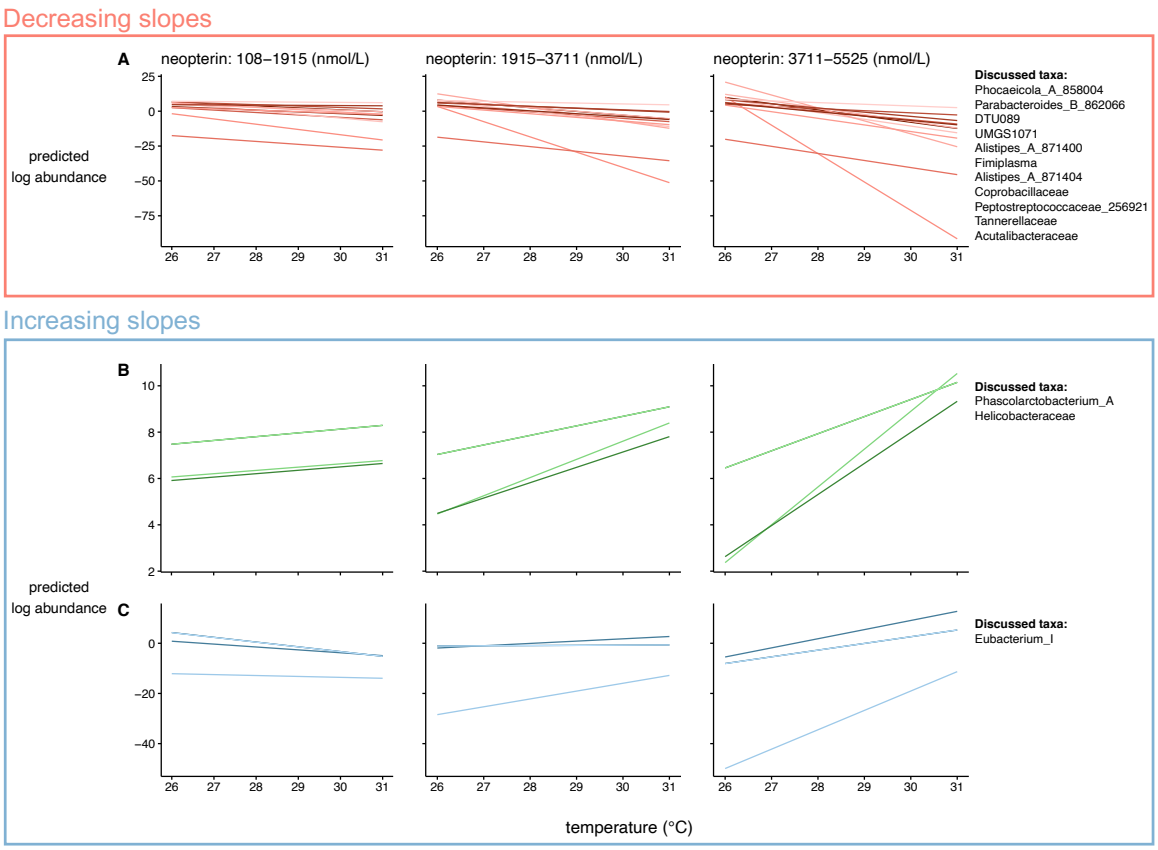



**Figure 4.4** The relationship between taxa abundance and temperature plotted across three distinct ranges of neopterin concentration. There are two main effects. Row A includes taxa for which the relationship between temperature and abundance becomes increasingly negative at higher neopterin concentrations. Rows B and C include taxa for which the relationship becomes increasingly positive, either through an increasingly positive slope (Row B) or a slope that switches from negative to positive (Row C). Taxa highlighted in the results and discussion are labeled on the right side of each row.

Neopterin concentration was also associated with the relationship between temperature and the abundance of individual metabolic pathways. The interaction model provided a better fit for 65 out of 116 pathways tested, accounting for at least 50% of the model weight in each case. To interpret the interaction effect, we plotted the relationship between abundance and temperature across two discrete ranges of neopterin values. These results indicated the same two effects as the taxonomic analysis (1) where slopes decreased at higher neopterin (Fig 4.5A-C) and (2) where slopes increased at higher neopterin (Fig 4.5D & E). The pathways that exhibited decreasing slopes showed three distinct patterns: one where a positive slope changed to negative, another where a positive slope became less positive, and one where a negative slope became steeper. This group included pathways involved in anti-inflammatory signaling, including synthesis of monounsaturated fatty acids (Hung et al., 2023) and tryptophan, as well as those typically associated with obligate anaerobes, including complex carb fermentation (Flint et al.,

2012) and Gram-positive cell wall biosynthesis characteristic of obligate anaerobes from the Firmicutes phylum (Rajilić-Stojanović & de Vos, 2014). The majority of pathways (37 out of 65) exhibited increasing slopes at higher neopterin concentrations. There were two patterns of increasing slopes: one where a negative slope changed to positive when neopterin was high, and another where a positive slope became steeper when neopterin was high. These groups included pathways commonly associated with facultative anaerobes, including the glyoxylate cycle (Ahn et al., 2016), Rubisco shunt (Badger & Bek, 2008), ubiquinone biosynthesis (Bekker et al., 2007), enterobacterial common antigen biosynthesis (Kuhn et al., 1988), and Lipid A biosynthesis (Raetz & Whitfield, 2002). They also included pathways involved in redox maintenance, including the synthesis of B vitamins (Hellmann & Mooney, 2010; Powers, 2003), menaquinol (Gout, 2018), and NADPH (Ying, 2008).

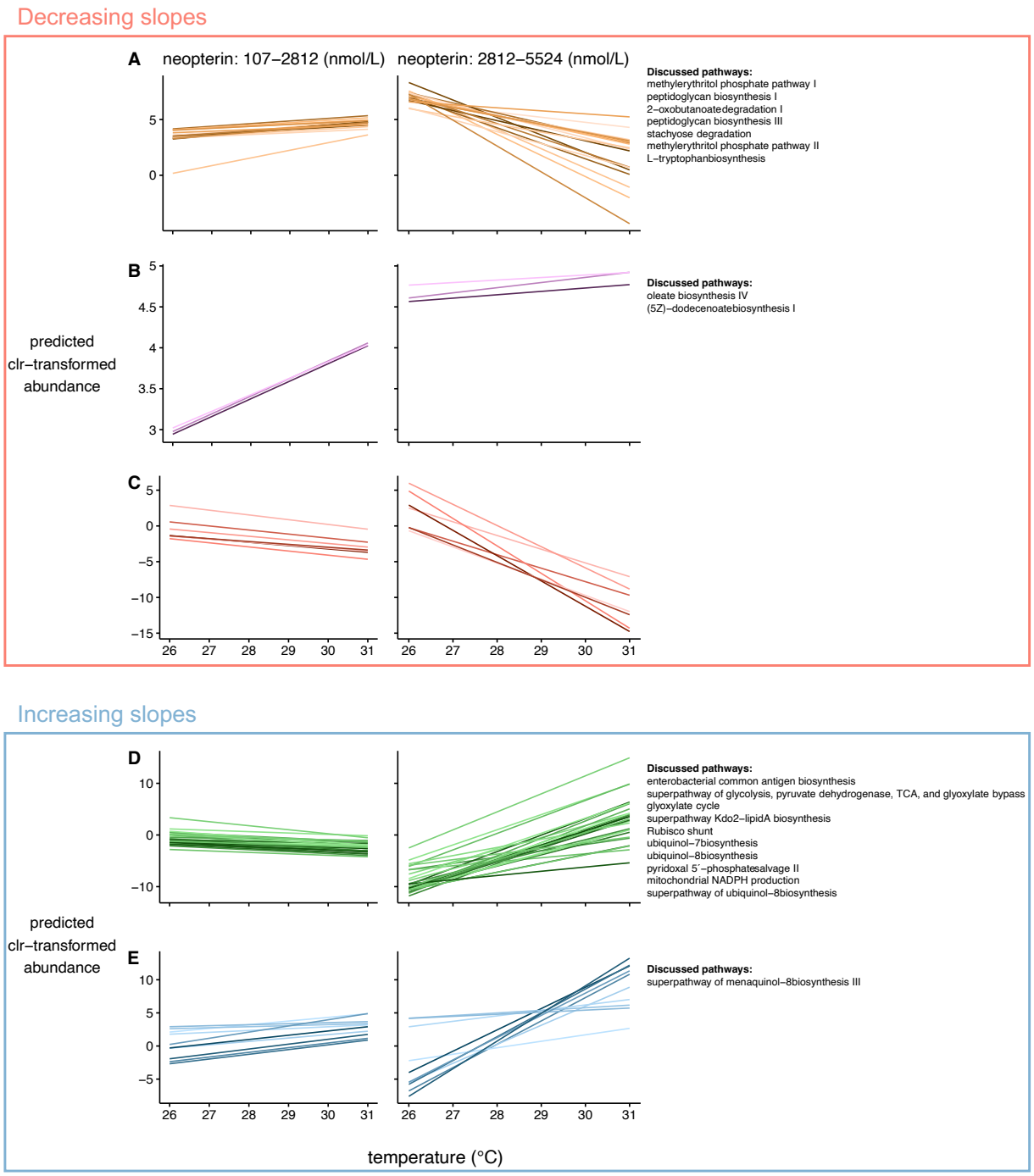



**Figure 4.5** The relationship between metabolic pathway abundance and temperature plotted across two distinct ranges of neopterin concentration. Pathway abundances are shown as centered log-ratio (clr)–transformed values. There are two main effects. Rows A-C include pathways for which the relationship becomes increasingly negative at higher neopterin, either through a slope that switches from positive to negative (Row A), a slope that becomes less positive (Row B), or an increasingly negative slope (Row C). Rows D and E include pathways for which the relationship between temperature and abundance becomes increasingly positive at higher neopterin, either through a slope that switches from negative to positive (Row D) or an increasingly positive slope (Row E). Pathways highlighted in the results and discussion are labeled on the right side of each row.

## 4.4 Discussion

Our study had two main goals: (1) to assess how temperature influences gut microbiome composition and function in wild, white-faced capuchins, and (2) to evaluate whether temperature-driven changes to the gut microbiome are related to host immunity. We found strong evidence that temperature is associated with community-level diversity, taxonomic composition, and metabolic capacity that may indicate the capuchins are undergoing periods of heat stress. Moreover, the microbiome's taxonomic and functional responses to temperature are linked to differences in urinary neopterin concentrations, suggesting that immunity is related to the gut microbiome's response to heat.

### *4.4.1 Gut microbiome response to temperature*

Our data suggest that the gut microbiome's response to high temperatures may affect its role in conferring colonization resistance— the ability to protect the host from exogenous pathogens. Colonization resistance arises from direct competition of commensals with pathogens for resources and microbial support for host immunity (Caballero-Flores et al., 2023). Community-level analysis reveals that taxonomic alpha diversity declines with increasing temperatures (Fig 4.2S), suggesting that heat may impair the gut microbiome's ability to resist colonization by reducing the diversity of taxa that are available to compete with pathogens (Spragge et al., 2023). High temperatures are also associated with the decline in the Bacteroidota and Firmicutes phyla, which contain taxa known to support host immunity such as *Clostridiaceae*, *Lactococcus*, and *Barnesiella* (Fig 4.2)(Buffie & Pamer, 2013), which may leave hosts more vulnerable to infection. However, functional alpha diversity appears more resilient to increasing temperatures (Fig 4.1), likely due to functional redundancy— when taxonomically distinct microbes perform similar functional roles (Louca et al., 2018). This redundancy may buffer the loss of taxonomic diversity and help preserve colonization resistance at high temperatures because remaining taxa fill enough functional niches to outcompete pathogens. Although we did not observe an increase in potentially pathogenic taxa, the phyla Proteobacteria and Campylobacteria, which are known to contain gut pathogens (Kaakoush et al., 2015; Shin et al., 2015), are less sensitive to heat compared to other phyla. Therefore, sustained high temperatures may continue to erode both taxonomic and functional diversity, eventually increasing the host's vulnerability to infection

Metabolic restructuring in response to rising temperatures suggests that the gut microbiome undergoes functional shifts that may support community stability. For example, the increase in lipid synthesis and remodeling pathways (Fig 4.3A) could reflect cytoplasmic membrane restructuring, a process bacteria use to preserve membrane stability at high temperatures (Mansilla et al., 2004; Sáenz, 2015). However, these changes may be insufficient to maintain membrane integrity during extended periods of high temperatures because the observed decline in lipid catabolism and recycling pathways likely compromises membrane repair mechanisms (Y.-M. Zhang & Rock, 2008). Limited repair capacity could leave gut microbes more vulnerable to peroxidative damage, a common consequence of heat stress driven by an accumulation of ROS that damages the lipid components of cell membranes (Belhadj Slimen et al., 2014; Lin et al., 2006). Similarly, the observed increase in anaerobic metabolic pathways and the decline in aerobic pathways (Fig 4.3B) likely reflect a response to maintain energy production under changing oxygen gradients due to redirected blood flow during heat exposure (Hall et al., 1999). However, these anaerobic pathways are primarily involved in simple carbohydrate metabolism typical of facultative taxa (Ng et al., 2013; Zeng et al., 2017), and coincide with a decline in complex polysaccharide fermentation typical of obligate taxa (Flint et al., 2012; Hooper et al., 2002; Maier et al., 2014). Therefore, this shift is likely driven by the relative persistence of facultative versus obligate anaerobes at high temperatures. Since fiber fermentation creates SCFAs, which are crucial energy sources (Donohoe et al., 2011) and immunomodulators (Furusawa et al., 2013; Klampfer et al., 2003), extended periods of high temperatures may eventually cause a decline in overall energy availability and affect immune function. While these metabolic shifts suggest that capuchin gut microbes actively respond to heat in ways that may support community stability, the decline in membrane repair and inferred loss of obligate fermenters indicate that this response is unlikely to be sustainable over longer periods of high temperatures.

The taxonomic response to temperature suggests that changes in gut physiology at high temperatures affect the persistence of obligate anaerobes compared to facultative anaerobes. There are broad declines in two phyla known to include obligate anaerobes, Bacteroidota and Firmicutes, including essential obligate taxa such as *Bacteroides*, *Parabacteroides*, *Clostridium*, *Ruminococcus*, *Eubacterium*, *Blautia*, *Anaerostipes*, and *Alistipes* (Fig 4.2). In contrast, Proteobacteria, comprised of primarily facultative taxa (Albenberg et al., 2014; Shin et al.,

2015), are relatively stable in response to rising temperatures. These patterns mirror compositional changes in response to heat stress in a wide range of vertebrates [e.g., fish (Neuman et al., 2016; Soriano et al., 2018), reptiles (Bestion et al., 2017; Moeller et al., 2020), amphibians, birds (Zhu et al., 2019), and mammals (Kohl & Yahn, 2016)]. The decrease in obligate anaerobes is likely due to redox imbalance induced by high temperatures, where the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the gut environment. Obligate anaerobes possess limited enzymatic defenses against ROS, and are therefore less able to tolerate changes in the oxygen environment compared to facultative taxa (Z. Lu et al., 2018; McCord et al., 1971). Although direct markers of redox balance are needed to assess the gut's redox environment, the observed decline in redox-maintenance pathways (Fig 4.3C) suggests that rising temperatures impair microbial antioxidant capacity, which can disrupt the redox environment (Kunst et al., 2023; Sun et al., 2024; Yardeni et al., 2019). We also observe an increase in DNA repair pathways (Fig 4.3D) that suggests cellular damage from the accumulation of ROS (Imlay, 2013). Together, these results provide compelling evidence that capuchins experience periods of redox imbalance at high temperatures that may affect the relative abundance of facultative versus obligate anaerobes.

Further, a decline in obligate anaerobes during heat stress may disrupt the gut microbiome's ability to regulate host immunity by impairing key immunomodulatory functions. Obligate anaerobes are essential SCFA producers that help regulate intestinal immunity and maintain host tolerance towards gut microbiomes, including the promotion of anti-inflammatory regulatory T-cell activity (Arpaia et al., 2013; Furusawa et al., 2013) and the suppression of proinflammatory cytokine signaling (Hung et al., 2023; Klampfer et al., 2003). The decrease in SCFA-producing pathways, particularly butyrate and pyruvate (Fig 4.3E), likely reflects the loss of obligate anaerobes at high temperatures due to redox imbalance and may decrease host tolerance towards gut microbiomes. The resulting increase in immune activity may exacerbate redox imbalance because activated immune cells, particularly phagocytes, release ROS as part of the inflammatory response (Nathan & Cunningham-Bussel, 2013).

These results may be driven by confounding variables related to seasonal ecology, such as changes in diet, ranging patterns, social structure, activity budgets, or water sources that can influence microbiome composition and function (E. K. Mallott et al., 2018; Orkin, Campos, et al., 2019; Orkin, Webb, et al., 2019). However, many of our results mirror those from

experimental studies evaluating the effects of high temperature on the microbiome, indicating that we have likely captured a true temperature effect rather than an indirect signature of seasonal ecology. This is likely because we assessed cumulative exposure to high temperatures rather than short-term fluctuations that are more likely to covary with other ecological variables. We also focused on interpreting broad patterns across taxonomic groups and metabolic pathways (Figs. 4.2 & 4.3) rather than interpreting individual features that may be more sensitive to ecological confounds.

Importantly, our results resemble those associated with heat stress in experimental work, including a decrease in obligate anaerobes relative to facultative anaerobes (Sepulveda & Moeller, 2020), evidence of redox imbalance (Fang et al., 2023; Liu, Ma, et al., 2023), and changes in immunomodulatory function (Chen et al., 2018; Tajima et al., 2007; Yu et al., 2024). Together, these findings suggest that the capuchins may be experiencing heat stress at the upper limit of their thermal range that could compromise essential microbiome functions. These effects also likely reinforce one another during extended periods of high temperatures via reciprocal interactions linking redox balance, microbial composition, and immune function (Li et al., 2023; Mostafavi Abdolmaleky & Zhou, 2024)(Fig 4.3F).

### *4.4.2 Gut microbiome response to temperature is related to microbiome-immune interactions*

Declines in the abundance of obligate anaerobes with temperature may impact host immunity. For example, stronger declines in obligate anaerobes at high temperatures, such as *Parabacteroides* and *Alistipes*, are associated with higher neopterin levels (Fig 4.4A). These taxa contribute to anti-inflammatory signaling through the production of SCFAs (Hiippala et al., 2020; Parker et al., 2020), suggesting that their loss may affect immune tolerance towards gut microbes. Similarly, stronger declines in metabolic pathways that produce anti-inflammatory immunomodulators are also associated with higher neopterin. This includes the synthesis of monounsaturated fatty acids (Hung et al., 2023) and tryptophan, a compound primarily synthesized by obligate anaerobes (Dodd et al., 2017; Zelante et al., 2013) (Fig 4.5A & B). Conversely, high neopterin could also contribute to a stronger decline in obligate anaerobes at high temperatures, likely because increased macrophage activity exacerbates heat-driven redox imbalance. Taxa that exhibit stronger declines when neopterin is high are primarily from the Bacteroidota and Firmicutes phyla, which include many obligate anaerobes (Fig 4A). We also observed a stronger decline in pathways commonly associated with obligate anaerobes including

complex carb fermentation (Flint et al., 2012) and Gram-positive cell wall biosynthesis characteristic of obligates from the Firmicutes phylum (Rajilić-Stojanović & de Vos, 2014)(Fig 4.5A).

In contrast, facultative anaerobes may be more resilient to these conditions. For example, *Helicobacteraceae* exhibits a stronger increase in abundance when neopterin is high (Fig 4.4B), suggesting they are better able to tolerate redox imbalance, and may exploit niches vacated by the loss of obligates. Similarly, several pathways commonly associated with facultative anaerobes exhibit a stronger increase at high neopterin levels and may help these taxa actively respond to changes in the gut environment (Fig 4.5D). For example, the glyoxylate cycle and Rubisco shunt both increase under high neopterin, which likely supports energy conservation under oxygen and energy-limited conditions (Ahn et al., 2016; Badger & Bek, 2008). In addition, redox maintenance pathways such as ubiquinone biosynthesis also increase which helps facultative anaerobes neutralize ROS (Bekker et al., 2007). Synthesis of enterobacterial common antigen (ECA) also increases, which is characteristic of *Enterobacteriaceae* (Kuhn et al., 1988). Conversely, an increase in facultative anaerobes at high temperatures could also affect host immunity. For example, lipid A synthesis, which is characteristic of many facultative taxa, increases at higher neopterin and forms pathogen-associated molecular patterns (PAMPs) that are recognized by Toll-like receptors (TLRs) and can initiate pro-inflammatory signaling (Raetz & Whitfield, 2002)(Fig 4.5D). *Helicobacteraceae* is also associated with inflammatory diseases and may promote macrophage activation by damaging the gut epithelium and disrupting the mucosal barrier (Chu et al., 2003).

Therefore, the decline of obligate anaerobes relative to facultative taxa at high temperatures may drive, and be driven by, increased immune activity in the gut. Although we cannot conclude directionality, these findings suggest that gut–immune interactions are an integral component of the gut microbiome's response to heat and may increase susceptibility to gut inflammation during prolonged high temperatures. These results may also reflect physiological heat stress in the capuchins, given the observed relationship between taxonomic shifts and immune activity that mirrors experimental findings, and the potential inflammatory consequences of long-term heat exposure (Chen et al., 2018; Sepulveda & Moeller, 2020; Tajima et al., 2007; Yu et al., 2024). Future work should incorporate additional immune biomarkers that directly assess inflammation, as neopterin primarily reflects macrophage activation and

represents only one of the many mechanisms linking immunity and the gut microbiome. Continued research should also assess whether these effects have measurable impacts on host health and fitness, providing critical insight into how climate warming may influence wildlife through gut–immune pathways.

## 4.5 Conclusion

Our results highlight the pivotal role of temperature in shaping the gut microbiome and its relationship with host immunity in a wild primate species. Many of the observed taxonomic and metabolic shifts resemble those characteristic of experimentally induced heat stress, including declines in obligate anaerobes relative to facultative anaerobes, evidence of redox imbalance, and altered immunomodulatory functions, suggesting that the capuchins may be experiencing heat stress that could compromise essential microbiome functions. We also found that gut–immune interactions are linked to the gut microbiome's response to heat and may increase susceptibility to gut inflammation during prolonged high temperatures. These results highlight the crucial role of temperature in shaping essential gut microbiome functions in a wild endotherm species and offer insight into how rising temperatures due to climate change may threaten species resilience through their microbiomes.

## 4.6 Methods

### *4.6.1 Site information and data collection*

We collected data from a wild population of *C. imitator* living in the Taboga Forest reserve in Guanacaste, Northwestern Costa Rica. The Taboga Forest Reserve is a 516-ha tropical dry forest fragment and forms an important piece of a fragmented biological corridor connecting the Tempisque River Basin to the Guanacaste Mountains (Tinsley Johnson et al., 2020). The Capuchinos de Taboga Research project has collected daily behavioral and endocrine data on the three groups of white-faced capuchins since 2018. All individuals are individually identified and habituated to human presence. Individual age is calculated by birth date for individuals younger than 7 years of age; for individuals older than 7 years, age was estimated based on body condition by a field team member (J.C.O.) with more than two decades of experience observing wild white-faced capuchins. A team of two or more trained field assistants collected data between 5:00 AM and 6:30 PM including detailed observations of feeding and foraging behavior using scan sampling. Scans occurred every 30 minutes for 10 minutes, where feeding and

foraging observations of individually identified monkeys were recorded. Individuals were recorded multiple times in one scan if they were observed engaging with different food items. Plant food items were identified on the family or genus level.

Between September 2022 and July 2023, we opportunistically collected fecal and urine samples in conjunction with behavioral data. All samples were collected from identified individuals who appeared healthy upon visual inspection. We placed fecal samples into 4 mL of 98% ethanol immediately after defecation (Amato et al., 2013). To avoid contamination, we wore gloves and only collected feces that had not contacted the soil. We discarded feces contaminated with urine to prevent contamination from the urogenital microbiome (Thomas-White et al., 2018). We collected urine samples using a clean catch method (Bergstrom et al., 2017) to avoid rainfall and fecal contamination. We also collected urine from leaves or the ground if it appeared free of soil or fecal contamination. We immediately stored fecal and urine samples in a portable cooler and transferred them to a –20°F freezer at the end of each field day. We kept fecal samples under refrigeration until shipment to the Amato Microbiome Lab at Northwestern University in Evanston IL for DNA extraction and sequencing. Urine samples remained in the freezer until we conducted assay analysis onsite in the Capuchinos de Taboga field laboratory (Beehner et al., 2022).

Temperature observations for this analysis came from the Palo Verde meteorological station, located at the Organization for Tropical Studies (OTS) Palo Verde research station (10.345°N, 85.339°E), approximately 20.81 km southwest of the Taboga Forest Reserve. Like Taboga, Palo Verde is one of Central America's last remaining tropical dry forest fragments (Stern et al., 2002). Palo Verde temperature data have been used in previous studies of the Taboga white-faced capuchin population (Lucore et al., 2024), given the habitat similarity and proximity to Taboga. The station records maximum and minimum air temperature every 15 minutes.

### *4.6.2 Neopterin analysis*

We measured the neopterin concentration of the urine samples using a commercially available competitive exclusion neopterin enzyme-linked immunosorbent assay (Neopterin ELISA, Ref. RE59321, IBL International GMBH, Hamburg, Germany) in the Capuchinos de Taboga field laboratory. The assay has been validated for use in the Taboga capuchin population (Lucore et al., 2022). We used specific gravity to control for sample water volume and diluted

samples using manufacturer assay buffer. We ran the assays using manufacturer protocol, see (Lucore et al., 2022, 2024) for details.

### *4.6.3 Sample processing for 16S rRNA amplicon and shotgun metagenomic sequencing*

#### *4.6.3.1 DNA extraction*

We conducted all lab work at the Amato Microbiome Lab at Northwestern University, Evanston IL. We extracted DNA from fecal samples using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD, USA), with minor modifications to manufacturer protocol. Specifically, we incubated samples at 65°C for 10 minutes after adding the C1 and PowerBead solutions, refrigerated the C2 and C3 incubation steps, and prewarmed the C6 solution before the final elution. These modifications align with other protocols designed for fecal samples containing high levels of plant fiber (Amato et al., 2020; E. k. Mallott et al., 2015; Pickett et al., 2012).

#### *4.6.3.2 16s rRNA amplicon sequencing and data processing*

We used 16s rRNA amplicon sequencing to describe the gut microbiome taxonomic composition. We amplified the hypervariable V4-V5 region of the 16s rRNA gene using the 515F/926R primers from the Earth Microbiome Project (www.earthmicrobiome.org) and a dual-indexing approach (Gohl et al., 2016); see previously described protocol for details (E. K. Mallott & Amato, 2018). We used negative controls to monitor contamination during the DNA extraction and PCR amplification. We purified and normalized PCR products using SequalPrep Normalization and sequenced them on the Illumina MiSeq V4 platform at the Rush Genomics and Microbiome Core Facility.

We analyzed the resulting data using the Quantitative Insights into Microbial Ecology (QIIME2) platform (Caporaso et al., 2010), v2023.9. Pair-end sequences from 900 samples yielded 28,869,202 raw sequence reads (average 32,077 per sample, range of 7 to 64851 reads). We trimmed forward and reverse reads to 216 bases and 206 bases, respectively, to remove low-quality base pairs using a minimum Phred score of 33. We quality-filtered and denoised sequences using the Divisive Amplicon Denoising Algorithm 2 (DADA2)(Callahan et al., 2016) via the q2-dada2 pipeline, which infers Amplicon Sequence Variants (ASVs) by identifying exact sequences. We assigned taxonomy to ASV's using the q2-feature classifier (Bokulich et al., 2018) against version 2022.10 of the Greengenes2 database (McDonald et al., 2012, 2024) by

applying the classify-sklearn model. We removed sequences assigned to chloroplasts and mitochondria using the q2-taxa-filter-table to retain only microbial sequences for downstream analysis. All negative controls contained fewer than 507 reads per sample (average of 29), and we excluded them after visually confirming that their taxonomic composition did not indicate contamination. We also excluded one fecal sample that contained fewer than 1,000 reads to avoid bias from low-coverage data. After filtering, we retained 797 samples, which contained an average of 16,898 reads (range: 4,944–38,941).

We imported the ASV count and taxonomy files generated by QIIME2 into R using the {qiime2R} package (Bisanz, 2018) and saved them as a phyloseq object using the {phyloseq} package (McMurdie & Holmes, 2013). Most ASV's were found at low frequencies; out of 11,916 total ASV's, 89% were only found in one sample, and 16% were not assigned to a phylum. To avoid analyzing taxa that are artifacts of the sequencing process (Cao et al., 2021), we removed ASV's found in less than 0.5% of total samples. After filtering, we removed 102 additional ASV's that were not assigned at the phylum level, resulting in 1159 total remaining ASV's. Most of the remaining ASV's could be assigned to class (100%), order (95%) and family (93%) levels but assignment decreased substantially at the genus (60%) level.

#### *4.6.3.3 Shotgun metagenomic sequencing and data processing*

We used metagenomic sequencing to characterize the functional capacity of the gut microbiome in a subset of 95 samples with sufficient DNA concentration for library preparation. We prepared sequencing libraries using the NEBNext Ultra™ II FS DNA PCR-free Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). We followed the manufacturer protocol for low inputs (50ng to 99ng) and selected library inserts around 350bp. We pooled the libraries and sequenced them on the Illumina NovaSeq platform at the Rush Genomics and Microbiome Core Facility. Pair-end sequences from 95 samples yielded 2,902,714,548 raw sequence reads (average 15,277,445 per sample, range of 531,829 to 25,348,795 reads).

We quality-filtered the raw metagenomic sequences using KneadData (v0.12.0) which uses Trimmomatic to remove adaptors, low-quality reads, and overrepresented or repetitive sequences, and Bowtie2 to remove host-derived sequences (Beghini et al., 2021). We ran KneadData using the standard parameters SLIDINGWINDOW:4:20 and MINLEN:90 to trim reads when the average quality score dropped below 20 and remove reads shorter than 90bp. To remove host-associated reads we used the GRCh38.p13 human reference genome (RefSeq

accession: GCF_009914755.1) which has been used to filter host genome data in previous capuchin metagenome studies (Orkin, Campos, et al., 2019). KneadData yielded 931,326,495 cleaned reads (average 98,034,376 per sample, range of 178,711 to 22,386,906 reads). We concatenated the forward and reverse reads and used HUMAnN3 (v3.9) for functional profiling. Gene families were assigned using the Uniref50 database and regrouped into MetaCyc reactions. The resulting table reported the abundance of each unique metabolic pathway normalized by the number of gene families contributing to that pathway. We imported the pathway abundance table into R and removed the unmapped values, which represent sequences that could not be assigned to any known gene family, as well as unintegrated values, which correspond to gene families not associated with any known pathway. After filtering, 548 unique metabolic pathways remained. We performed all subsequent analyses using R v4.3.1.

#### *4.6.4 Metadata assembly*

We matched each 16s rRNA and shotgun metagenomic sample with the relevant metadata to facilitate subsequent analyses. We paired samples with the average neopterin value from a 7-day window, encompassing three days before, the day of, and three days after sample collection. Our time window provides a general measure of immune performance associated with the microbiome composition, enabling us to assess the relationship between the two. We also averaged the maximum temperature (collected every 15 minutes) 15 days before sample collection. This time frame allowed us to evaluate the relationship between the microbiome and long-term trends in temperature. This temperature predictor has also been associated with changes in immune performance in this population (Lucore et al., 2024).

We also paired each sample with all diet observations from the same individual within two weeks before sample collection, following time frames used in previous white-faced capuchin gut microbiome studies (E. K. Mallott et al., 2018; Orkin, Campos, et al., 2019). We quantified diet diversity by modeling diet richness (i.e., the total number of unique food items recorded) as a function of the total number of feeding observations. We then used the residuals as a measure of diet diversity, with negative values indicating lower-than-expected diversity and positive values indicating higher-than-expected diversity (Marshall et al., 2021). We calculated the percent consumption of each food type by dividing the total number of feeding observations for each food category (animal, flower, fruit, fungi, insect, leaves, nectar, nurse, pith, seeds) by the total number of feeding observations. We then plotted each food type against temperature to

identify diet categories that vary with temperature to control for these factors when assessing the direct effect of temperature on the microbiome. We found that percent fruit consumption has a strong, positive relationship with temperature (Fig 4.4S) and controlled for this in subsequent analyses. After matching each sample with the appropriate metadata, we were left with 277 16s rRNA samples and 71 shotgun metagenomic samples.

### *4.6.5 Community-level composition analysis*

To evaluate how gut microbiome taxonomic composition and functional capacity are related to temperature, we generated metrics of community composition including alpha and beta diversity. To measure alpha diversity, we calculated two widely used indices: Shannon diversity index (which accounts for both richness and evenness) and observed richness (the total number of ASVs or pathways in a sample) using the estimate_richness() function from the {phyloseq} package. To measure beta diversity (i.e., community dissimilarity between samples), we generated Aitchison distances by computing the Euclidean distance between pairwise samples after performing a centered log-ratio (clr) transformation on the raw count data (Aitchison & Greenacre, 2002). We added a pseudo count to all zeros before the clr transformation using the cmultRepl() function from the {zCompositions} package, which uses Bayesian-multiplicative replacement to preserve the structure of the data and minimize distortion compared to adding a fixed value (Palarea-Albaladejo & Martín-Fernández, 2015). We used Aitchison distances because microbiome datasets are compositional, meaning that observed read counts are constrained by sequencing depth and do not reflect absolute DNA abundances (Gloor & Reid, 2016). Unlike traditional beta diversity metrics that were designed for absolute counts (e.g., Bray-Curtis, Unifrac, Jensen-Shannon divergence), the clr transformation projects samples into Euclidean space where distances reflect log-ratio relationships among taxa, avoiding misinterpretations that may arise from using absolute differences to interpret compositional data (Gloor et al., 2017). We then performed a Principal Components Analysis (PCA) on the Aitchison distance matrix and extracted the scores for the first three principal components (PC1–PC3) to capture major axes of variation in community composition. Components one through three were selected because they explained greater than 5% of the variation (Fig 4.5S).

To evaluate the relationship between temperature and community composition, we built mixed models using alpha diversity indices and the first three principal components as the outcome variables. We modeled each community metric twice, and compared model fit using

Akaike information criterion adjusted for small sample sizes (AICc) (Akaike, 1998; Hurvich & Tsai, 1989). The baseline model, m_main, included predictors for social group, age, sex, rainfall, diet diversity and fruit consumption. The temperature model, m_full, included all of the same predictors with an additional predictor for temperature (Fig 4.1S). Both models included a random intercept for individual to control for repeated sampling and an offset for the log-transformed number of reads to control for differences in sequencing depth between samples. We included fruit consumption as a predictor because it increased with rising temperatures (Fig 4.4S), and we wanted to control for this effect to better isolate the relationship between temperature and community features. We scaled all continuous predictors (i.e., age, rainfall, diet diversity, fruit consumption, temperature) for ease of coefficient interpretation. We fit all models as linear mixed models (LMMs) with a Gaussian distribution except for observed taxonomic richness, which was fit using a Gamma distribution with a log link function because the variable was continuous and positive with a long right tail. We used the model weights from the AICc comparison to evaluate the importance of temperature as a predictor of community composition.

Since rarefaction to account for differences in sequencing depth (i.e., choosing a fixed sequencing depth and randomly subsampling all samples to that depth) is strongly discouraged because it discards valuable data (McMurdie & Holmes, 2014), we used alternative methods to leverage the full dataset (i.e., clr transformation, modeled offset for sequencing depth). Further, we chose to use an Information-theoretic approach for our analysis because, unlike null-hypothesis significance testing, it allows for the comparison of multiple alternative hypotheses to assess biological significance (Anderson et al., 2000; Burnham et al., 2011). This allowed us to evaluate if adding temperature to our model improved the explanation of community-level taxonomic and metabolic diversity compared to other known predictors of microbiome composition.

### *4.6.6 Temperature differential abundance analysis*

We also used a comparative modeling approach to identify taxa and metabolic pathways associated with temperature. For the taxonomic analysis, we aggregated the number of reads per taxa on the family and genus levels using the tax_glom() function in the {phyloseq} package. We used this to calculate the relative abundance of each taxon by dividing the number of reads per taxon by the total number of reads per sample and removed taxa with less than 0.2% average

relative abundance across all samples to avoid analyzing extremely low-abundance taxa. This resulted in 134 genera and 79 families to be used for differential abundance testing.

We built two generalized linear mixed models (GLMMs) using a negative binomial distribution with a log link function and used individual family and genera abundances as our outcome variables. We used negative binomial GLMMs because they can effectively model over-dispersed count data with an abundance of zeros, a pattern typical of microbiome datasets . We modeled each taxon twice and compared model fit using AICc. The baseline model, m_main, included predictors for social group, age, sex, rainfall, and fruit consumption. The temperature model, m_full, included all of the same predictors with an additional predictor for temperature (Fig 4.1S). Both models included a random intercept for individuals to control for repeated sampling and an offset for the log-transformed number of reads to control for differences in sequencing depth between samples. Similar to our community feature analysis, fruit consumption was included to better isolate the relationship between temperature and individual taxa while accounting for diet components also related to temperature. However, we did not include diet diversity as a predictor to reduce model complexity and facilitate model convergence. We scaled all continuous predictors. Model weights were used to evaluate the importance of temperature as a predictor of individual taxon abundance. Taxa where m_full held greater than 50% of the model weight were considered temperature sensitive. We then used the m_full model weights of all genera included in the analysis to perform ancestral state reconstruction using the ace() function in the {ape} package. Internal node values represent the maximum likelihood estimates of the temperature model weight under a Brownian motion model of trait evolution.

The modeling approach for the metabolic pathway data mirrored that used for the taxonomic data, except we modeled clr-transformed abundances instead of raw counts. This allowed us to use a Gaussian distribution and omit the offset term for log-transformed read depth, as the clr transformation already accounts for differences in sequencing depth. We used clr-transformed abundances because pathway abundance values are normalized by the number of contributing gene families, meaning they are no longer raw counts and are therefore inappropriate for modeling with a negative binomial distribution. We modeled each of the 547 pathways twice, once with the baseline model, m_main, and once with the temperature model, m_full (Fig 4.1S). We compared the models using AICc. As in the taxonomic analysis, pathways

for which m_full accounted for more than 50% of the model weight were considered temperature sensitive.

### *4.6.7 Immune and temperature interaction analysis*

To assess whether the relationship between temperature and community composition is associated with immune performance, we employed a second comparative modeling approach and used alpha diversity and the first three principal components as outcome variables. Similar to our previous approach, we modeled each community feature twice, once with each model, and compared model fit using AICc. The baseline model, m_immune_main, included the same predictors as m_full (i.e., social group, age, sex, rainfall, fruit consumption, temperature, random effect for ID, log total reads offset). The interaction model, m_immune_full, included all of the same predictors with an additional interaction effect between temperature and neopterin (Fig 4.1S). The interaction effect allowed us to test if the relationship between community composition changes depending on neopterin levels. We scaled all continuous predictors, including neopterin. All models were fit as linear mixed models (LMMs) with a Gaussian distribution except for observed taxonomic richness, which was fit using a Gamma distribution with a log link function because the variable was continuous, positive, and left-skewed with a long right tail.

To assess whether the relationship between temperature and individual taxa or pathways is associated with immune performance, we employed the same model comparison approach but used taxon and pathways abundances as outcome variables. First, we filtered for taxa and pathways that were temperature-sensitive (i.e., m_full held greater than 50% of the model weight). Then, we modeled each feature twice, once with m_immune_main and once with m_immune_full, and compared model fit using AICc. Importantly we fit the models with taxa as outcome variables as GLMMs with a negative binomial distribution, log link function, and included a log total reads offset. We fit the models with pathways as outcome variables as LMMs with a Gaussian distribution and did not include a log total reads offset. Features where m_immune_full held greater than 50% of the model weight were considered to have a relationship between temperature and abundance that changes depending on neopterin levels. We visualized these features using a coplot, where the relationship between feature abundance and temperature was plotted over distinct ranges of neopterin values.

## 4.7 Supplementary Material

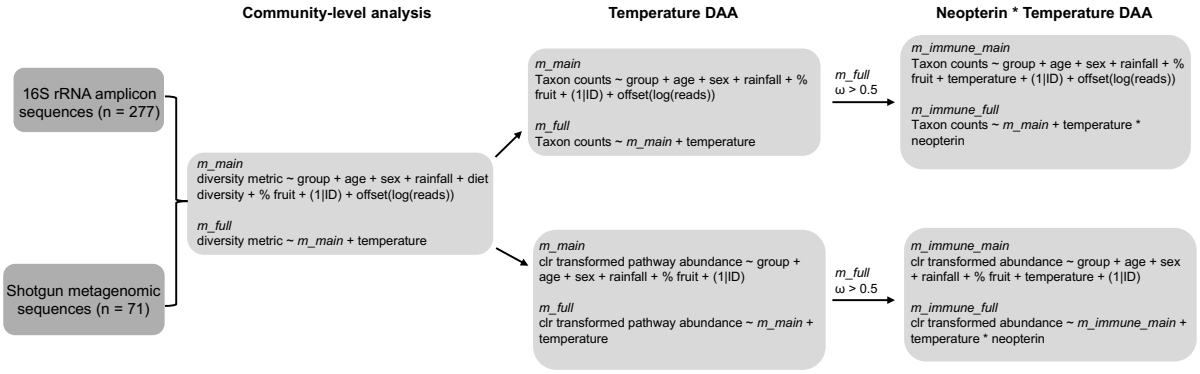



**Figure S4.1** Flowchart outlining the model selection approach for each component of the analysis. Dark grey boxes represent the two data types: 16S rRNA amplicon sequences and shotgun metagenomic sequences. Light grey boxes specify the model formulas applied to each analysis. While the community-level modeling approach used the same model structure for both data types, the models for the temperature and interaction differential abundance analyses differed. Only features for which *m_full* accounted for more than 50% of the model weight were included in the neopterin–temperature interaction analysis.

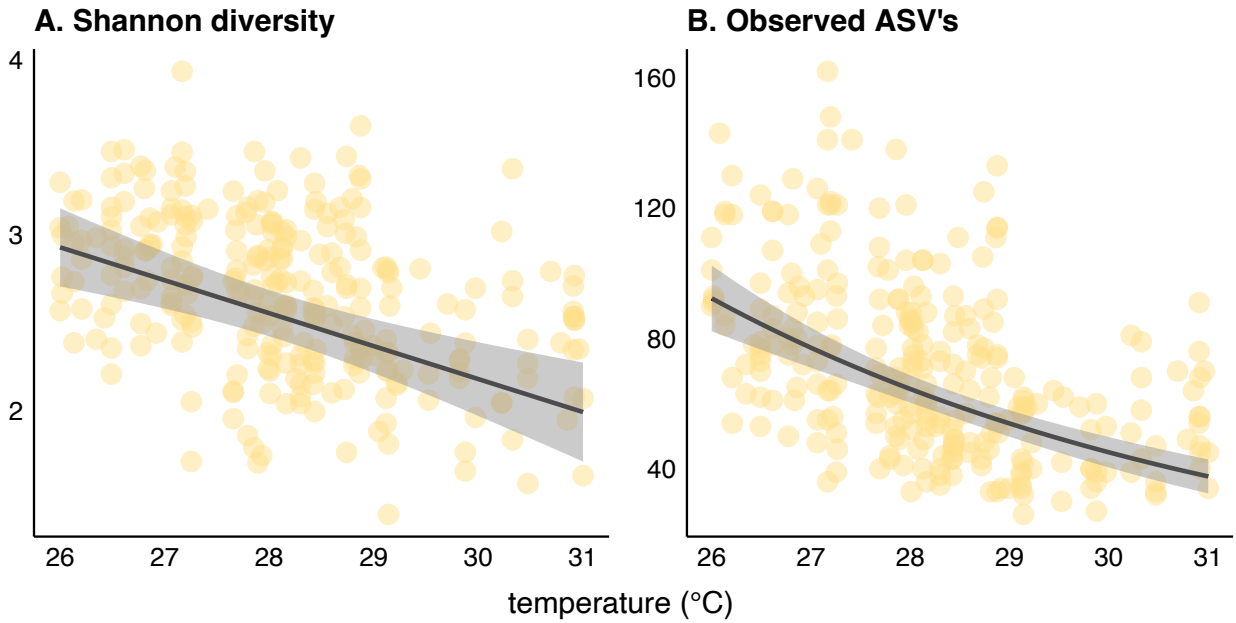



**Figure S4.2** Plotted model results showing the relationship between taxonomic alpha diversity and temperature. Panel (A) measures alpha diversity using Shannon Index, while panel (B) uses the total number of ASVs. Both metrics demonstrate a reliable decrease in taxonomic alpha diversity as temperature increases.

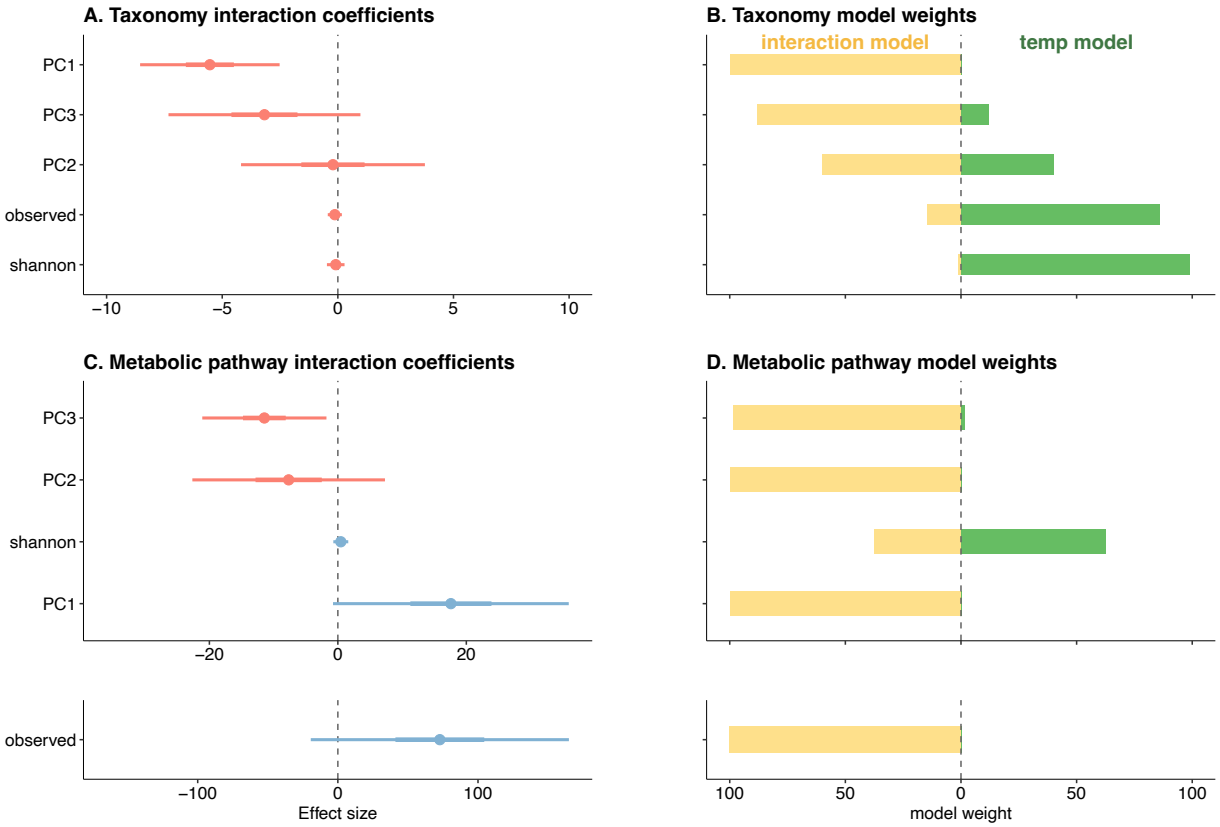



**Figure S4.3** Model comparison results assessing whether adding an interaction between temperature and neopterin to the temperature model (model with base predictors plus temperature) improves the explanation of community-level taxonomic and metabolic diversity. Panels A and C show the coefficients of the interaction effect for community diversity metrics including beta diversity (measured by principal components 1-3) and alpha diversity (measured by the Shannon Index and the observed number of ASVs or metabolic pathways). The thick bars represent 50% confidence intervals (CI), while the thin bars represent 95% CI. Panels B and D compare the model weights of the temperature model (in green) to the interaction model (in yellow). The weights of the temperature model plus the interaction model equal 100%. The top panels A and B show the results for community-level taxonomic diversity while the bottom panels C and D show the results for metabolic pathway diversity. Results show that the relationship between temperature and beta diversity is associated with neopterin concentration for both taxonomic and metabolic community composition, but the relationship between alpha diversity and temperature is independent of neopterin concentration.

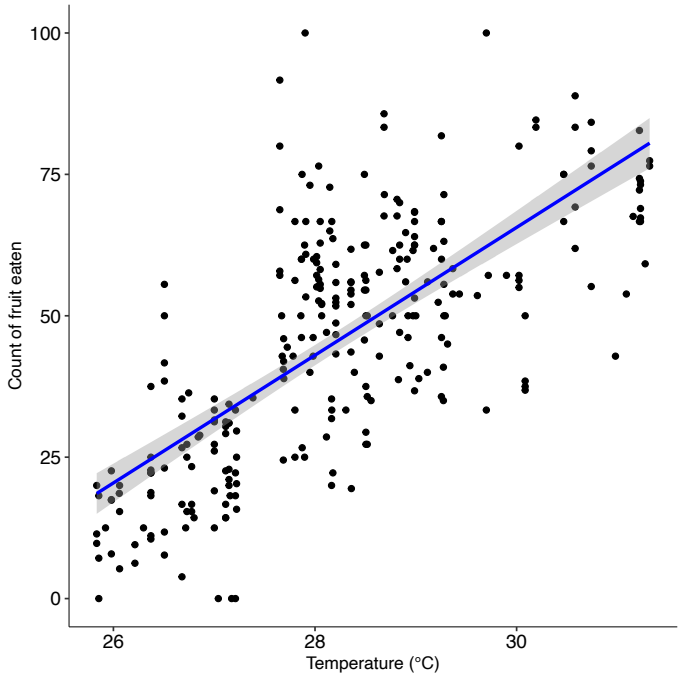



**Figure S4.4** Scatter plot of the count of fruit feeding observations two weeks before sample collection and its relationship with temperature. The plot demonstrates a clear positive relationship where fruit consumption increases with rising temperatures.

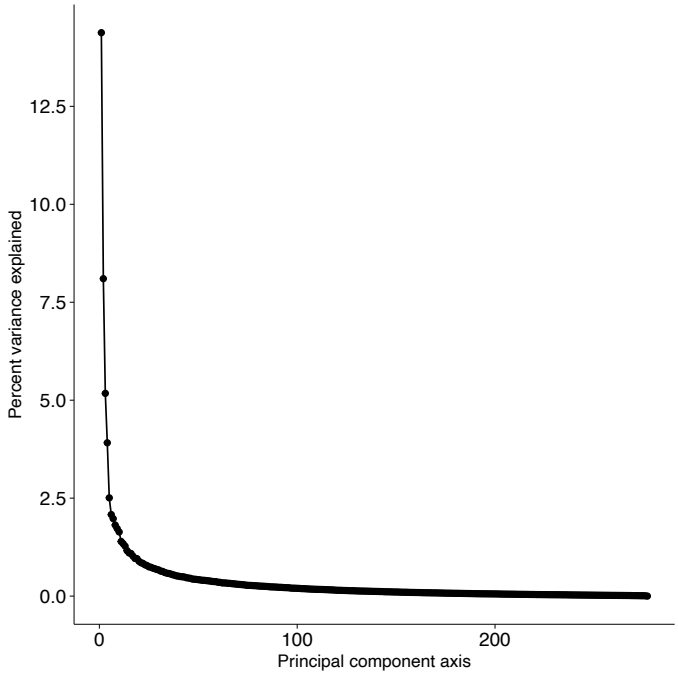



**Figure S4.5** Visualization of the eigenvalues of the taxonomic data for each principal component in descending order. Principal components 1 through 3 were used for analysis because they explain >5% of the variation.

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# Chapter 5 Ambient Heat Predicts Conserved Changes in Gut Microbiome Composition and Metabolic Capacity Independent of Immune Activation in a Wild Primate

## 5.1 Introduction

Temperature is a ubiquitous environmental stressor that has shaped the evolution of diverse behavioral and physiological mechanisms for regulating internal conditions in response to ambient environments (Micheal & Angilletta, 2009). Elevated temperatures, in particular, impose strong constraints on physiology by destabilizing cells and increasing oxygen demands (Pörtner, 2002; Somero, 2020). Consequently, understanding responses to environmental temperature variation is essential for predicting how heat shapes physiological processes that influence health and fitness. Emerging evidence indicates that heat exposure also alters the microbial community of the gastrointestinal tract, the gut microbiome, in ways that are central to understanding responses to elevated temperatures. For example, heat-associated changes in the gut microbiome are linked to alterations in host digestion, metabolism, and immune function (Chen et al., 2018; Fontaine et al., 2018; Zhu et al., 2019). The gut microbiome may therefore play a key role in shaping host heat tolerance (Baldassarre et al., 2022; Jaramillo & Castañeda, 2021; Moghadam et al., 2018; Ziegler et al., 2017), and changes in community composition and function at high temperatures have also been associated with reduced survivorship and fitness (Zhang et al., 2019). Notably, changes to the microbiome with heat exposure show striking convergence across host taxa. Experimental studies in both invertebrates and vertebrates consistently report declines in Firmicutes and expansions of Proteobacteria under high temperatures (reviewed in Huus & Ley, 2021; Sepulveda & Moeller, 2020). This convergence suggests that heat alters the gastrointestinal environment in ways that consistently favor some bacteria over others. However, it remains unclear why heat exposure repeatedly produces these patterns. Identifying the mechanisms underlying these responses is essential for understanding how temperature reshapes the microbiome and for predicting the consequences of heat exposure.

One plausible mechanism is heat-driven alteration of the intestinal redox environment, determined by the balance between reactive oxygen and nitrogen species (RONS) and

antioxidant defenses. When RONS production exceeds antioxidant defenses, RONS can damage cells, causing oxidative stress (Finkel & Holbrook, 2000; Hall et al., 2001). Temperature is a well-known driver of this process, because heat increases metabolic rate and mitochondrial respiration, accelerating RONS leakage from the electron transport chain (Belhadj Slimen et al., 2014). Experimental studies of heat stress show that prolonged heat exposure increases oxidative stress within the gastrointestinal tract (Hall et al., 2001). These shifts in the redox environment during heat exposure may favor bacteria with greater tolerance to oxygen and oxidative stress, such as facultative anaerobes and aerobes (Winter et al., 2010, 2013), over obligate anaerobes that generally lack robust defenses against RONS (Lu et al., 2018; McCord et al., 1971). Because oxygen-tolerant taxa are disproportionately represented within Proteobacteria, whereas many obligate anaerobes belong to the Firmicutes phylum (Albenberg et al., 2014; Shin et al., 2015), shifts in the redox environment provide a plausible mechanism linking heat exposure to the observed changes in the microbiome.

Immune activity may also contribute to heat-driven changes in the intestinal redox environment because innate immune cells generate RONS as part of antimicrobial defense (Andrés et al., 2022). The immune system is also highly responsive to heat, where even moderate, short-term heat exposure can increase immune cell activation (Leijh et al., 1981; Ohlsson et al., 2020). Because immune processes play a central role in regulating gut microbial homeostasis (Hooper et al., 2012; Hooper & Macpherson, 2010), heat-driven immune activation could contribute to the conserved microbiome shifts observed at high temperatures. Consistent with this, experimental studies in diverse host species show that heat stress is associated with increased inflammatory biomarkers and changes to microbiome composition and function (Alhenaky et al., 2017; Bai et al., 2022; Chen et al., 2018; Fang et al., 2023). Further, conserved taxonomic shifts observed at high temperatures are also a hallmark of inflammatory bowel disorders (Frank et al., 2007; Rizzatti et al., 2017), particularly the expansion of Protobacteria that includes many pathobionts (i.e., taxa commonly associated with disease)(Rizzatti et al., 2017; Shin et al., 2015). This suggests that inflammation alters the gastrointestinal environment in ways that consistently favor certain bacteria over others and are similar to patterns associated with heat exposure.

However, much of what we know about gut microbiome responses to heat comes from experimental studies that may not fully reflect responses under natural conditions. In the wild,

individuals move among microclimates (Kearney et al., 2009; Tull et al., 2001), weather conditions fluctuate over short timescales (Kearney et al., 2012), and multiple factors interact to shape experienced heat, including life-history stage (Kingsolver et al., 2011), behavioral thermoregulation (Campos & Fedigan, 2009; Iii et al., 2006), and prior thermal exposure (Sinclair et al., 2016). This complexity is difficult to reproduce under experimental conditions. In parallel, much of what is known about gut–immune interactions derives from pathological contexts, such as inflammatory bowel disorders (Ferreira et al., 2014; Neish, 2009), which involve chronic immune dysregulation and do not necessarily reflect processes occurring in otherwise healthy individuals. Therefore, studying natural populations allows us to assess the relative importance of temperature as a driver of gut microbiome variation and to evaluate the extent to which gut–immune interactions shape these effects under non-pathological conditions.

Here, we assess how natural variation in ambient temperature predicts gut microbiome taxonomic composition and functional potential in wild capuchin monkeys (Cebus imitator). Our study population lives in a tropical dry forest fragment in northwestern Costa Rica characterized by extreme seasonality between the wet and dry seasons that exposes individuals to a wide range of ambient temperatures (approximately 20–35 °C average daily max temperature; Melin et al., 2014). Because high temperatures covary with drastic seasonal ecological change, our analyses capture responses to the hot, dry season rather than the effects of temperature alone. Although this limits inference about the effects of heat in isolation, it enables us to assess whether microbiome responses observed under experimental heat stress are reproduced in ecologically relevant conditions. White-faced capuchins are well suited for this study because, like many tropical taxa, they have evolved within a narrow thermal niche (Bennett et al., 2021; Tewksbury et al., 2008), but appear more heat-sensitive than other sympatric primate species (Fedigan & Jack, 2001; Johnson & Kerry, 2018). Previous work in this population also shows that ambient temperature is an important predictor of gut microbiome composition compared to other ecological and demographic factors (Lucore et al., in review), motivating further investigation of the mechanisms underlying these changes.

We used urinary neopterin to measure immune activation and evaluate the importance of immunity in producing conserved microbiome changes during heat exposure. Neopterin reflects macrophage activity during Th1-type immune responses (Murr et al., 2002; Widner et al., 1999) and is well-suited for evaluating gut–immune interactions because intestinal macrophages play a

central role in containing the gut microbiome to the intestinal lumen (Kelsall, 2008) and maintaining tolerance to commensal taxa through interactions with microbial metabolites (Corrêa-Oliveira et al., 2016; Wang et al., 2020). In this population, ambient temperature is an important predictor of urinary neopterin levels, indicating that immune activation is sensitive to natural variation in heat exposure (Lucore et al., 2024). We paired urinary neopterin samples with fecal samples to characterize gut microbiome taxonomic composition using 16S rRNA gene amplicon sequencing (n = 219 pairs) and functional capacity using shotgun metagenomic sequencing (n = 71 pairs). Using this framework, we tested four predictions. First, ambient temperature will predict shifts in the gut microbiome, and these shifts will mirror conserved changes observed at high temperatures across a wide range of taxa. Second, changes predicted by temperature will reflect altered redox conditions that favor oxygen-tolerant bacteria. Third, ambient temperature will predict variation in urinary neopterin, with higher temperatures associated with elevated immune activation. Fourth, immune activation will mediate the relationship between temperature and gut microbiome composition.

Importantly, mediation analysis allows us to evaluate whether immune responses to temperature shape microbiome responses to temperature, rather than simply testing if temperature effects on the microbiome are associated with immunity. This approach decomposes the relationship between ambient temperature and the gut microbiome into a direct effect of temperature and an indirect effect mediated by immune activation (Fig. 5.1)(Imai et al., 2010; Rosenbaum et al., 2020) Together, our results expand on experimental work by evaluating how ambient temperature explains variation in the gut microbiome in a natural environment and by testing whether gut–immune interactions influence conserved microbiome responses to heat.

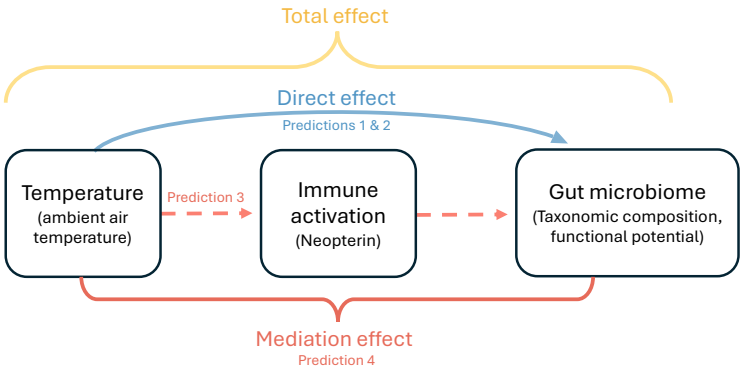



**Figure 5.1 Conceptual schematic of the mediation modeling framework.** This framework tests whether heat-driven immune activation contributes to changes in the gut microbiome at elevated temperatures. The mediation approach decomposes the relationship between ambient temperature and the gut microbiome into a direct effect of

temperature (blue) and a mediation effect of immune activation (red). The sum of these components represents the total effect of temperature on the gut microbiome (yellow).

## 5.2 Methods

### *5.2.1 Site information and data collection*

We collected data from a wild population of C. imitator in the Taboga Forest Reserve. The reserve is a 516-ha tropical dry forest fragment in Guanacaste, northwestern Costa Rica, that forms part of a biological corridor connecting the Tempisque River Basin to the Guanacaste Mountains (Tinsley Johnson et al., 2020). The Capuchinos de Taboga has collected daily behavioral and endocrine data on three habituated groups of white-faced capuchins since 2018. All individuals were uniquely identified. Age was determined using known birth dates for monkeys younger than seven years and estimated for older individuals by a field team member (J.C.O.) with over two decades of experience observing wild capuchins. Daily behavioral observations and sample collection were conducted by a team of two or more trained field assistants between 5:00 AM and 6:30 PM. Behavioral observations included feeding and foraging behavior using a scan sampling procedure every 30 minutes for 10 minutes. During the scan, feeding and foraging observations of individually identified monkeys were recorded, with individuals recorded multiple times if they engaged with different food items. Plant food items were identified on the family or genus level.

In conjunction with behavioral observations, we opportunistically collected fecal and urine samples between September 2022 and July 2023. All samples were collected from identified individuals that appeared healthy upon visual inspection. Fecal samples were placed in 4 mL of 98% ethanol immediately after defecation (Amato et al., 2013). To minimize contamination with other microbiomes (e.g., skin, soil, urogenital), we wore gloves, collected feces that had not contacted the soil, and discarded samples contaminated with urine. Urine samples were collected using a clean-catch method (Bergstrom et al., 2017) to avoid fecal or water contamination, and samples were stored immediately in a portable cooler before transfer to a –20 °C freezer at the end of each field day. We also collected urine from leaves or the ground if it appeared free of soil and feces. Fecal samples were refrigerated until shipment to the Amato Microbiome Lab at Northwestern University (Evanston, IL), and urine samples remained frozen until assay analysis in the Capuchinos de Taboga field laboratory (Beehner et al., 2022).

We used temperature observations from the Palo Verde meteorological station, located ~20.81 km southwest of the Taboga Forest Reserve at the Organization for Tropical Studies (OTS) Palo Verde research station (10.345°N, 85.339°E). Like Taboga, Palo Verde is one of Central America's last remaining tropical dry forest fragments (Stern et al., 2002). Because of its habitat similarity and proximity, temperature measurements from Palo Verde have been used in previous studies of the Taboga capuchin population (Lucore et al., 2024). Maximum and minimum temperature were recorded every 15 minutes.

### *5.2.2 Neopterin analysis*

We measured urinary neopterin concentrations using a commercially available competitive exclusion enzyme-linked immunosorbent assay (Neopterin ELISA, Ref. RE59321, IBL International GMBH, Hamburg, Germany). The assay was previously validated for use in this capuchin population (Lucore et al., 2022). To control for differences in urine concentration, we measured specific gravity and diluted samples using the manufacturer's assay buffer. We ran the assays according to the manufacturer protocol, see Lucore et al. (2022, 2024) for details.

### *5.2.3 Sample processing for 16S rRNA amplicon and shotgun metagenomic sequencing*

#### *5.2.3.1 DNA extraction*

All lab work was conducted at the Amato Microbiome Lab at Northwestern University, Evanston IL. We extracted DNA from the fecal samples using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD, USA). We made minor modifications to the manufacturer protocol, see Lucore et al., in review for details.

#### *5.2.3.2 16s rRNA amplicon sequencing and data processing*

To characterize the taxonomic composition of the gut microbiome, we performed 16s rRNA amplicon sequencing targeting the hypervariable V4-V5 region of the 16s rRNA gene. Amplification was conducted with primers 515F/926R, recommended by the Earth Microbiome Project (www.earthmicrobiome.org), using a dual-indexing strategy see Mallot & Amato 2018 for details. We included negative controls to monitor contamination during DNA extraction and PCR amplification, and purified PCR products using SequalPrep Normalization. Sequencing was performed on the Illumina MiSeq V4 platform at the Rush Genomics and Microbiome Core Facility.

We analyzed sequences using the Quantitative Insights into Microbial Ecology (QIIME2) platform (Caporaso et al., 2010), v2023.9. Pair-end sequences from 900 samples produced 28,869,202 raw reads (average 32,077 per sample, range of 7 to 64851 reads). We trimmed reads at a minimum Phred score of 33 and truncated forward and reverse reads to 216 bases and 206 bases, respectively. We then used the Divisive Amplicon Denoising Algorithm 2 (DADA2)(Callahan et al., 2016) via the q2-dada2 pipeline to quality filter and denoise sequences. DADA2 inferred Amplicon Sequence Variants (ASVs), which we taxonomically assigned using the q2-feature classifier (Bokulich et al., 2018) against version 2022.10 of the Greengenes2 database (McDonald et al., 2012, 2024). To retain only microbial sequences, we removed those assigned to chloroplasts and mitochondria with the q2-taxa-filter-table. We removed all negative controls after confirming their taxonomic composition did not indicate contamination, as well as one fecal sample with fewer than 1,000 reads to avoid bias from shallow sequencing depth. We retained 797 samples that contained an average of 16,898 reads (range: 4,944–38,941).

We imported the ASV count and taxonomy outputs from QIIME2 into R using the {qiime2R} package (Bisanz, 2018) and created a phyloseq object using the {phyloseq} package (McMurdie & Holmes, 2013). Most ASVs were rare: of 11,916 total ASVs, 89% occurred only in one sample and 16% lacked phylum level classification. We removed ASVs present in fewer than 0.5% of samples as well as those without a phylum-level classification. After filtering, 1,159 ASVs remained, all classified at the class level, 95% at the order level, 93% at the family level, and 60% at the genus level.

#### *5.2.3.3 Shotgun metagenomic sequencing and data processing*

To characterize the functional capacity of the gut microbiome, we performed metagenomic sequencing on a subset of 95 samples with sufficient DNA for library preparation. We used the NEBNext Ultra™ II FS DNA PCR-free Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) to prepare sequencing libraries, following the manufacturer's protocol for low inputs (50ng to 99ng). We selected library inserts around 350bp and pooled libraries for sequencing on the Illumina NovaSeq platform at the Rush Genomics and Microbiome Core Facility. Pair-end sequencing of the 95 samples produced 2,902,714,548 raw reads (average 15,277,445 per sample, range of 531,829 to 25,348,795 reads).

We quality filtered sequences with KneadData (v0.12.0) using standard parameters SLIDINGWINDOW:4:20 and MINLEN:90, and removed host-associated reads using the GRCh38.p13 human reference genome (RefSeq accession: GCF_009914755.1)(Beghini et al., 2021). This reference genome has been used to filter host reads in previous work on this population (Lucore et al., in review) and in other capuchin metagenomic studies (Orkin, Campos, et al., 2019). After quality filtering, 931,326,495 cleaned reads remained (average 98,034,376 per sample, range of 178,711 to 22,386,906 reads). We concatenated the forward and reverse reads and used HUMAnN3 (v3.9) for functional profiling, assigning gene families with the Uniref50 database, and regrouping results into MetaCyc reactions. The final table reported the abundance of each unique metabolic pathway normalized for the number of contributing gene families. We imported the pathway abundance table into R and removed unmapped values (sequences not assigned to any gene family) and unintegrated values (gene families not associated with any pathway). After filtering, 548 unique metabolic pathways remained. All subsequent analyses were conducted in R v4.3.1.

### *5.2.4 Metadata assembly*

We matched gut microbiome samples (taxonomic and metabolic pathways) and neopterin samples with the relevant metadata for downstream analyses. For both sample types, we averaged the maximum temperature (recorded every 15 minutes) across the 15 days preceding sample collection. This approach is designed to capture responses to longer-term thermal conditions, rather than acute temperature spikes that are more likely to be confounded by short-term shifts in diet, ranging behavior, or social interactions. Importantly, this temperature predictor has previously been linked to changes in immune activation and the gut microbiome in this population (Lucore et al., in review, 2024). We also averaged the rainfall over the four days before sample collection.

Gut microbiome samples were further paired with dietary observations two weeks before sample collection, a time frame consistent with previous studies of this population (Lucore et al., in review) and other white-faced capuchin microbiome research (Mallott et al., 2018; Orkin, Campos, et al., 2019). To construct dietary predictors, we calculated the percent consumption of common food types (e.g., animal, flower, fruit, pith, seeds) by dividing the total number of feeding observations for each food category by the total number of feeding observations per individual. Neopterin samples were not matched with diet variables, as diet has not been

identified as a predictor of neopterin in this population (Lucore et al., 2022, 2024). To attempt to control for large temperature-driven changes in diet, we visualized the relationship between each food type and temperature. Percent fruit consumption showed a strong positive association with temperature (Fig. S5.1), so we included it as a covariate in the models.

We paired each taxonomic and metabolic pathway sample with the closest neopterin sample collected on the same day or within three days before or after fecal sample collection. This ensured a 1:1 sample match required for mediation modeling (see next section). We then excluded any pair in which the neopterin value was five standard deviations above the mean (Lucore et al., 2024). The final dataset included 218 paired taxonomic samples and 71 paired metabolic pathway samples.

### *5.2.5 Mediation analysis*

We conducted mediation modeling to evaluate whether neopterin's response to temperature affects the relationship between temperature and the gut microbiome (Fig. 5.1). We used the {mediation} package to fit the mediation models, which differs from traditional structural equation modeling (SEM) approaches by implementing a nonparametric bootstrapping protocol. This protocol repeatedly simulates potential mediator values and the corresponding potential outcomes to generate summary statistics and confidence intervals (Imai et al., 2010). A key advantage of this approach is its ability to estimate mediation effects from non-linear models, whereas SEM formulas typically rely on linear beta coefficients (Imai et al., 2010).

To implement the mediation analysis, we built two models: (1) a mediator model with neopterin concentration as the outcome, and (2) an outcome model with gut microbiome features as the outcome. We tested mediation across multiple microbiome features (i.e., computed metrics capturing different aspects of microbiome composition and function), which are described in the following sections. For the mediator model, we log-transformed neopterin to approximate a Gaussian distribution and fit a generalized additive model (GAM) with predictors for temperature, rainfall, age, and social group. Temperature and age were modeled with splines to capture their known nonlinear relationships with neopterin, consistent with a previous analysis of this population that also evaluated predictors such as sex and reproductive state (Lucore et al., 2024). For the outcome model, we fit a linear mixed model with predictors for temperature, rainfall, social group, age, sex, percent fruit consumption, and neopterin. All linear predictors (temperature, rainfall, age, fruit consumption, and neopterin) were scaled to facilitate the

interpretation of coefficients. Although repeated sampling of individuals would ideally be modeled with a random intercept for individual, this term was excluded because it increased model complexity to the point that mediation models failed to converge.

To conduct the mediation analysis, we used the mediate() function with temperature as the treatment variable and neopterin as the mediator. This function requires two treatment values, a reference and a comparison, between which effects are averaged. We specified the minimum and maximum observed temperatures as the reference and comparison values to approximate effects across the full temperature range.

#### *5.2.5.1 Community feature mediation*

To evaluate whether neopterin mediates temperature-driven changes in microbiome community composition, we generated alpha and beta diversity metrics for both the taxonomic and metabolic pathway data as outcome variables in the mediation framework. To measure alpha diversity, we calculated the Shannon diversity index and observed richness (the total number of ASVs or pathways in a sample) with the estimate_richness() function from the {phyloseq} package. Since microbiome datasets are compositional and read counts reflect relative rather than absolute abundances (Gloor & Reid, 2016), abundances must be adjusted for sequencing depth. We chose not to use rarefaction to account for differences in sequencing depth because it discards valuable data (McMurdie & Holmes, 2014). Instead, we modeled each alpha diversity metric as a function of the total number of reads per sample and extracted the residuals, which provided depth-corrected measures of alpha diversity. Negative residuals indicated lower-than-expected diversity, while positive residuals indicated higher-than-expected diversity (Marshall et al., 2021). We used a residual-based approach because the mediation framework does not accommodate log-offset variables, the common method of controlling for sequencing depth on unrarefied data (Baniel et al., 2022; Lucore et al., in review).

We also generated beta diversity metrics to serve as outcome variables in the mediation framework. To account for the compositional nature of the data, we computed Aitchison distances, which transform abundances into log-ratio space and reduce biases from sequencing depth (Gloor et al., 2017). Specifically, we added a pseudo count to all zeros using the cmultRepl() function from the {zCompositions} package, which applies Bayesian-multiplicative replacement to preserve the structure of the data (Palarea-Albaladejo & Martín-Fernández, 2015). We then performed a centered log-ratio (clr) transformation and calculated the Euclidean

distance between pairwise samples (Aitchison & Greenacre, 2002). To summarize the major axes of variation in community composition, we conducted a Principal Components Analysis (PCA) on the Aitchison distances and retained the first three principal components (PC1–PC3), each of which explained more than 5% of the variation (Fig S5.2). We used the depth-corrected alpha diversity metrics and beta diversity principal components as community-level outcome variables in the mediation framework.

#### *5.2.5.2 Taxonomic mediation*

We used taxonomic abundances to test whether ambient temperature was associated with shifts in taxa expected to be temperature sensitive. First, we aggregated reads at the genus level using the tax_glom() function in the {phyloseq} package. To exclude extremely rare genera, we calculated relative abundance (reads per genus divided by total reads per sample) and removed genera with an average relative abundance below 0.2%, leaving 113 genera for analysis. Rather than modeling individual genera separately, we constructed three log-ratios from the remaining taxa to test our predictions. The first ratio captured shifts observed in heat-stress studies by comparing the relative abundance of Proteobacteria in the numerator to Firmicutes in the denominator. The second ratio reflected oxygen sensitivity, with strict aerobes and facultative anaerobes (i.e., oxygen-tolerant genera) grouped in the numerator and obligate anaerobes in the denominator (Table 1A, Appendix A). The third ratio compared genera that may include pathogenic species (i.e., pathobionts) in the numerator to core genera, defined as those present in at least 70% of samples, in the denominator (Table 2A, Appendix A). We then used the three log ratios as outcome variables in the mediation outcome model.

To calculate the log ratios, we first replaced zeros with a pseudocount using multiplicative zero replacement via the cmultRepl() function (Palarea-Albaladejo & Martín-Fernández, 2015), preparing the data for log transformation. For each ratio, we then calculated the log of the geometric mean abundance of the numerator taxa relative to the denominator taxa. This approach is appropriate for compositional data because it projects abundances into log-ratio space, reducing bias from sequencing depth differences (Gloor et al., 2017).

#### *5.2.5.3 Metabolic pathway mediation*

Similarly, we used metabolic pathway abundances to test our predictions about how the gut microbiome responds to heat. We first removed pathways present in fewer than 30% of

samples (i.e., <21 samples) to retain only the most common pathways. We then calculated relative abundances and excluded pathways with an average relative abundance below 0.2%. After filtering, 270 pathways remained for analysis.

In line with the taxonomic data, we computed log ratios from the remaining pathways to avoid using single pathways as outcome variables. The first ratio captured functional differences across oxygen niches, with pathways typical of strict aerobes and facultative anaerobes in the numerator and those typical of obligate anaerobes in the denominator (Table 3A, Appendix A). These groupings reflected characteristic metabolic functions of the taxa (e.g., simple sugar utilization vs. fiber fermentation) and excluded pathways specific to aerobic or anaerobic respiration, as our goal was to capture functional shifts associated with changes in taxonomic composition rather than respiratory mode. The second ratio captured contrasted pathways linked to antagonistic immune effects (e.g., host glycan foraging, lipid A biosynthesis) typical of pathogenic bacteria with those associated with immune regulation and anti-inflammatory functions (e.g., SCFA production, antioxidant pathways, polyamine synthesis) typical of commensal bacteria (Table 4A, Appendix A). The third ratio reflected redox balance, contrasting pathways involved in DNA repair and nucleotide mobilization to those that help maintain redox balance by neutralizing RONS (e.g., oxygen cycling and cofactor synthesis; Table 5A , Appendix A). We then used the three log ratios as outcome variables in the mediation outcome model.

### *5.2.6 Neopterin temperature modeling*

We used an information-theoretic model selection approach to evaluate whether temperature meaningfully predicts variance in neopterin concentrations. We compared the mediator model, with neopterin concentration as the outcome that included a spline for temperature, to an otherwise identical model without temperature. We then used Akaike information criterion adjusted for small sample sizes (AICc) to compare these models and used the model weights to evaluate the importance of temperature as a predictor of neopterin concentration (Akaike, 1998; Hurvich & Tsai, 1989). We modeled neopterin samples associated with 16s rRNA samples separately from neopterin samples paired with shotgun metagenomic samples to assess the relationship between neopterin and temperature for each dataset.

## 5.3 Results

### *5.3.1 Temperature predicts conserved changes in taxonomic composition and functional capacity*

We found that both taxonomic structure and functional capacity of the microbiome are strongly associated with ambient temperature, supporting our first prediction that temperature is an important driver of microbiome composition. Temperature had a strong direct effect on both taxonomic and functional community-level diversity (blue arrow in Fig. 5.1; blue effects in Fig. 5.2). For taxonomic composition, temperature was strongly associated with variation along all three major axes of beta diversity: PC1 ($\beta_{\text{direct effect}} = 7.51$), PC2 ($\beta_{\text{direct effect}} = 10.51$), and PC3 ($\beta_{\text{direct effect}} = 16.26$), with all confidence intervals excluding zero (Fig. 5.2A). Patterns for functional composition were broadly similar. Temperature was strongly associated with PC2 ($\beta_{\text{direct effect}} = 24.08$) and PC3 ($\beta_{\text{direct effect}} = -17.07$), while the magnitude of the effect on PC1 was less certain, as its 95% confidence interval crossed zero ($\beta_{\text{direct effect}} = -15.44$; Fig. 5.2B). Overall this suggests that increasing temperature drives shifts in taxonomic and functional composition that are substantial enough to affect community-level beta diversity.

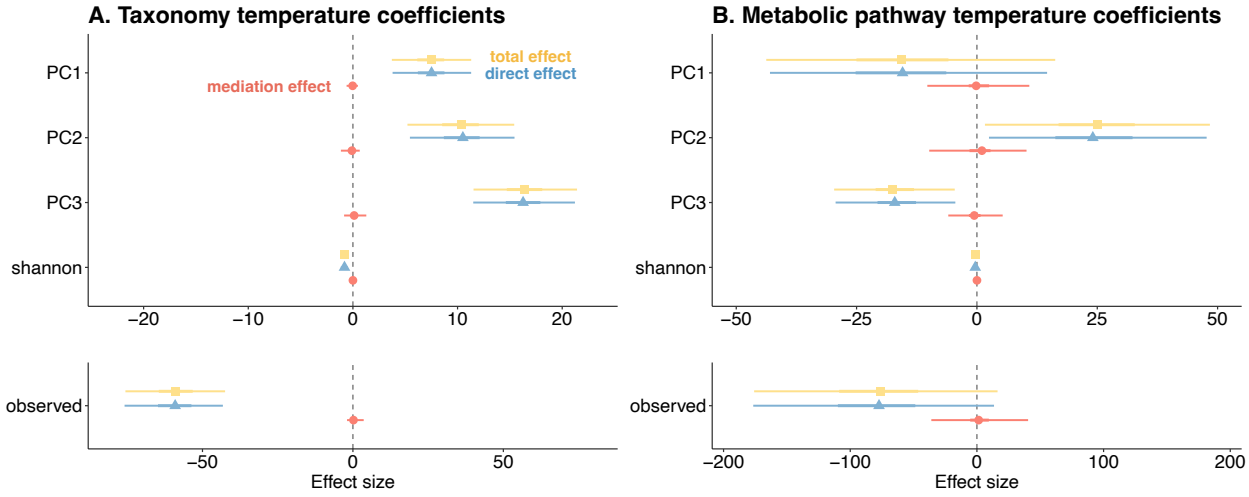



**Figure 5.2 Predicted effects of temperature on taxonomic and functional community-level composition.** Panel A shows that temperature has a reliable effect on taxonomic alpha and beta diversity. Mediation analyses indicate that this relationship is driven entirely by the direct effect of temperature (in blue), with no detectable mediation by neopterin (in red). Consequently, the total effect (in yellow) is equal to the direct effect. Panel B shows that temperature similarly affects functional alpha and beta diversity, although the effect on alpha diversity is weaker and less consistent. As in panel A, neopterin does not mediate these relationships.

Temperature also influenced alpha diversity, though the magnitude of effects differed between taxonomic and functional metrics. For taxonomic alpha diversity, temperature was

associated with a reliable decrease in both Shannon's diversity ($\beta_{\text{direct effect}} = -0.80$) and observed richness ($\beta_{\text{direct effect}} = -59.08$), but the effect on Shannon's diversity was much smaller. These results are consistent with prior studies showing that high temperatures are associated with reduced taxonomic diversity (Bestion et al., 2017; Fontaine et al., 2018; Zhu et al., 2019), but suggest that heat primarily affects taxonomic richness rather than evenness. In contrast, effects on functional alpha diversity were less consistent. Temperature was associated with a small negative effect on Shannon diversity ($\beta_{\text{direct effect}} = -0.34$), but this effect was not reliable, as confidence intervals overlapped zero (Fig. 5.2B). Temperature was also associated with a larger negative effect on observed pathway richness but the 95% CI's overlapped with zero ($\beta_{\text{direct effect}} = -77.89$; Fig. 5.2B). These results suggest that high temperatures reduce the number of distinct metabolic pathways, but have little effect on pathway evenness.

We also found that ambient temperature predicted changes in the relative abundance of specific taxa. For example, increasing temperature was associated with higher relative abundances of Proteobacteria compared to Firmicutes ($\beta_{\text{direct effect}} = 0.60$; Fig. 5.3). This pattern closely matches conserved gut microbiome responses to heat [e.g., reptiles (Bestion et al., 2017), birds (He et al., 2019; Zhu et al., 2019), mammals (Tajima et al., 2007)], likely because heat affects the gut redox environment in ways that favor oxygen-tolerant bacteria. Consistent with this prediction, temperature was also associated with changes in the relative abundances of taxa differing in oxygen tolerance. With increasing temperature, the relative abundance of aerobic and facultatively anaerobic taxa increased and obligate anaerobes declined ($\beta_{\text{direct effect}} = 0.75$; Fig. 5.3). Rising temperatures also predict an increase in the abundance of pathobiont taxa relative to core taxa ($\beta_{\text{direct effect}} = 0.77$; Fig. 5.3). The observed increase in pathobionts is consistent with the expansion of Proteobacteria and oxygen-tolerant genera, as many pathobionts belong to Proteobacteria and are favored under more oxidative conditions (Winter et al., 2010, 2013).

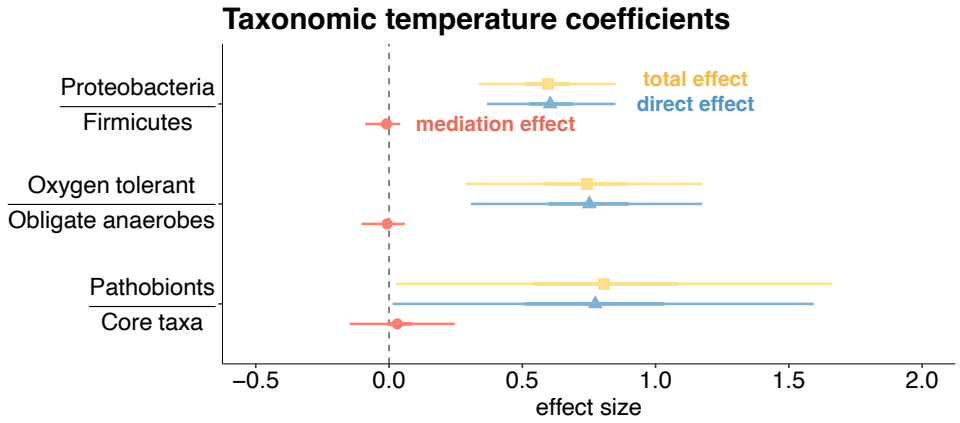



**Figure 5.3 Predicted effects of temperature on taxonomic composition.** Ambient temperature is associated with taxonomic shifts consistent with patterns observed during experimental heat stress. This is likely driven by the expansion of oxygen-tolerant taxa, including pathobionts. Mediation analysis shows that this relationship is explained entirely by the direct effect of temperature (blue), with no detectable mediation effect through neopterin (red). Accordingly, the total effect (yellow) is approximately equal to the direct effect.

Metabolic pathway data further supported our prediction that heat alters gut redox conditions in ways that favor oxygen-tolerant taxa compared to obligate anaerobes. We found that the abundances of metabolic pathways characteristic of oxygen-tolerant taxa increased compared to pathways typical of obligate anaerobes ($\beta_{\text{direct effect}} = 3.46$; Fig. 5.4). Specifically, higher temperatures were associated with increased abundances of pathways involved in simple sugar utilization, which are commonly enriched in facultative anaerobes and aerobes, relative to pathways involved in fiber fermentation that are characteristic of obligate anaerobes (Table 5.3S). Pathway data also indicated increase abundances of metabolic functions commonly associated with pathobiont taxa relative to commensals ($\beta_{\text{direct effect}} = 0.97$; Fig. 5.4). Specifically, higher temperatures were associated with increased abundances of pathways involved in host glycan foraging and lipid A biosynthesis, whereas pathways linked to immunomodulatory functions typical of commensal microbes, such as short-chain fatty acid production, were relatively reduced (Table 5.4S). These patterns further support the relative expansion of Proteobacteria observed in the taxonomic data.

Pathway analyses further indicated functional changes consistent with increasing oxidative stress with rising temperatures. Specifically, higher temperatures were associated with greater relative abundance of pathways involved in molecular repair, including nucleotide repair and de novo nucleotide synthesis, relative to pathways supporting redox maintenance, such as antioxidant metabolism ($\beta_{\text{direct effect}} = 0.92$; Fig. 5.4). This pattern suggests increased investment

in damage repair under elevated oxidative pressure, likely because RONS production outpaces antioxidant mechanisms Although oxidative damage was not directly measured in this study, these shifts provide functional evidence that oxidative stress increases with temperature and support the observed shift toward an oxygen-tolerant community composition.

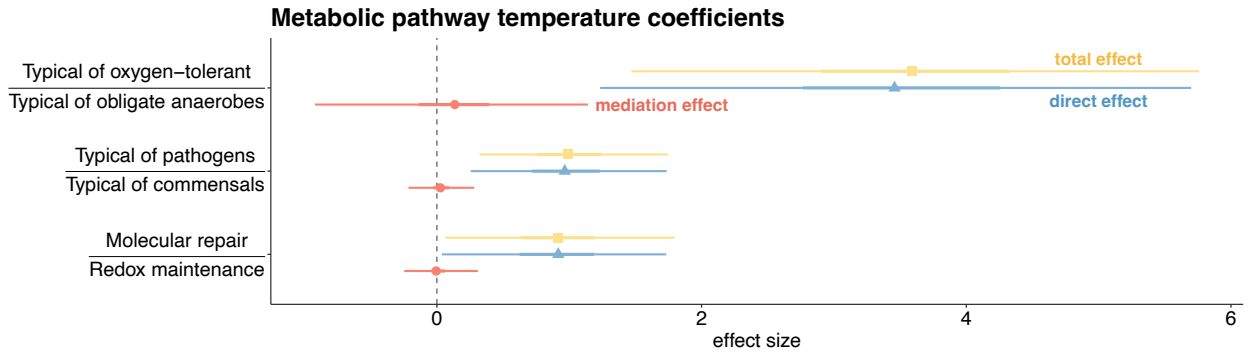



**Figure 5.4 Predicted effects of temperature on metabolic potential.** Ambient temperature is associated with shifts in microbial metabolic pathways consistent with the taxonomic patterns observed at higher temperatures. Functional changes suggest altered gut redox conditions that favor oxygen-tolerant taxa, including potential pathogens. Mediation analysis indicates that these relationships are explained entirely by the direct effect of temperature (blue), with no detectable indirect effect through neopterin (red). Accordingly, the total effect (yellow) is approximately equal to the direct effect.

### *5.3.2 Taxonomic and functional changes predicted by temperature are not mediated by neopterin*

In contrast, our results do not support the prediction that heat-driven immune activation contributes to the conserved microbiome changes observed at high temperatures. Across all microbiome metrics, the direct effect of temperature was nearly identical to the total effect (yellow brackets in Fig. 5.1; yellow effect estimates in Figs. 5.2–5.4). Because the total effect is defined as the sum of the direct and mediation effects, this indicates that the mediation of temperature effects via neopterin was negligible (red bracket in Fig. 5.1). Consistent with this interpretation, estimated mediation effects were close to zero and their 50% confidence intervals overlapped zero for all models (Figs. 5.2–5.4), providing no evidence that heat-driven immune activation explains changes in microbiome composition. Wider confidence intervals for pathway-based models (Figs. 5.2B & 5.4) relative to taxonomic models (Figs. 5.2A & 5.3) likely reflect greater variance due to smaller sample sizes rather than greater potential for mediation effects for functional composition.

The absence of mediation is not explained by a lack of a relationship between temperature and neopterin. Neopterin associated with 16S rRNA samples was best predicted by the model including temperature ($\omega = 100\%$). The relationship between temperature and

neopterin was nonlinear (edf = 4.39), with neopterin increasing across moderate temperatures and declining above approximately 30 °C (Fig. S5.3A). This pattern closely matches previously reported relationships in this population based on a substantially larger (~700 samples) and longer-term dataset (Lucore et al., 2024), and supports our prediction that rising temperatures are associated with elevated immune activation. Although temperature was a weaker predictor of neopterin in the shotgun-associated dataset, the model including temperature still received the majority of model support (ω = 55%) and exhibited a similarly shaped nonlinear relationship (edf = 2.53, Fig. S5.3B). The reduced strength of this association compared to the 16s rRNA data is likely due to increased variance arising from smaller sample sizes rather than a biologically distinct temperature–neopterin relationship for these samples. Together, these results indicate that the absence of a mediation effect arises from a weak relationship between neopterin and the gut microbiome responses, rather than from a lack of association between neopterin and temperature.

**5.4 Discussion**

We found that increasing ambient temperature predicts gut microbiome shifts that closely mirror patterns observed in experimental heat-stress studies across a wide range of taxa. In our study population, exposure to high temperatures occurs primarily during the dry season, where daily temperatures typically exceed 30 °C (Melin et al., 2014). However, the dry season is also characterized by food and water scarcity, which can alter diet, ranging behavior, social structure, and activity budgets (Campos & Fedigan, 2009) that are shown to influence the gut microbiome (Orkin, Campos, et al., 2019; Orkin, Webb, et al., 2019). As a result, high temperatures are experienced in tandem with drastic ecological changes rather than as an isolated perturbation in our study population. Despite this environmental complexity, the convergence between our results and experimental findings suggests that temperature itself is an important driver of gut microbiome composition in ecologically relevant contexts. Importantly, individuals in this population also have relatively stable food and water availability during the dry season because they exploit secondary forest and agricultural resources (Tinsley Johnson et al., 2020). Consistent resource availability likely reduces the physiological stress typically associated with the dry season compared to other nearby populations (Beehner et al., 2022; Campos & Fedigan, 2009). Therefore, our results are consistent with ambient temperature acting as a key driver of gut microbiome shifts, rather than reflecting a secondary consequence of resource scarcity. More

broadly, our findings underscore the importance of explicitly considering temperature in studies of wild animal microbiomes, particularly in primates, where much research has focused on the influence of diet.

Consistent with our predictions, we showed that conserved shifts in microbiome composition at high temperatures may be driven by changes in the redox environment. Specifically, higher temperatures were associated with a pronounced expansion of oxygen-tolerant taxa relative to obligately anaerobic taxa, and changes in metabolic pathways provided evidence consistent with increased oxidative stress. These shifts suggest that increased oxidative pressure at higher temperatures favors oxygen-tolerant bacteria (Lupp et al., 2007; Stecher et al., 2007; Zeng et al., 2017), providing a mechanistic explanation for why heat stress produces conserved shifts in gut microbiome composition. Contrary to our prediction, however, changes to the microbiome were not mediated by immune activation. Instead, our findings indicate that temperature exerts strong, largely independent effects on both immune activation and the gut microbiome in wild capuchin monkeys. These results suggest that changes in the redox environment of the gut are primarily a consequence of elevated metabolic activity from heat exposure (Belhadj Slimen et al., 2014), rather than changes in immune activation.

The absence of mediation could reflect limitations in our sampling procedure. Immune effects on the gut microbiome can occur over short timescales (hours to days) and during brief episodes of extreme heat (Hall et al., 2001; Koch et al., 2019; Slawinska et al., 2019), which may be missed by opportunistic sampling and the use of two-week average temperature predictors. In addition, neopterin reflects only one aspect of immune activity, Th1 macrophage activation (Murr et al., 2002; Widner et al., 1999), and may not reflect broader inflammatory responses or processes occurring locally in the gastrointestinal tract. Experimental studies linking heat stress to microbiome disruption have used tissue-based measures that more directly reflect intestinal inflammation (Slawinska et al., 2019). Future studies may benefit from using shorter-term temperature predictors and incorporating complementary immune biomarkers that capture broader or more generalized inflammatory activity, such as urinary soluble urokinase-type plasminogen activator receptor (suPAR) (Higham et al., 2020; Thunø et al., 2009). This could offer greater sensitivity for detecting immune-mediated pathways linking heat exposure to changes in gut microbiome composition.

Our results also have important implications for the health and potential fitness consequences of hosts exposed to high temperatures. In particular, the temperature-associated increase in Proteobacteria aligns with taxa frequently implicated in inflammatory bowel diseases (Lupp et al., 2007; Rizzatti et al., 2017; Stecher et al., 2007), along with enrichment of potentially pathogenic taxa that may gain a relative advantage during heat exposure. Together, these patterns suggest that the gut microbiome may play an important role in shaping host resilience to elevated temperatures and determining heat tolerance. Although these microbiome shifts were not mediated by immune activation as measured here, the immune system may nonetheless contribute to these effects through mechanisms or timescales that were not captured in this study. More broadly, our findings provide strong evidence for the ecological relevance of heat as a driver of gut microbiome variation in wild animals and offer insight into how microbiome responses may contribute to the physiological consequences of heat exposure. Understanding these dynamics in natural environments is increasingly important as anthropogenic climate change raises global temperatures, intensifies heat exposure, and creates more challenging thermal environments.

## 5.5 Supplementary materials

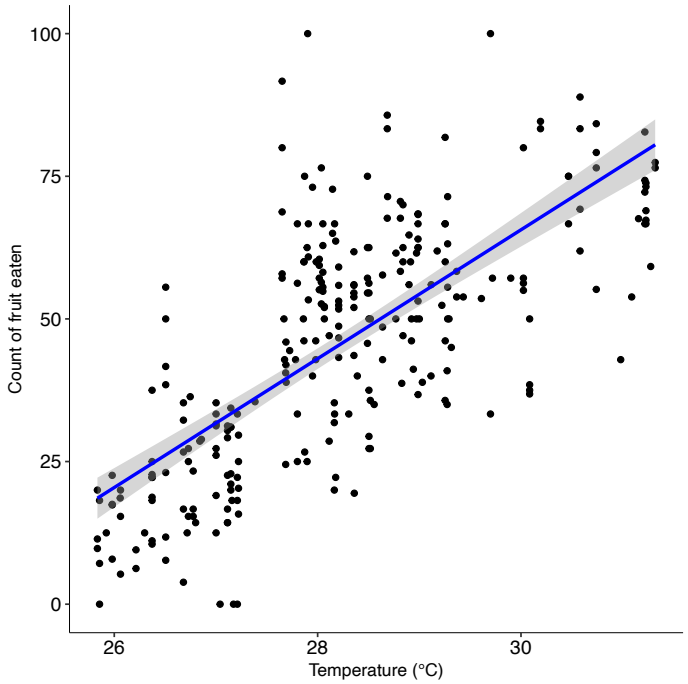



**Figure S5.1** Scatter plot of the count number of fruit feeding observations two weeks before sample collection and its relationship with temperature. The plot demonstrates a clear positive relationship where fruit consumption increases with rising temperatures.

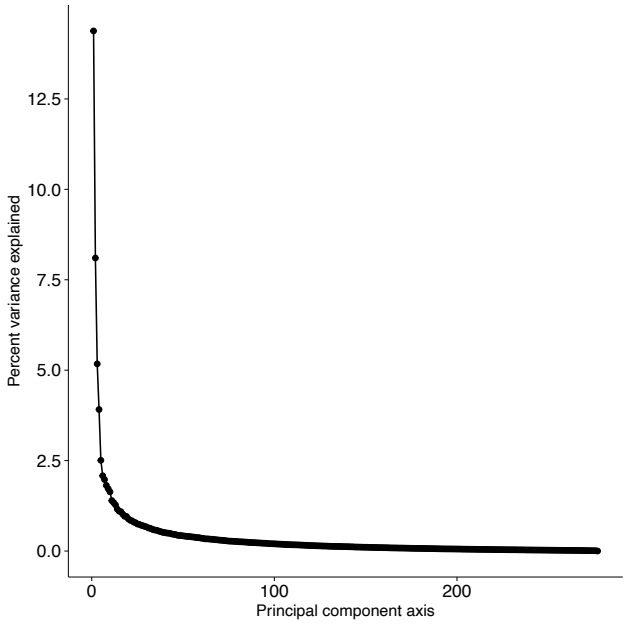



**Figure S5.2** Visualization of the eigenvalues of the taxonomic data for each principal component in descending order. Principal components 1 through 3 were used for analysis because they explain >5% of the variation.

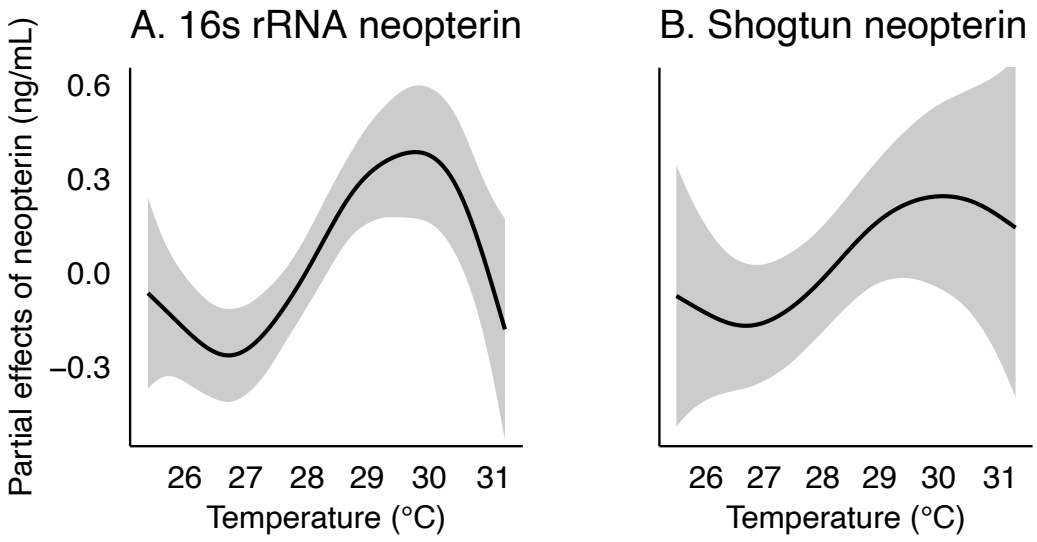



**Figure S5.3** Partial effects plot of the generalized additive model that evaluates the nonlinear relationship between neopterin concentration and average temperature in the 15 days before sample collection. For both datasets, neopterin reliably increases from 27-30°C, indicating that moderate elevation in temperature increases immune activation.

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# Chapter 6 Conclusion

## 6.1 Introduction

Understanding how organisms respond to ambient heat is essential for predicting how thermal environments shape health and fitness. Research on thermal tolerance has traditionally emphasized thermoregulatory adaptations that buffer body temperature from environmental change. However, heat tolerance is also constrained by the ability of essential biological processes to function at elevated temperatures. This dissertation advances our understanding of these constraints by examining how vulnerability of the immune system and gut microbiome to high temperature may affect heat tolerance. Because these systems play critical roles in disease resistance and metabolism, their sensitivity to heat has important implications for the evolution of thermal tolerance. Many have proposed that heat-induced dysfunction in these systems may increase vulnerability to high temperatures by affecting disease risk. However, support for this idea has been largely limited to experimental systems. My findings show some of the first evidence of these processes in a natural population, providing evidence that these mechanisms are relevant in real-world contexts. In this concluding chapter, I synthesize my key findings and outline directions for future research.

## 6.2 Heat-driven changes in immunity and the gut microbiome may influence health

Ambient temperature was a strong predictor of immune activity and gut microbiome composition compared to commonly considered demographic and ecological factors (e.g., age, sex, social group, diet, and rainfall). Because these data were collected from a wild population, these patterns could reflect responses to confounding ecological variables that co-occur with hot periods rather than direct responses to temperature. However, the observed changes in immune activity and the gut microbiome closely resemble responses documented in experimental studies of heat stress (Sepulveda & Moeller, 2020; Zhang et al., 2008). This suggests that the patterns observed here reflect the effects of ambient heat and underscore the importance of temperature in influencing the function of these systems. These results are important because temperature has

not historically been considered a major predictor of these systems outside of laboratory settings. Most studies of the immune system and gut microbiome in wild animals have instead focused on ecological variables such as diet and seasonality to explain variation. This work demonstrates that temperature is a critical, yet previously underappreciated, driver of these systems in natural environments.

The relationship between immune activity and ambient temperature has important health implications. Neopterin concentration exhibited a thermal performance pattern, peaking at approximately 30 °C and declining sharply at higher temperatures. Because biological performance (i.e., rate or efficacy of a process) becomes compromised shortly after reaching a thermal optima (Sinclair et al., 2016), the decline suggests that the capuchins may experience periods where TH1 cellular immunity is impaired. This could increase vulnerability to infection and negatively affect long-term health outcomes. These consequences may be most pronounced in young individuals, whose immune systems are particularly sensitive to high temperatures. In early life, immunity relies more heavily on cell-mediated, nonspecific responses while the adaptive immune system develops (Simon et al., 2015). Compromised macrophage activation may therefore leave young individuals especially susceptible to infection. Together, these findings suggest that decreased immune activation at high temperatures may constrain heat tolerance by increasing disease risk. While experimental studies have shown that heat can cause immunosuppression and proposed increased disease risk as a key mechanism linking heat exposure to fitness, these findings provide some of the first evidence of this process in a natural population.

The relationship between the gut microbiome and ambient temperature raises similar concerns for host health. Rising temperatures predicted shifts in microbial composition and function that may increase disease risk by (1) favoring taxa associated with infection or inflammatory disorders and (2) reducing functions that support host health. Specifically, higher temperatures were associated with an expansion of oxygen-tolerant taxa relative to obligate anaerobes, likely reflecting heat-driven increases in oxidative conditions within the gastrointestinal tract. Oxygen-tolerant taxa are frequently linked to dysbiosis and include many potential pathogens (Hughes et al., 2017), whereas obligate anaerobes are key commensals that contribute to host energy balance and immune regulation (Corrêa-Oliveira et al., 2016; Flint et al., 2012). Consistent with this pattern, functional results showed that increasing temperatures

were associated with reduced microbial metabolism of immunomodulatory compounds, including short-chain fatty acids and other lipids that help maintain intestinal homeostasis (Arpaia et al., 2013; Hung et al., 2023). Similarly, higher temperatures were associated with reduced microbial metabolism of compounds involved in redox maintenance, including cofactors and antioxidant precursors (Hellmann & Mooney, 2010; Lu, 2013), which may contribute to altered oxidative conditions at elevated temperatures. Together, these results suggest that heat-driven changes in the gut microbiome may increase susceptibility to gastrointestinal infection and inflammation, representing an additional constraint on heat tolerance. As with the immune system, the effects of heat on the gut microbiome have largely been demonstrated in experimental studies, leading to the hypothesis that microbiome sensitivity to heat may constrain host heat tolerance. However, these results provide some of the first evidence that this process occurs in a natural environment.

### 6.3 Quantifying the health and fitness consequences of heat-driven changes in immunity and the gut microbiome

To build on these results, we would ideally test whether the physiological consequences of heat translate into differences in health, survival, and reproductive outcomes. Establishing these links is essential for determining the fitness consequences of physiological sensitivity to heat and its implications for thermal tolerance. In the following section, I outline approaches to quantify the health impacts of these changes and to connect them to variation in fitness.

One limitation of this dissertation is that immune activity was assessed using a single mechanism, macrophage activation associated with Th1 cellular immunity. This constraint largely reflects the limited availability of noninvasive immune biomarkers that can be collected from wild animal populations. However, future studies could incorporate some additional urinary markers to capture a broader range of immune processes. For example, urinary soluble urokinase plasminogen activator receptor (suPAR) provides a measure of systemic sterile inflammation (Higham et al., 2020) and may be particularly useful for assessing inflammatory responses driven by heat exposure rather than infection. Urinary cytokines could also be measured to evaluate specific innate inflammatory pathways, such as TNF-α, IL-1β, and IL-6 (Taha et al., 2003). Expanding immune sampling could help identify which components of the immune system are most sensitive to heat and clarify whether high ambient temperatures suppress immune function broadly in the capuchins. Evidence of suppression of multiple immune

mechanisms would indicate more generalized vulnerability to infection and provide stronger evidence for immune constraints on thermal tolerance.

Another important future direction would be to incorporate metabolomic or proteomic approaches to better link microbial functional potential to actual metabolic activity of the gut microbiome. A limitation of shotgun metagenomics is that it quantifies the abundance of genes associated with specific metabolic processes, but does not indicate whether these genes are actively expressed. In contrast, metabolomics and proteomics measure the abundance of microbial metabolites and proteins, providing direct insight into microbial activity (Tsutaya et al., 2021; Worsley et al., 2024). These approaches would allow stronger inference about how heat exposure alters microbial metabolism in ways that directly influence host physiology. Although reference databases for microbially derived metabolites and proteins in nonhuman animals remain limited, recent studies demonstrate that these methods are feasible in wild populations (Amato et al., 2015; Orkin et al., 2025). Such information would provide a more mechanistic understanding of how microbiome sensitivity to high temperatures affects host health and constrains heat tolerance.

Future studies could also use gastrointestinal parasite burden as an indicator of infection vulnerability. My results suggest that changes to immunity and gut microbiome with heat exposure will increase susceptibility to infection. Testing whether parasite abundance or diversity increases with ambient temperature and is correlated with heat-related changes in immunity and the gut microbiome could help evaluate this prediction. Importantly, both immune function and gut microbiome composition influence susceptibility to parasitic infection (Anthony et al., 2007; Martínez-Mota et al., 2021). Therefore, incorporating longitudinal measures of parasite burden could help link heat exposure with specific health outcomes. This would strengthen inferences about how physiological vulnerability to heat translates to disease risk and affects thermal tolerance.

Ultimately, linking heat-driven changes in immune function and the gut microbiome to survival and reproductive success will determine whether physiological sensitivity to high temperatures carries fitness costs. Addressing this question requires long-term datasets that integrate physiological measures with lifetime reproductive outcomes. Longitudinal field projects are uniquely positioned to meet this need, as many include decades of demographic records often paired with biological samples and climate data (Alberts & Altmann, 2012;

Lonsdorf et al., 2022). These data make it possible to test whether individuals that exhibit greater immune suppression or microbiome disruption during periods of high temperature experience reduced survival or lower reproductive success. These data would also enable identification of the temperature thresholds and exposure durations at which heat produces lasting health and fitness consequences.

### 6.4 Research Contributions

This dissertation demonstrates that ambient heat consistently alters two systems fundamental to health: the immune system and the gut microbiome. Although these systems are widely expected to be temperature-sensitive, evidence for heat-related effects has largely been limited to experimental settings. By showing that ambient temperature is a strong predictor of immune function and microbiome composition in a natural population, this work provides some of the first evidence that these processes are relevant in real-world contexts. These findings also provide some of the first evidence from a natural population that heat-induced changes in these systems may constrain host heat tolerance by increasing disease risk. As global temperatures continue to rise, understanding these mechanisms will be critical for predicting how organisms respond to environmental change. Together, this work highlights key processes through which climate change may shape the health, survival, and fitness of wild animal and human populations.

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## Appendix A: Chapter 5 Supplementary Tables

**Table A.1** Genera included in the oxygen tolerance ratio used to compare oxygen-tolerant to obligately anaerobic taxa. Each genus is classified according to oxygen requirement (aerobe, facultative anaerobe, or obligate anaerobe). Aerobic and facultatively anaerobic genera were combined to form the numerator of the ratio, while obligately anaerobic genera formed the denominator. A source is provided for the classification of each genus. See chapter 5 supplementary references for full citation.

| Taxa | Oxygen_tolerance | Source |
|---|---|---|
| Abiotrophia | facultative_anaerobe | (Ruoff, 2011) |
| Actinomyces | facultative_anaerobe | (Gillespie, 2006) |
| Alistipes_A_871400 | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Alistipes_A_871404 | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Anaerobiospirillum_A | obligate_anaerobe | (Davis et al., 1976) |
| Anaeroglobus | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Anaerostipes | obligate_anaerobe | (Whitman & Parte, 2009) |
| Anaerovibrio | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Bacteroides_H | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Barnesiella | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Bifidobacterium_388775 | obligate_anaerobe | (Shimamura et al., 1992) |
| Bilifractor | obligate_anaerobe | (Whitman & Parte, 2009) |
| Bilophila | obligate_anaerobe | (Galushko & Kuever, 2020) |
| Blautia_A_141780 | obligate_anaerobe | (Whitman & Parte, 2009) |
| Blautia_A_141781 | obligate_anaerobe | (Whitman & Parte, 2009) |
| Butyricicoccus_A_77030 | obligate_anaerobe | (Geirnaert et al., 2014) |
| Campylobacter_D | facultative_anaerobe | (Kaakoush et al., 2007) |
| Capnocytophaga_820688 | facultative_anaerobe | (Leadbetter et al., 1979) |
| Cardiobacterium | facultative_anaerobe | (Moore et al., 2014) |
| Catenibacterium | obligate_anaerobe | (Nakano & Zuber, 2004) |

| | | |
|---|---|---|
| Citrobacter_A_692098 | facultative_anaerobe | (Octavia & Lan, 2014) |
| Clostridium_P | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Clostridium_T | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Collinsella | obligate_anaerobe | (Ben Khedher et al., 2021) |
| Coprobacter | obligate_anaerobe | (Shkoporov et al., 2013) |
| Desulfovibrio_R_446353 | obligate_anaerobe | (Galushko & Kuever, 2020) |
| Eisenbergiella | obligate_anaerobe | (Togo et al., 2018) |
| Enterocloster | obligate_anaerobe | (Magdy Wasfy et al., 2023) |
| Erysipelatoclostridium | obligate_anaerobe | (Yutin & Galperin, 2013) |
| Eubacterium_I | obligate_anaerobe | (Whitman & Parte, 2009) |
| Eubacterium_R | obligate_anaerobe | (Beuscher & Andreesen, 1984) |
| Flavonifractor | obligate_anaerobe | (Zamora Diaz & Llanes Somellera, 2025) |
| Fusobacterium_A | obligate_anaerobe | (Brazier et al., 1991) |
| Fusobacterium_C | obligate_anaerobe | (Brazier et al., 1991) |
| Gemella | facultative_anaerobe | (M. D. Collins et al., 1998) |
| Klebsiella_724518 | facultative_anaerobe | (Octavia & Lan, 2014) |
| Lactiplantibacillus | facultative_anaerobe | (Zheng et al., 2020) |
| Lactobacillus | facultative_anaerobe | (Zheng et al., 2020) |
| Lactococcus_A_343473 | facultative_anaerobe | (Teuber, 1995) |
| Lactococcus_A_346120 | facultative_anaerobe | (Teuber, 1995) |
| Lautropia | facultative_anaerobe | (Gerner-Smidt et al., 1994) |
| Lawsonibacter | obligate_anaerobe | (Sakamoto et al., 2018) |
| Leuconostoc_B | facultative_anaerobe | (Zheng et al., 2020) |
| Massilia | aerobic | (La Scola et al., 1998) |
| Megasphaera_A_38685 | obligate_anaerobe | (Lanjekar et al., 2014) |
| Methylobacterium | aerobic | (Patt et al., 1976) |
| Moraxella_C_651924 | aerobic | (Baumann & Doudoroff, 1968) |
| Morganella | facultative_anaerobe | (Octavia & Lan, 2014) |
| Neisseria_561456 | aerobic | (Bennett et al., 2014) |

| | | |
|---|---|---|
| Neisseria_563205 | aerobic | (Bennett et al., 2014) |
| Oxalobacter_566322 | obligate_anaerobe | (Duncan et al., 2002) |
| Pantoea_A_680069 | facultative_anaerobe | (Octavia & Lan, 2014) |
| Pantoea_A_680430 | facultative_anaerobe | (Octavia & Lan, 2014) |
| Parabacteroides_B_862066 | obligate_anaerobe | (Gajdács & Urbán, 2020) |
| Paraprevotella | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Phascolarctobacterium_A | obligate_anaerobe | (Wu et al., 2017) |
| Phocaeicola_A_858004 | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Pigmentiphaga | facultative_anaerobe | (Chen et al., 2009): |
| Porphyromonas_A_859423 | obligate_anaerobe | (Diaz et al., 2006) |
| Prevotella | obligate_anaerobe | (Nakano & Zuber, 2004) |
| Proteus | facultative_anaerobe | (Octavia & Lan, 2014) |
| Pseudoflavonifractor_80958 | obligate_anaerobe | (Zamora Diaz & Llanes Somellera, 2025) |
| Pseudomonas_B_650451 | aerobic | (Peix et al., 2009) |
| Pseudomonas_E_647464 | aerobic | (Peix et al., 2009) |
| Rodentibacter_A_734545 | facultative_anaerobe | (Christensen et al., 2014) |
| Rothia | facultative_anaerobe | (West et al., 2024) |
| Ruminococcus_B | obligate_anaerobe | (Whitman & Parte, 2009) |
| Sellimonas | obligate_anaerobe | (Whitman & Parte, 2009) |
| Serratia_D_727363 | facultative_anaerobe | (Octavia & Lan, 2014) |
| Stenotrophomonas_A_615274 | facultative_anaerobe | (Zgair & Chhibber, 2011) |
| Streptococcus | facultative_anaerobe | (Toit et al., 2014) |
| Succinatimonas | obligate_anaerobe | (Santos & Thompson, 2014) |
| Terrisporobacter | obligate_anaerobe | (Böer et al., 2024) |
| Turicibacter | obligate_anaerobe | (Bosshard et al., 2002) |
| Veillonella_A | obligate_anaerobe | (Ding et al., 2024) |
| Weissella_A_338544 | facultative_anaerobe | (Zheng et al., 2020) |

**Table A.2** Genera included in the pathobiont ratio. Each genus is classified as either a pathobiont or a core taxon. Core genera are defined as those present in ≥70% of samples. Pathobiont genera were used to form the numerator of the ratio, while core genera formed the denominator. A source is provided for the classification of each pathobiont genus. See chapter 5 supplementary references for full citation.

| **Taxa** | **Category** | **Source** |
|---|---|---|
| Porphyromonas_A_859423 | pathobiont | (Mysak et al., 2014) |
| Campylobacter_D | pathobiont | (Janssen et al., 2008) |
| Bilophila | pathobiont | (Zhou et al., 2017) |
| Desulfovibrio_R_446353 | pathobiont | (Singh et al., 2023) |
| Ruminococcus_B | pathobiont | (Hall et al., 2017) |
| Erysipelatoclostridium | pathobiont | (Huang et al., 2025) |
| Fusobacterium_A | pathobiont | (Huh & Tae-Young, 2020) |
| Fusobacterium_C | pathobiont | (Huh & Tae-Young, 2020) |
| Anaerobiospirillum_A | pathobiont | (Malnick et al., 1990) |
| Citrobacter_A_692098 | pathobiont | (J. W. Collins et al., 2014) |
| Klebsiella_724518 | pathobiont | (Pötgens et al., 2018) |
| Morganella | pathobiont | (Clayton et al., 2024) |
| Proteus | pathobiont | (Hamilton et al., 2018) |
| Pseudomonas_B_650451 | pathobiont | (de Bentzmann & Plésiat, 2011) |
| Pseudomonas_E_647464 | pathobiont | (de Bentzmann & Plésiat, 2011) |
| Serratia_D_727363 | pathobiont | (Tavares-Carreon et al., 2023) |
| Stenotrophomonas_A_615274 | pathobiont | (Brooke, 2012) |
| Phascolarctobacterium_A | core | present in ≥70% of samples |
| Sutterella | core | present in ≥70% of samples |
| Bifidobacterium_388775 | core | present in ≥70% of samples |
| Prevotella | core | present in ≥70% of samples |
| Anaerobiospirillum_A | core | present in ≥70% of samples |
| Streptococcus | core | present in ≥70% of samples |
| Phocaeicola_A_858004 | core | present in ≥70% of samples |

| | | |
|---|---|---|
| Bacteroides_H | core | present in ≥70% of samples |
| Rothia | core | present in ≥70% of samples |
| JC017 | core | present in ≥70% of samples |

**Table A.3** Metabolic pathways included in the oxygen tolerance ratio. Each pathway is classified as typical of oxygen-tolerant taxa (aerobes or facultative anaerobes) or obligately anaerobic taxa. Pathways characteristic of oxygen-tolerant taxa were used to form the numerator of the ratio, while pathways typical of obligately anaerobic taxa formed the denominator. Classifications reflect characteristic metabolic functions (e.g., simple sugar utilization versus fiber fermentation) and exclude pathways specific to aerobic or anaerobic respiration to capture functional shifts associated with changes in taxonomic composition rather than respiratory mode. A source is provided for the classification of each pathway. See chapter 5 supplementary references for full citation.

| **Pathway** | **Category** | **Source** |
|---|---|---|
| PWY0-1477: ethanolamine utilization | facultative | (Garsin, 2010; Severi et al., 2007; Tailford et al., 2015) |
| OANTIGEN-PWY: O-antigen building blocks biosynthesis (E. coli) | facultative | (Garsin, 2010; Severi et al., 2007; Tailford et al., 2015) |
| P461-PWY: hexitol fermentation to lactate, formate, ethanol and acetate | facultative | (Lengeler, 1975; Mandrand-Berthelot et al., 2004) |
| HEXITOLDEGSUPER-PWY: superpathway of hexitol degradation (bacteria) | facultative | (Lengeler, 1975; Mandrand-Berthelot et al., 2004) |
| PWY-8187: L-arginine degradation XIII (reductive Stickland reaction) | obligate | (Flint et al., 2012; Maier et al., 2014) |
| P441-PWY: superpathway of N-acetylneuraminate degradation | facultative | (Garsin, 2010; Severi et al., 2007; Tailford et al., 2015) |
| COLANSYN-PWY: colanic acid building blocks biosynthesis | facultative | (Garsin, 2010; Severi et al., 2007; Tailford et al., 2015) |
| PWY-7013: (S)-propane-1,2-diol degradation | facultative | (Garsin, 2010; Severi et al., 2007; Tailford et al., 2015) |
| PWY-7456: β-(1,4)-mannan degradation | obligate | (Flint et al., 2012; Maier et al., 2014) |
| KETOGLUCONMET-PWY: ketogluconate metabolism | facultative | (Lengeler, 1975; Mandrand-Berthelot et al., 2004) |

| | | |
|---|---|---|
| GALACTITOLCAT-PWY: galactitol degradation | facultative | (Lengeler, 1975; Mandrand-Berthelot et al., 2004) |
| PWY-5676: acetyl-CoA fermentation to butanoate II | obligate | (Flint et al., 2012; Maier et al., 2014) |

**Table A.4** Metabolic pathways included in the immune interaction ratio. Each pathway is classified as either immune-antagonistic (typical of pathogenic taxa) or immune-regulatory (typical of commensal taxa). Immune-antagonistic pathways were used to form the numerator of the ratio, while immune-regulatory pathways formed the denominator. Example immune-antagonistic functions include host glycan foraging and lipid A biosynthesis, whereas immune-regulatory functions include short-chain fatty acid production, antioxidant metabolism, and polyamine synthesis. A source is provided for the classification of each pathway. See chapter 5 supplementary references for full citation.

| **Pathway** | **Category** | **Source** |
|---|---|---|
| COLANSYN-PWY: colanic acid building blocks biosynthesis | antagonist | (Vuong et al., 2004; Whitfield, 2006) |
| 1CMET2-PWY: folate transformations III (E. coli) | regulatory | (Kolb & Petrie, 2013; Mölzer et al., 2021) |
| GLCMANNANAUT-PWY: superpathway of N-acetylglucosamine, N-acetylmannosamine and N-acetylneuraminate degradation | antagonist | (Severi et al., 2007; Tailford et al., 2015) |
| NAGLIPASYN-PWY: lipid IVA biosynthesis (E. coli) | antagonist | (Raetz & Whitfield, 2002) |
| ARG+POLYAMINE-SYN: superpathway of arginine and polyamine biosynthesis | regulatory | (Nakamura & Matsumoto, 2025) |
| COA-PWY: coenzyme A biosynthesis I (prokaryotic) | regulatory | (Gout, 2018) |
| OANTIGEN-PWY: O-antigen building blocks biosynthesis (E. coli) | antagonist | (Vuong et al., 2004; Whitfield, 2006) |
| P441-PWY: superpathway of N-acetylneuraminate degradation | antagonist | (Severi et al., 2007; Tailford et al., 2015) |
| PEPTIDOGLYCANSYN-PWY: peptidoglycan biosynthesis I (meso-diaminopimelate containing) | antagonist | (Boneca, 2005) |

| | | |
|---|---|---|
| PPGPPMET-PWY: ppGpp metabolism | antagonist | (Dalebroux et al., 2010) |
| FOLSYN-PWY: superpathway of tetrahydrofolate biosynthesis and salvage | regulatory | (Kolb & Petrie, 2013; Mölzer et al., 2021) |
| HOMOSER-METSYN-PWY: L-methionine biosynthesis I | regulatory | (Kunst et al., 2023) |
| HSERMETANA-PWY: L-methionine biosynthesis III | regulatory | (Kunst et al., 2023) |
| MET-SAM-PWY: superpathway of S-adenosyl-L-methionine biosynthesis | regulatory | (Kunst et al., 2023) |
| PWY-1269: CMP-3-deoxy-D-manno-octulosonate biosynthesis | antagonist | (Raetz & Whitfield, 2002) |
| METSYN-PWY: superpathway of L-homoserine and L-methionine biosynthesis | regulatory | (Kunst et al., 2023) |
| PANTOSYN-PWY: superpathway of coenzyme A biosynthesis I (bacteria) | regulatory | (Gout, 2018) |
| POLYAMSYN-PWY: superpathway of polyamine biosynthesis I | regulatory | (Nakamura & Matsumoto, 2025) |
| PWY-3841: folate transformations II (plants) | regulatory | (Kolb & Petrie, 2013; Mölzer et al., 2021) |
| PWY-4041: γ-glutamyl cycle | regulatory | (Diotallevi et al., 2017) |
| PWY-4984: urea cycle | regulatory | (Chi et al., 2021) |
| PWY-5345: superpathway of L-methionine biosynthesis (by sulfhydrylation) | regulatory | (Kunst et al., 2023) |
| PWY-5347: superpathway of L-methionine biosynthesis (transsulfuration) | regulatory | (Kunst et al., 2023) |

| | | |
|---|---|---|
| PWY-5676: acetyl-CoA fermentation to butanoate II | regulatory | (Klampfer et al., 2003) |
| PWY-6385: peptidoglycan biosynthesis III (mycobacteria) | antagonist | (Boneca, 2005) |
| PWY-6151: S-adenosyl-L-methionine salvage I | regulatory | (Kunst et al., 2023) |
| PWY-6612: superpathway of tetrahydrofolate biosynthesis | regulatory | (Kolb & Petrie, 2013; Mölzer et al., 2021) |
| PWY-6386: UDP-N-acetylmuramoyl-pentapeptide biosynthesis II (lysine-containing) | antagonist | (Boneca, 2005) |
| PWY-6387: UDP-N-acetylmuramoyl-pentapeptide biosynthesis I (meso-diaminopimelate containing) | antagonist | (Boneca, 2005) |
| PWY-702: L-methionine biosynthesis II | regulatory | (Kunst et al., 2023) |
| PWY-7323: superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis | antagonist | (Vuong et al., 2004; Whitfield, 2006) |
| PWY-7328: superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis | antagonist | (Vuong et al., 2004; Whitfield, 2006) |
| PWY-7953: UDP-N-acetylmuramoyl-pentapeptide biosynthesis III (meso-diaminopimelate containing) | antagonist | (Boneca, 2005) |
| PWY-7204: pyridoxal 5-phosphate salvage II (plants) | regulatory | (Diotallevi et al., 2017) |
| PWY-8073: lipid IVA biosynthesis (P. putida) | antagonist | (Raetz & Whitfield, 2002) |
| PWY0-1261: anhydromuropeptides recycling I | antagonist | (Boneca, 2005) |
| PWY0-1477: ethanolamine utilization | antagonist | (Severi et al., 2007; Tailford et al., 2015) |
| PWY-7851: coenzyme A biosynthesis II (eukaryotic) | regulatory | (Gout, 2018) |
| PWY-7977: L-methionine biosynthesis IV | regulatory | (Kunst et al., 2023) |
| PWY-I9: L-cysteine biosynthesis VI (from L-methionine) | regulatory | (Kunst et al., 2023) |

| | | |
|---|---|---|
| PWY0-845: superpathway of pyridoxal 5-phosphate biosynthesis and salvage | regulatory | (Diotallevi et al., 2017) |
| PWY1ZNC-1: assimilatory sulfate reduction IV | regulatory | (Diotallevi et al., 2017) |
| PYRIDOXSYN-PWY: pyridoxal 5-phosphate biosynthesis I | regulatory | (Diotallevi et al., 2017) |
| PWY0-1586: peptidoglycan maturation (meso-diaminopimelate containing) | antagonist | (Boneca, 2005) |
| SO4ASSIM-PWY: assimilatory sulfate reduction I | regulatory | (Diotallevi et al., 2017) |
| SULFATE-CYS-PWY: superpathway of sulfate assimilation and cysteine biosynthesis | regulatory | (Diotallevi et al., 2017) |

**Table A.5** Metabolic pathways included in the redox balance ratio. Each pathway is classified as either involved in molecular repair or redox maintenance. Molecular repair pathways were used to form the numerator of the ratio, while redox maintenance pathways formed the denominator. Molecular repair pathways include those involved in nucleotide repair and de novo nucleotide synthesis, whereas redox maintenance pathways include functions that help neutralize reactive oxygen and nitrogen species (RONS), such as oxygen cycling, cofactor biosynthesis, and antioxidant metabolism. A source is provided for the classification of each pathway. See chapter 5 supplementary references for full citation.

| **Pathway** | **Category** | **Source** |
|---|---|---|
| 1CMET2-PWY: folate transformations III (E. coli) | redox | (Belenky et al., 2007; Ying, 2008) |
| ARG+POLYAMINE-SYN: superpathway of arginine and polyamine biosynthesis | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| DENOVOPURINE2-PWY: superpathway of purine nucleotides de novo biosynthesis II | nucleotide | (Imlay, 2013) |
| PPGPPMET-PWY: ppGpp metabolism | nucleotide | (Imlay, 2013; Vinayak & Pathak, 2009) |
| PRPP-PWY: superpathway of histidine, purine, and pyrimidine biosynthesis | nucleotide | (Imlay, 2013) |

| | | |
|---|---|---|
| COA-PWY-1: superpathway of coenzyme A biosynthesis III (mammals) | redox | (Gout, 2018) |
| PWY-5686: UMP biosynthesis I | nucleotide | (Imlay, 2013) |
| PWY-5695: inosine 5-phosphate degradation | nucleotide | (Imlay, 2013) |
| PWY-6121: 5-aminoimidazole ribonucleotide biosynthesis I | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-6122: 5-aminoimidazole ribonucleotide biosynthesis II | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-6123: inosine-5-phosphate biosynthesis I | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-6124: inosine-5-phosphate biosynthesis II | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| COA-PWY: coenzyme A biosynthesis I (prokaryotic) | redox | (Gout, 2018) |
| FOLSYN-PWY: superpathway of tetrahydrofolate biosynthesis and salvage | redox | (Belenky et al., 2007; Ying, 2008) |
| PWY-6125: superpathway of guanosine nucleotides de novo biosynthesis II | nucleotide | (Imlay, 2013) |
| PWY-6126: superpathway of adenosine nucleotides de novo biosynthesis II | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-6277: superpathway of 5-aminoimidazole ribonucleotide biosynthesis | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| HEME-BIOSYNTHESIS-II-1: heme b biosynthesis V (aerobic) | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-6353: purine nucleotides degradation II (aerobic) | nucleotide | (Imlay, 2013) |
| PWY-6545: pyrimidine deoxyribonucleotides de novo biosynthesis III | nucleotide | (Imlay, 2013) |
| PWY-6606: guanosine nucleotides degradation II | nucleotide | (Imlay, 2013) |

| | | |
|---|---|---|
| PWY-6608: guanosine nucleotides degradation III | nucleotide | (Imlay, 2013) |
| PWY-6609: adenine and adenosine salvage III | nucleotide | (Imlay, 2013) |
| HEME-BIOSYNTHESIS-II: heme b biosynthesis I (aerobic) | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-6700: queuosine biosynthesis I (de novo) | nucleotide | (Imlay, 2013; Vinayak & Pathak, 2009) |
| HEMESYN2-PWY: heme b biosynthesis II (oxygen-independent) | redox | (LeBlanc et al., 2011; Powers, 2003) |
| HOMOSER-METSYN-PWY: L-methionine biosynthesis I | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY-6703: preQ0 biosynthesis | nucleotide | (Imlay, 2013; Vinayak & Pathak, 2009) |
| PWY-7184: pyrimidine deoxyribonucleotides de novo biosynthesis I | nucleotide | (Imlay, 2013) |
| PWY-7185: UTP and CTP dephosphorylation I | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-7187: pyrimidine deoxyribonucleotides de novo biosynthesis II | nucleotide | (Imlay, 2013) |
| HSERMETANA-PWY: L-methionine biosynthesis III | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| MET-SAM-PWY: superpathway of S-adenosyl-L-methionine biosynthesis | redox | (Belenky et al., 2007; Ying, 2008) |
| METSYN-PWY: superpathway of L-homoserine and L-methionine biosynthesis | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |

| | | |
|---|---|---|
| NONOXIPENT-PWY: pentose phosphate pathway (non-oxidative branch) I | redox | (Belenky et al., 2007; Ying, 2008) |
| P4-PWY: superpathway of L-lysine, L-threonine and L-methionine biosynthesis I | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY-7198: pyrimidine deoxyribonucleotides de novo biosynthesis IV | nucleotide | (Imlay, 2013) |
| PWY-7199: pyrimidine deoxyribonucleosides salvage | nucleotide | (Imlay, 2013) |
| PWY-7208: superpathway of pyrimidine nucleobases salvage | nucleotide | (Imlay, 2013) |
| PANTOSYN-PWY: superpathway of coenzyme A biosynthesis I (bacteria) | redox | (Gout, 2018) |
| PENTOSE-P-PWY: pentose phosphate pathway | redox | (Belenky et al., 2007; Ying, 2008) |
| POLYAMSYN-PWY: superpathway of polyamine biosynthesis I | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY-7210: pyrimidine deoxyribonucleotides biosynthesis from CTP | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-241: C4 photosynthetic carbon assimilation cycle, NADP-ME type | redox | (Lu, 2013) |
| PWY-3841: folate transformations II (plants) | redox | (Belenky et al., 2007; Ying, 2008) |
| PWY-5188: tetrapyrrole biosynthesis I (from glutamate) | redox | (Gout, 2018) |
| PWY-5189: tetrapyrrole biosynthesis II (from glycine) | redox | (Gout, 2018) |
| PWY-7211: superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis | nucleotide | (Imlay, 2013) |

| | | |
|---|---|---|
| PWY-5345: superpathway of L-methionine biosynthesis (by sulfhydrylation) | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY-5347: superpathway of L-methionine biosynthesis (transsulfuration) | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY-5838: superpathway of menaquinol-8 biosynthesis I | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-7220: adenosine deoxyribonucleotides de novo biosynthesis II | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-5861: superpathway of demethylmenaquinol-8 biosynthesis I | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-5897: superpathway of menaquinol-11 biosynthesis | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-5898: superpathway of menaquinol-12 biosynthesis | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-7221: guanosine ribonucleotides de novo biosynthesis | nucleotide | (Imlay, 2013) |
| PWY-5899: superpathway of menaquinol-13 biosynthesis | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-5918: superpathway of heme b biosynthesis from glutamate | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-7222: guanosine deoxyribonucleotides de novo biosynthesis II | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-6147: 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I | redox | (Belenky et al., 2007; Ying, 2008) |
| PWY-7228: superpathway of guanosine nucleotides de novo biosynthesis I | nucleotide | (Imlay, 2013) |
| PWY-6151: S-adenosyl-L-methionine salvage I | redox | (Belenky et al., 2007; Ying, 2008) |

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| PWY-6168: flavin biosynthesis III (fungi) | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-7229: superpathway of adenosine nucleotides de novo biosynthesis I | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-7234: inosine-5-phosphate biosynthesis III | nucleotide | (Imlay, 2013; Lane & Fan, 2015) |
| PWY-6549: L-glutamine biosynthesis III | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY-6612: superpathway of tetrahydrofolate biosynthesis | redox | (Belenky et al., 2007; Ying, 2008) |
| PWY-6823: molybdopterin biosynthesis | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-7282: 4-amino-2-methyl-5-diphosphomethylpyrimidine biosynthesis II | nucleotide | (Imlay, 2013) |
| PWY-702: L-methionine biosynthesis II | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY-7115: C4 photosynthetic carbon assimilation cycle, NAD-ME type | redox | (Lu, 2013) |
| PWY-7117: C4 photosynthetic carbon assimilation cycle, PEPCK type | redox | (Lu, 2013) |
| PWY-7204: pyridoxal 5-phosphate salvage II (plants) | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-724: superpathway of L-lysine, L-threonine and L-methionine biosynthesis II | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY-7761: NAD salvage pathway II (PNC IV cycle) | redox | (Belenky et al., 2007; Ying, 2008) |

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| PWY-7851: coenzyme A biosynthesis II (eukaryotic) | redox | (Gout, 2018) |
| PWY-7790: UMP biosynthesis II | nucleotide | (Imlay, 2013) |
| PWY-7977: L-methionine biosynthesis IV | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY-7791: UMP biosynthesis III | nucleotide | (Imlay, 2013) |
| PWY-7992: superpathway of menaquinol-8 biosynthesis III | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-8178: pentose phosphate pathway (non-oxidative branch) II | redox | (Belenky et al., 2007; Ying, 2008) |
| PWY-I9: L-cysteine biosynthesis VI (from L-methionine) | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |
| PWY0-1415: superpathway of heme b biosynthesis from uroporphyrinogen-III | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY0-845: superpathway of pyridoxal 5-phosphate biosynthesis and salvage | redox | (LeBlanc et al., 2011; Powers, 2003) |
| PWY-841: superpathway of purine nucleotides de novo biosynthesis I | nucleotide | (Imlay, 2013) |
| PWY0-1296: purine ribonucleosides degradation | nucleotide | (Imlay, 2013) |
| PWY0-1297: superpathway of purine deoxyribonucleosides degradation | nucleotide | (Imlay, 2013) |
| PWY0-1298: superpathway of pyrimidine deoxyribonucleosides degradation | nucleotide | (Imlay, 2013) |
| PWY1ZNC-1: assimilatory sulfate reduction IV | redox | (Lu, 2013) |
| PWY3O-4107: NAD salvage pathway V (PNC V cycle) | redox | (Belenky et al., 2007; Ying, 2008) |

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| PWY0-1479: tRNA processing | nucleotide | (Imlay, 2013; Vinayak & Pathak, 2009) |
| PWY0-166: superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis (E. coli) | nucleotide | (Imlay, 2013) |
| PWY66-409: superpathway of purine nucleotide salvage | nucleotide | (Imlay, 2013) |
| PYRIDNUCSYN-PWY: NAD de novo biosynthesis I (from aspartate) | redox | (Belenky et al., 2007; Ying, 2008) |
| SALVADEHYPOX-PWY: adenosine nucleotides degradation II | nucleotide | (Imlay, 2013) |
| PYRIDOXSYN-PWY: pyridoxal 5-phosphate biosynthesis I | redox | (Belenky et al., 2007; Ying, 2008) |
| TRNA-CHARGING-PWY: tRNA charging | nucleotide | (Imlay, 2013; Vinayak & Pathak, 2009) |
| RIBOSYN2-PWY: flavin biosynthesis I (bacteria and plants) | redox | (LeBlanc et al., 2011; Powers, 2003) |
| SO4ASSIM-PWY: assimilatory sulfate reduction I | redox | (Lu, 2013) |
| SULFATE-CYS-PWY: superpathway of sulfate assimilation and cysteine biosynthesis | redox | (Lu, 2013; Nakamura & Matsumoto, 2025; Timmons et al., 2012) |