AN ABSTRACT OF THE DISSERTATION OF

Michael James Sieler Jr. for the degree of Doctor of Philosophy in Microbiology presented on August 25, 2025.

Title: Defining the Context Dependence of How the Zebrafish (*Danio rerio*) Gut Microbiome Responds to Environmental Stressors

Abstract approved:

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The Anthropocene has upended the epochal cadence of Earth's changing environments, accelerating the pace of global change from geological time to human time scales, presenting unprecedented environmental challenges that test the resilience of host-microbiome holobiont systems. Given that microbial communities can serve as early indicators of ecosystem health before macroscopic symptoms appear, understanding how holobionts navigate complex, sequential environmental stressors is critical for predicting and mitigating the cascading effects of anthropogenic change. This dissertation leverages the zebrafish (*Danio rerio*) model system to investigate context-dependent principles of microbiome response to environmental stressors, specifically examining how diet modulates microbiome sensitivity to pathogen exposure, how temperature and parasite exposure interact to shape infection outcomes, and how historical

contingency fundamentally reshapes host-microbiome system resilience and recovery. Through novel analytical frameworks and multi-omic data integration, this work clarifies context-dependence of host-microbiome-environmental interactions to anthropogenic stressors. Chapter 2 demonstrates that diet actively shapes the successional pathways along which zebrafish gut communities develop, with different laboratory diets producing distinct diversity and compositional trajectories that predict community response to *Mycobacterium chelonae* exposure. These findings reveal diet as a potential hidden driver behind inconsistent microbiome compositions across facilities, motivating diet standardization as a prerequisite for reproducible zebrafish microbiome research and highlighting how climate-driven disruptions to global food supply could alter vertebrate microbiomes in ways that erode natural defenses against disease. Chapter 3 disentangles direct thermal effects from host-microbe interactions in a changing climate, revealing that water temperature and *Pseudocapillaria tomentosa* exposure independently alter gut diversity, yet only the highest temperature tested suppresses worm development. The work challenges current expectations regarding warming waters increasing disease burden by demonstrating that microbiome-mediated responses may result in unanticipated outcomes, with apparent dysbiosis reflecting unmeasured environmental context rather than intrinsic instability. Chapter 4 extends beyond single stressors by demonstrating that past exposures define both resistance and resilience to new perturbations, with sequential exposure to antibiotics, heat, and parasites showing that mortality climbs with added stressors while final infection burden is not strictly proportional to stress history. Multi-omic integration uncovers stress-specific gene-taxon networks, confirming that host-microbiome responses are historically contingent and implying that variation among studies may stem from unrecorded exposure histories, necessitating dynamic, history-aware models to predict system state from

stressor sequence and spacing. Collectively, these projects deepen our understanding of how organisms navigate environmental change, revealing that host-microbiome relationships are shaped not only by present stressors but also by the history and specific dimensions—modality, magnitude, and timing—of those stressors.

Defining the Context Dependence of How the Zebrafish (*Danio rerio*) Gut Microbiome Responds to Environmental Stressors

by

Michael James Sieler Jr.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Michael James Sieler Jr., Author

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CONTRIBUTION OF AUTHORS

Chapter 2: TS and MK conceived the study, oversaw project administration and supervision, and provided resources. MS curated the dataset, performed the formal analyses and investigations, developed the software and methodological pipeline, validated results, and generated visualizations. CA and KK carried out additional investigations and contributed to methodological development. MS drafted the initial manuscript.

Chapter 3: TJS and MLK conceived and designed the study. CES performed the experiments. MJS, TJS, and KDK conducted the gut microbiome and integrated analyses. MJS prepared the figures. MJS, TJS, MLK, CES, KDK, and ZMV wrote and edited the manuscript. All authors read and approved the final manuscript.

Chapter 4: TJS and MLK conceived and designed the study. CL and MJS performed the experiments. MJS, TJS, and KDK conducted the gut-microbiome and integrated analyses. MJS prepared the figures. MJS, TJS, MLK, CL, and KDK wrote and edited the manuscript.

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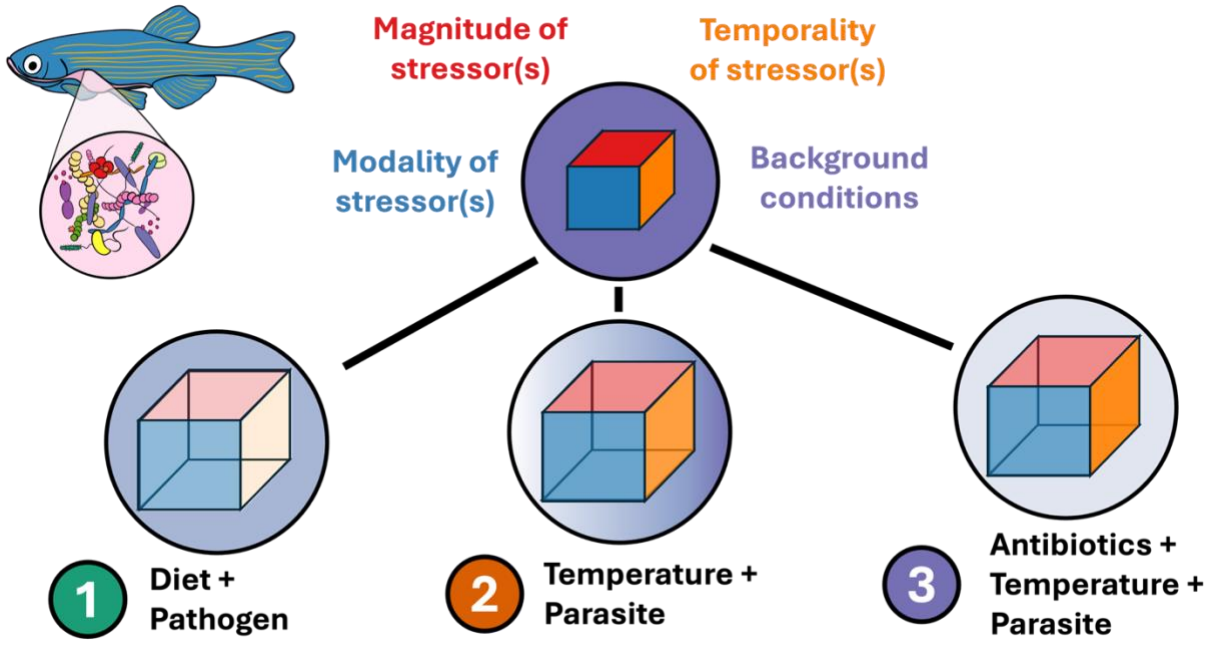
To Amanda and Sofie, whose undying support and encouragement know no bounds.

CHAPTER 1

INTRODUCTION



The Anthropocene and Accelerating Environmental Change

Life on Earth has been a delicate evolutionary dance, a dynamic back-and-forth in which organisms step to the rhythms of shifting environments' soils, seas, and skies, while also subtly shaping the stage beneath their feet [1,2]. For most of the planet's history, the tempo of change was measured in epochs; evolutionary partners adjusting their choreography at a pace matched by drifting continents and cyclical climates [3]. Recently, the Anthropocene [4,5]—the geological period of time where humans have risen as a major force of global change—has upended this epochal cadence [6]. Industrial emissions, the spread of novel diseases, habitat fragmentation, and climate change are now accelerating the pace of global change on the order of centuries. Ecologists fear that many organisms cannot match these hastened steps [7,8], as

evidenced by dramatic species losses [9], abrupt turnover in community structure [10], and the unraveling of ecological functions that once provided stability [11,12]. Predicting, mitigating, or reversing such impacts is challenging because global change factors are not unidimensional [13,14]; instead, they vary in modality (e.g., sequential or concurrent)[15,16], magnitude (e.g., degrees of strength)[15], and temporality (e.g., short pulses or sustained presses)[15]. Together, these dimensions of stressors define an ecosystem's contextual landscape: the environmental backdrop shaped by the timing, magnitude, and modality of disturbances. This landscape, in turn, governs the range of trajectories along which organisms may respond to perturbations [17]. Survival, therefore, hinges on how well an organism's inherited traits match the contours of that landscape, shaping its ability to persist as conditions shift. Clarifying how these dimensions of stressors shape an ecosystem's contextual landscape is critical for predicting whether ecosystems, and the life they host, can keep pace with the shifting rhythms of the Anthropocene and dance another day.

Microbial Ecosystems in a Changing World

One of the most important ecosystems that we also understand nearly the least about in terms of their contextual landscapes is those occupied by microorganisms. Human activity in the Anthropocene has disrupted microbial ecosystems at every scale, reshaping both where microbes reside and how their communities function [18]. Chemical pollution is a major driver: antibiotics, heavy metals, microplastics, and other persistent compounds enter soils, waterways, and even animal tissues, where they can slow or accelerate growth, redirect metabolic pathways, and reorganize microbial interactions [19–21]. Physical landscape change intensifies the pressure. When habitats are cleared or fragmented, dispersal routes break down, populations

become isolated, and genetic diversity erodes [22,23]. In addition to these direct effects, climate change is altering temperature regimes, precipitation patterns, and seasonal cycles that have long governed microbial community assembly [24]. Microbes must either adapt quickly or disappear, and their fates reverberate through the hosts and ecosystems they support [25]. Because many microbes live in close association with plants and animals, stressors that impair host fitness or drive population declines can indirectly disrupt their microbiomes [26,27]. For example, host range shifts may expose hosts to novel microbes (e.g., pathogens)[28], while chronic physiological stress can alter host immunity and gut conditions [29], reshaping the microbiome's composition and functioning [18]. These changes may lead to dysbiosis, which can further undermine host health and amplify the cascading effects of global change.

Microbiome disruption can intensify the ecological pressures of the Anthropocene, triggering cascading impacts on ecosystem function, host health, and resilience that extend well beyond the initial disturbance [26]. Microbial communities, and their metabolic products, drive processes such as nutrient turnover, carbon storage, pathogen suppression, and the maintenance of host health [30]. When these microbial communities are disrupted, the loss of their micro-ecosystem services has the potential to ripple through entire macro-ecosystems [31]. Disruption of soil microbiomes, for example, limits nutrient availability to plants, which in turn affects herbivores and progressively restructures food webs [32]. Disturbances to microbial communities in aquatic environments can compromise water quality, disrupt essential biogeochemical processes, and reduce ecosystem resilience to additional environmental pressures [28,33]. The implications for host organisms are similarly concerning: microbiome disruption has been associated with increased disease susceptibility, diminished reproductive success, and compromised immune function across diverse taxa [34]. Together, these impacts

threaten population stability and may raise extinction risk, especially for organisms already vulnerable to habitat loss and climate change [35]. Perhaps most troubling is the potential for vicious feedback loops, as stressed hosts foster dysbiotic microbiomes that further erode host resilience, setting off a downward spiral that could hasten population decline [36]. Because human activities may set these loops in motion, microbiome disruption could be a critical yet often under-recognized driver of global change.

Microbiomes as Early Indicators and Intervention Targets

Understanding microbiome responses to anthropogenic stressors is crucial for developing effective mitigation strategies [37]. Because microbial communities often shift well before macroscopic symptoms appear, they may be able to serve as early indicators of host health or disease, signaling degradation at an early stage while still recoverable [38]. Monitoring these shifts can reveal when microbial communities are approaching resilience thresholds—points beyond which they can no longer recover their original state—and detect impending tipping points where small changes trigger abrupt, potentially irreversible ecosystem transformations [39,40]. This predictive capacity is important for conservation planning in rapidly changing landscapes. For instance, researchers quantified the abundance of black howler monkey microbiota and identified reductions in microbial taxa indicative of suboptimal habitats which may link to poor health outcomes [41]. Furthermore, microbiome research also reveals direct avenues for intervention. For example, manipulating community composition has shown promise in soil cleanup, coral reef recovery, and other ecological restoration efforts [42]. At the host level, microbial-targeted therapeutics could reinforce immunity and reproduction, buffering populations against anthropogenic stress [14,42]. Additionally, microbiome-informed

conservation strategies that safeguard microbial diversity alongside plants and animals are therefore more likely to preserve the processes on which ecosystems depend [43]. Moreover, insights gained at one level of organization, from individual hosts to entire populations, can be scaled up to strengthen ecosystem resilience. Taken together, microbiome science holds promise to equip us with both immediate and long-term tools for confronting the challenges of the Anthropocene while protecting the biological functions that sustain life on Earth.

The Gut Microbiome: A Critical Host-Associated Ecosystem

Microbial organisms can be found virtually everywhere [44,45], but nowhere is their abundance, diversity, and complexity more pronounced than in vertebrate animal gastrointestinal tracts [46], where trillions of bacterial, archaeal, fungal, and viral cells form intricate, host-specialized communities [47]. These microbial communities, known as the gut microbiome, provide beneficial ecosystem services to their hosts [48,49]. For instance, gut microbiota are central to host metabolism, in that they can liberate otherwise indigestible nutrients [50], produce beneficial short-chain fatty acids [51–53], and regulate circulating nutrients and hormones [54]. Although diet is a primary driver of microbiome composition, we still know little about how dietary change modifies the microbiome's ability to resist or recover from stressors, for instance, pathogenic invasion, especially when other environmental pressures co-occur. Additionally, gut microbiota can support the host's immune system [48,55,56], for example, through competitive exclusion of pathogens [57] and immune cell priming [58,59]. However, the durability of this protection offered by gut microbiota across varying environmental conditions or multiple stressors is unclear. Lastly, beyond the local effects, gut microbiota facilitate cross-organ communication, for instance between the gastrointestinal tract and the nervous [60] and

endocrine systems [61]. Yet, many of these studies are correlational and lack metrics for predicting how environmental perturbations will decouple these cross-organ signals [62,63]. Moreover, accumulating research links microbiome disruption to disorders ranging from metabolic to neurodevelopmental [29,34,48,64,65]. The concern is that if the microbes that perform these health-sustaining functions are lost due to anthropogenic stressors, the host may suffer adverse health consequences, as evidenced by the rising prevalence of inflammatory bowel diseases, colon cancer, and other microbiome-associated disorders [66–68]. Furthermore, the loss of these functions could have devastating long-term evolutionary consequences to the fitness and survival of humans and other vertebrate animals [66,69]. Understanding how these critical host-microbiome relationships maintain stability under environmental pressure is therefore essential for predicting and potentially mitigating these health and evolutionary risks.

Buffering Capacity and Stability in Host-Microbiome Systems

While the specific mechanisms maintaining the stability of the host-microbiome relationship are not known [31,70–73], one hypothesized safeguard is the gut microbiota's buffering capacity [14]. The gut microbiota's buffering capacity refers to its ability to shield the host from harmful environmental stressors while maintaining its key microbiome functions that support host health, even under pressure [14]. This capacity is thought to stabilize the host-microbiome systems through functional redundancy. For instance, community membership can shift under external stressors, yet as long as the remaining or newly recruited microbes carry out essential functions, host health and homeostasis remain intact [74–76]. However, buffering is not guaranteed; disturbances that disrupt community composition can erode functional capacity and precipitate disease [29]. Although a limited number of studies document microbiome-mediated

buffering [14,77], we do not know whether it is a general property of host-microbiome systems, nor how the modality, magnitude, or timing of stressors modulate it. Crucially, we still lack a comprehensive understanding of how different dimensions of environmental stressors shape microbiome responses and influence host health outcomes, an issue complicated by the microbiome's inherent temporal variability.

Moving Beyond Homeostasis: Toward a Homeorhetic Framework

The gut microbiome is a dynamic system that can fluctuate over the course of hours, days, months, and even years [78–81]. These dynamics arise from the reciprocal interaction of host, microbial, and environmental factors that influence its taxonomic abundance, microbial composition and diversity. For instance, temperature [82], pH [83], oxygen gradients [84], nutrient flux [85], and xenobiotics impose steep and often rapid selection gradients [86]. At the same time, age [78], diet [87], stress reactivity [60], and circadian regulation reshape gut architecture [88], immune system [55,56], and gut transit [89]. Together, these influences create a moving ecological backdrop in which microbiomes continually respond to and reorganize. Furthermore, temporal sampling reveals regular diurnal oscillations [90,91], seasonal cycling [92,93], and developmental transitions that redefine “normal” community structure [78,94,95]. Cross-sectional studies, which capture only a single moment, can blur individual differences with temporal change [96], overlook autocorrelation [38,39], and miss the size, timing, and duration of responses to disturbance [81,97].

The prevailing homeostatic model of microbiome stability [55,65,72,98], that a healthy community rebounds to one fixed state after perturbation, fails under such temporal fluidity. Baseline microbiome states drift among hosts, life stages, and environmental contexts [78,81,99],

and multiple distinct microbiome configurations can support comparable host health [100–102]. For example, healthy infant gut consortia are compositionally and metabolically distinct from healthy adult communities [103,104]. Furthermore, efforts to define a universal "healthy" microbiome are confounded by genetic, cultural, and socioeconomic heterogeneity [34,105–109]. Rather than a homeostatic framework, ecological principles point to a different view of stability, one comprising historical contingency [16], priority effects [110], succession [78], drift [111], and dispersal that set path-dependent trajectories and alternative stable states [101,111]. This may explain why models of the microbiome that assume linearity or only track taxonomy instead of function fall short of capturing these realities [112]. Instead, a more suitable framework is homeorhesis, which defines a stable system as one with the capacity to keep pace with, or realign to, a shifting trajectory rather than returning to a fixed point [113,114]. To date, homeorhetic models have had limited applications in microbiome research, partly due to the complexity of conducting longitudinal microbiome studies [114]. Therefore, making homeorhesis operational will require longitudinal and multifactorial microbiome experiments that capture the multidimensional influences across various host-microbiome systems.

Advances in Microbiome Research: From 16S to Multi-omics

Approaches for studying microbial communities have evolved considerably over the past century, culminating in the methodological ability to rigorously test the fundamental ecological theories and host-microbiome questions outlined above [115,116]. Early microbiologists relied on culture-based methods, isolating individual strains on selective media to characterize their properties. However, the discovery of the 16S rRNA gene and subsequent environmental microbial amplicon sequencing revealed that very few bacteria were culturable [117,118],

leaving the vast majority of microbial diversity invisible to cultivation approaches [119,120]. The breakthrough of 16S amplicon sequencing ushered in a new era of culture-independent microbial surveying, revolutionizing our view of the tree of life and opening microbial ecology to previously unculturable taxa [121–123]. Building on this foundation, contemporary gut microbiome studies typically follow a standardized workflow: collecting fecal samples, extracting community DNA, amplifying hypervariable regions of the 16S gene, and analyzing reads through denoising pipelines, such as DADA2, to generate amplicon sequence variants (ASV) that are then taxonomically annotated [115,116,124]. While the 16S amplicon sequencing approach excels at answering the fundamental question of "who is there," it cannot reveal "what they are doing" in terms of functional capacity or metabolic activity [34,63,125]. Additionally, its resolution is constrained by primer bias, short read lengths, and an inability to discriminate between closely related strains [126]. Furthermore, there may be nuanced interaction patterns between “-omics” methods that are not perceptible individually but are revealed when integrated [97,127–129]. To bridge these conceptual and technical gaps, the field is increasingly embracing multi-omic workflows that integrate complementary analytical approaches [97,127–129]. For instance, shotgun metagenomics provides a deeper insight into taxonomy at the strain level as well as the functional capacity [130,131], transcriptomics captures active gene expression patterns [129], and metabolomics reveals the biochemical products of microbial activity or their host [112]. Together, these approaches offer richer portraits of microbiome ecology and host-microbe interactions, but at the cost of greater logistical and analytical complexity [132–134]. Executing such integrative studies requires a tractable and scalable model system whose biology, husbandry, and genomic resources all support longitudinal, multivariate, and multi-omic sampling experimental schemes.

Zebrafish as a Model for Host-Microbiome-Environment Interactions

Zebrafish are the second most popular biomedical model [135–138]. Their established history as a model organism has contributed to their increasing popularity as a uniquely powerful ecological model for host-microbiome-environment interactions, offering experimental advantages that enable scientific discoveries beyond what is possible in humans or other vertebrate model systems [137–139]. First, zebrafish are highly economical both financially and logistically: they are inexpensive to rear, require minimal space, and can be maintained in large numbers, making it feasible to conduct experiments with hundreds or thousands of individuals simultaneously [135,139]. This scalability allows for robust statistical power and the ability to implement complex, multi-factorial microbiome experimental studies where multiple treatments can be applied individually and in combination to tease out the relationships between host-microbiome-environmental interactions [140]. Second, zebrafish are well-suited for longitudinal studies due to their rapid development and tolerance for repeated, non-invasive sampling, such as fecal collection, which enables researchers to track microbiome and host changes over both short and long timescales [95,141–143]. Third, decades of research in developmental biology, genetics, and toxicology have firmly established zebrafish as a model organism, providing researchers a rich foundation of biological knowledge, established husbandry protocols, and high-throughput molecular and analytical experimental methods to build upon [135]. This established foundation promotes experimental consistency and enables meaningful comparisons across studies, accelerating progress toward generating biological discoveries and developing novel microbiome-targeted therapeutics [144,145]. Furthermore, zebrafish benefit from a well-annotated genome and a suite of genetic and molecular tools, which enable precise manipulation

and mechanistic microbiome studies [146,147]. This genetic tractability opens the door to linking microbial traits with host phenotypes to illuminate host-microbiome relationships. Fourth, discoveries in the zebrafish system are highly translatable. Zebrafish share considerable genetic homology with humans and other vertebrates [146]. For example, zebrafish share 70% orthologous protein-coding genes with humans [146]. While Zebrafish microbiomes are taxonomically distinct from those of humans, they perform many of the same functional roles [148]. This functional convergence makes zebrafish an attractive system for identifying fundamental principles of host-microbiome interaction that may apply across other vertebrate systems.

Additionally, an important benefit of zebrafish is that they are a particularly powerful model for studying host-microbiome-parasite interactions, which represent a significant source of mortality in both animal and human populations worldwide [149–151]. For example, two intestinal infectious agents of particular relevance to zebrafish research are the pathogenic bacterium *Mycobacterium chelonae* and the parasitic nematode *Pseudocapillaria tomentosa* [151,152]. Both organisms are common in zebrafish facilities and may represent an underappreciated source of unintended biological variation in zebrafish microbiome study outcomes [153]. *M. chelonae*, a rapidly growing mycobacterium, can cause chronic infections in zebrafish characterized by granulomatous lesions and systemic inflammation, and can infect animals broadly [154–156]. *P. tomentosa*, an intestinal nematode, which establishes chronic infections that can lead to increased mortality, reduced fecundity, and altered gut physiology [157]. Additionally, both *M. chelonae* and *P. tomentosa* have been previously shown to disrupt the functional capacity, and assembly and successional development of zebrafish gut microbiomes [158–161]. In addition to their prevalence in zebrafish facilities, both *P. tomentosa*

and *M. chelonae* serve as well-established and reproducible laboratory infection models. *P. tomentosa* infections are initiated by exposure to larvated eggs, which reliably establish chronic intestinal infections with dose–response relationships and overdispersed worm burdens typical of macroparasites [157]. *M. chelonae* infections can be generated by precise intraperitoneal injection, providing controlled systemic disease, or by the natural oral route through ingestion of contaminated feed or tank biofilms, which more closely reflects environmental transmission [162,163]. These complementary approaches make both *P. tomentosa* and *M. chelonae* robust and reliable zebrafish disease models, providing a strong foundation for studying host–microbiome–parasite interactions Therefore, clarifying the impact of these infectious microbes on zebrafish microbiome studies is essential for understanding how they may impact microbiome composition and inadvertently skew experimental results, while also providing opportunities to discover potential microbiome-targeted interventions to treat these and other intestinal infectious agents in human and animal populations. Collectively, these attributes of the zebrafish create a uniquely powerful model system for generating fundamental insights into host-microbiome-environmental dynamics that can be translated across vertebrate systems.

Outstanding Challenges and Knowledge Gaps in Host-Microbiome Research

Although recent technological advances have accelerated host-microbiome research, many challenges remain that limit our ability to disentangle host-microbiome-environmental interactions to gain molecular insights that connect to ecological and evolutionary processes critical to host-microbiome health. These limitations fall broadly into two major categories: experimental and analytical. Experimentally, investigating questions of host-microbiome-environmental interactions is challenged by the substantial resources required to conduct

longitudinal and multivariate experiments with large sample sizes [127]. Analytically, microbiome data is heterogenous, sparse, compositional, and temporally variable [127]. Further compounding these issues are the lack of standardized methods for integrating multi-omic microbiome data [127,132]. For example, variation in model organism husbandry practices, sample collection, sample preparation, sequence processing, and data analysis can contribute to inconsistencies in study outcomes. My dissertation addresses some of these challenges by leveraging the zebrafish's unique advantages as a model organism to interrogate how different dimensions of environmental stressors impact the microbiome to influence host health. Specifically, my work integrates longitudinal and multi-variate multi-omic data with ecologically informed models and methods of microbiome measurement. In particular, my dissertation focuses on three key questions, which are motivated by the above discussion:

1. Do different diets affect host-associated microbiome's sensitivity to pathogen exposure? Diet is a major driver of gut microbiota composition. Anthropogenically driven ecosystem collapse threatens global food supplies. Clarifying diet-driven changes in microbiome-mediated disease resistance addresses the current gap in understanding how nutritional context modulates host sensitivity to emerging pathogens.

2. Does varying the background conditions across a gradient of increasing heat stress impact the microbiome's response to an intestinal parasite and the host's infection outcomes? Increasing water temperatures are caused by anthropogenic climate change. Testing how increasing water temperature affects host-microbiome-parasite dynamics fills a critical gap in predicting how warming ecosystems will reshape infection risk and microbiome stability.

3. Is the host-microbiome system's response to parasite exposure or recovery historically contingent upon exposure to prior stressors? Organisms often experience multiple stressors. Furthermore, these stressors may occur within the context of past stress exposure. Uncovering how stress history influences microbiome resistance and resiliency addresses a critical gap in how host-microbiome systems will respond to rapidly changing and increasingly stressful environments.

Research Aims and Dissertation Overview

This dissertation explores these three questions across the subsequent three chapters (Chapters 2, 3, and 4). In Chapter 2, I assessed how diets differentially impacted the zebrafish gut microbiome's sensitivity to bacterial pathogen infection to clarify the role of diet impacting microbiome structure and response to pathogen infection [158]. Despite zebrafish's long history as a model organism and increasing popularity in microbiome studies, zebrafish studies do not utilize a standard reference diet [164–167]. This is in stark contrast to mouse models that have used standard reference diets for over 50 years [167]. Given that diet is known to substantially influence the gut microbiome [168,169], inter-study differences in diet could inadvertently drive variation in zebrafish microbiome study outcomes [144]. Furthermore, microbiome function is coupled to the composition of gut microbial members [112]. Therefore, diet-driven gut microbiome variation could be challenging efforts to understand how the gut microbiome mediates intestinal pathogen outcomes [170]. Our analysis finds that diet has a substantial impact on gut microbiome assembly and development, where fish fed particular diets differed in their gut microbial diversity and composition, suggesting that one explanation for differences in microbiome composition across facilities could be in part due to husbandry practices involving

diet. I next investigated whether the microbiome's sensitivity to pathogen infection varied as a function of diet. I found that zebrafish fed particular diets were more sensitive to pathogen infection in terms of changes in gut microbial diversity than fish fed other diets. Together, this work demonstrates that diet drives the successional development of the gut microbiome as well as its sensitivity to pathogen exposure, and potentially affects the interpretation of study outcomes. Consequently, investigators should carefully consider the role of diet in their microbiome investigations and scientific studies broadly, especially when integrating results across studies. Furthermore, our study underscores that a host's context (e.g., diet) can shape host-microbiome responses to environmental stressors.

In chapter 3, I longitudinally investigated whether the gut microbiome is resistant or resilient to the stressors of increased water temperature, parasitic helminth exposure, and their combination, and whether gut microbiome variation is linked to infection outcomes in order to investigate how these combined environmental stressors affect microbiome stability and host health outcomes [159]. Anthropogenic climate change is raising concerns about the potential of increased parasite burden on aquatic organisms driven by rising global water temperatures. Climate experts anticipate global water temperatures to increase between 1.6 and 6 degrees Celsius in the coming years [171,172]. These temperature shifts are already altering aquatic habitats and disrupting traditional migratory patterns and food harvesting practices across marine and freshwater ecosystems [173]. Furthermore, the warming waters create new opportunities for emergent diseases to proliferate in regions where they previously could not survive, expanding the geographic range of many parasitic organisms [172]. Pathogen expansion could threaten local ecosystems and food webs by disrupting native species that lack defenses against these newly arrived pathogens. Furthermore, the effects of pathogen expansion could cascade to affect

not only local ecosystems but also disrupt global food supply chains. In particular, it affects communities that rely heavily on aquatic resources for nutritional and economic sustenance. While some evidence has been conducted on the effects of rising water temperatures on host-associated microbiomes [82], to date, no study has investigated how rising water temperature and intestinal parasite exposure together shape both gut-microbiome dynamics and infection outcomes in an aquatic vertebrate host. Our analysis revealed that parasite exposure and water temperature independently alter gut microbiome diversity, where fish exposed to different temperature conditions showed distinct microbial community responses to parasitic helminth infection. I next investigated whether the microbiome's sensitivity to parasite infection varied as a function of water temperature. I found that increasing water temperature reduced *P. tomentosa* worm development and overall infection burden in zebrafish, suggesting that elevated temperatures may constrain parasite success through either direct thermal effects or temperature-mediated host-microbiome interactions. Together, our findings demonstrate that water temperature establishes the ecological context for the gut microbiome and significantly impacts how the microbial community responds to helminth infection, with direct implications for host health in a warming world. Additionally, these findings also challenge the common assumption that higher temperatures boost parasite success by demonstrating that temperature-driven changes in host-microbiome interactions can lead to outcomes that defy current expectations about the effects of climate change.

In chapter 4, I conducted a longitudinal experiment where I sequentially exposed eight cohorts of adult zebrafish to antibiotics, elevated temperature, or parasite exposure—individually or in combination—to investigate how stress history shapes host-microbiome system resilience and recovery. The mounting pressures of the Anthropocene create complex scenarios in which

organisms face multiple environmental stressors rather than isolated challenges [14,16]. Historically contingent events, defined as past exposures that alter community assembly and response to future perturbations [16], can fundamentally reshape host-microbiome system trajectories [110]. While historical contingency has been studied in environmental-associated microbial systems [174,175], little is known about whether vertebrate host-associated microbial communities' response to future perturbations depends on past stressor exposure, and if they do, for how long do these effects persist [110]. Moreover, the specific mechanisms underlying how prior stressors might buffer or exacerbate host-microbiome responses to subsequent challenges remain unclear [110]. Our analysis reveals five key findings that demonstrate the historical contingency of the host-microbiome system responses to a perturbation. First, host mortality increased with added stressors, with mortality rates escalating from 9.4% in fish with no stressors to 27.8% in those exposed to two stressors. These results suggest that multiple stressors may overwhelm the host's ability to maintain stability in the face of increasing stress. Second, the gut microbiome's response to parasite exposure is historically contingent, where prior antibiotic exposure amplifies parasite-associated taxonomic changes, heat stress alone produces minimal compositional shifts, and combined stressors create unique microbial assemblages that persist beyond stress cessation with elevated stochastic assembly processes. These findings suggest the microbiome's capacity to respond to a future perturbation or recover from prior stress exposure depends upon stress history. Third, host intestinal gene expression varies dramatically across exposure regimes, with parasite exposure alone triggering the largest transcriptional response (3,753 genes), prior heat stress nearly doubling these changes, and dual stressors markedly dampening the response. These transcriptional patterns suggest that stress history impacts the host's molecular response capacity, where prior stressors amplify or suppress the host's ability to

mount effective immune and metabolic responses to subsequent challenges. Fourth, multi-omic integration reveals stress-specific taxon-gene coupling patterns, where parasite exposure alone creates dense gene-taxon networks (11,681 correlations), prior stressors either enhance, redirect, or completely disrupt this coupling, and recovery phases show stress-specific patterns with the bacteria *Culicoidibacter* emerging as a key hub across all exposure regimes. Finally, mortality correlations identify putative health and disease indicators, with *Culicoidibacter* acting as a putative health indicator (negative correlation with mortality), while the microbial genera *Tundrisphaera* and *Cloacibacterium* serve as putative disease risk indicators (positive correlations with mortality). Together, this work demonstrates that historical contingency operates at multiple levels within host-microbiome systems, fundamentally shaping both resistance to future perturbations and resilience during recovery, with important implications for predicting organismal responses to the sequential stressors characteristic of the Anthropocene. Moreover, these findings suggest that stress history must be considered when predicting host-microbiome system resilience and recovery potential in an increasingly stressed world.

Dissertation Synthesis and Objectives

In summary, this dissertation deepens our understanding of host-microbiome stability by systematically testing how different dimensions of environmental stressors impact microbial community dynamics and host health. Chapter 2 shows that dietary context drives gut microbiome assembly and modulates the community's sensitivity to bacterial pathogen invasion. These findings expose an often-overlooked source of variation that can confound cross-study comparisons. Chapter 3 reveals that incremental warming can constrain parasite success through nonlinear, temperature-mediated host-microbiome interactions, thereby challenging the

prevailing expectation that higher temperatures will amplify parasitic infections. Chapter 4 introduces a longitudinal, multi-omic framework that uncovers historical contingency as a key determinant of future resistance and recovery, with stress history dictating host-microbiome response.

Taken together, the analytical approaches employed here map the multidimensional fabric of the host-microbiome system's contextual landscape—defined by nutrition, climate, and sequential stress—onto measurable shifts in microbial diversity, host transcription, and infection outcomes. Collectively, this work advances our fundamental understanding of how organisms navigate the shifting rhythms of environmental change, revealing that the dance between hosts and their microbial partners is not merely reactive but fundamentally shaped by the choreography of the environments they inhabit. As we face increasingly complex anthropogenic challenges, these insights provide essential frameworks for predicting and potentially mitigating the cascading effects of environmental stressors on host-microbiome systems across scales from individual health to ecosystem stability.

CHAPTER 2

DISENTANGLING THE LINK BETWEEN ZEBRAFISH DIET, GUT MICROBIOME SUCCESSION, AND *MYCOBACTERIUM CHELONAE* INFECTION

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ZIRC
100% REAL FLAKES

Abstract

Despite the long-established importance of zebrafish (Danio rerio) as a model organism and their increasing use in microbiome-targeted studies, relatively little is known about how husbandry practices involving diet impact the zebrafish gut microbiome. Given the microbiome's important role in mediating host physiology and the potential for diet to drive variation in microbiome composition, we sought to clarify how three different dietary formulations that are commonly used in zebrafish facilities impact the gut microbiome. We compared the composition of gut microbiomes in approximately 60 AB line adult (129- and 214-day-old) zebrafish fed each diet throughout their lifespan. Our analysis finds that diet has a substantial impact on the composition of the gut microbiome in adult fish, and that diet also impacts the developmental variation in the gut microbiome. We further evaluated how 214-day-old fish microbiome compositions respond to exposure of a common laboratory pathogen, Mycobacterium chelonae, and whether these responses differ as a function of diet. Our analysis finds that diet determines the manner in which the zebrafish gut microbiome responds to M. chelonae exposure, especially for moderate and low abundance taxa. Moreover, histopathological analysis finds that male fish fed different diets are differentially infected by M. chelonae. Overall, our results indicate that diet drives the successional development of the gut microbiome as well as its sensitivity to exogenous exposure. Consequently, investigators should carefully consider the role of diet in their microbiome zebrafish investigations, especially when integrating results across studies that vary by diet.

Introduction

In the effort to understand how the gut microbiome mediates vertebrate health, zebrafish (*Danio rerio*) have emerged as an important microbiome experimental model organism[139]. Despite the increasing use of zebrafish in microbiome research, key knowledge gaps remain about how different zebrafish husbandry practices, especially diet, influences microbiome composition[164,176]. For example, in contrast to mice, zebrafish do not have a standard reference diet[167]. Instead, zebrafish research facilities vary by dietary husbandry practice, which can impact physiological and reproductive outcomes[166,177,178]. Diet plays an important role in shaping the composition of the gut microbiome in humans and across vertebrate and invertebrate animal models, such as mice and honeybees[168,179–183]. Therefore, we hypothesize that variation in dietary husbandry practice also impacts the composition of the zebrafish gut microbiome. Quantifying this association is important because it could explain why, despite the existence of a core gut microbiome, gut microbiome composition differs across research facilities[144,145], improve efforts to integrate data across investigations, and clarify how dietary variation manifests as physiological variation.

Relatively little is known about how variation in dietary husbandry practice impacts the zebrafish gut microbiome. Prior studies that measured the impact of diet on the zebrafish gut microbiome have largely considered how substantial variation in specific macronutrients impacts the gut microbiome (e.g., high fat versus low fat diets)[177,184–186]. This variation is not typically representative of the variation in nutrient content observed across standard dietary husbandry practices[166,167]. Additionally, these studies have typically reared fish on a singular diet up to the point of experimentation, at which point fish are exposed to alternative diets. While insightful about acute effects, such experimental designs do not model the chronic dietary exposure that fish experience through husbandry. This prior work also does not typically

consider how diet impacts the microbiome at different fish developmental periods, or whether dietary variation affects other characteristics of the gut microbiome, such as its sensitivity (i.e., changes in community composition) in response to exogenous agents (e.g., pathogens).

In this study, we sought to determine how the gut microbiome of early adult (129 days post fertilization, dpf) and fully mature (214 dpf) zebrafish is influenced by rearing them on different common facility diets. To do so, we reared fish throughout their lifespan on one of three different diets: fish were fed either (1) the Gemma (Skretting, Fontaine-les-Vervins, France) diet, which is a commercial feed widely used in zebrafish research facilities, (2) the ZIRC diet, a compound diet mixed and adopted by the Zebrafish International Research Center (ZIRC), which is one of the largest zebrafish stock centers in the world, or (3) a precisely defined laboratory grade diet developed by Watts[166]. Overall, these diets are relatively similar from a macronutrient perspective, though they differ by formulation, ingredient sourcing, manufacturing process details, and consequently also by exact nutritional content. (Table S4.1.1). In particular, we evaluated how the gut microbiome differed across these groups of fish as well as over development. We also determined if these differences in the microbiome link to variation in fish body size (weight and body condition score length normalized measure of weight). Lastly, we determined if fish fed different diets manifested differences in extraintestinal infection outcomes to one of the most common infection agents of zebrafish research facilities, *Mycobacterium chelonae*[151], as well as how the gut microbiome of fish fed different diets responds *M. chelonae* exposure.

Results

Diet differentially influences physiology and gut microbiome at 129 days post fertilization

To determine how common zebrafish diets differently impact fish size (weight and body condition score) and the gut microbiome, we reared 179 zebrafish that were assigned one of three diets from 30- to 214 days-post fertilization (dpf; Figure 1): Gemma, Watts and ZIRC diets. Prior to diet assignment, fish were fed a nursery diet (see methods). At 129 dpf, we selected 89 individuals across these three cohorts and collected fecal samples from each fish for microbiome profiling prior to measuring their weight and body condition score (BCS). Wilcoxon Signed-Rank Tests found that diet and sex significantly associated with weight and BCS (Figure 2A & B). Female fish had higher weight ($Z = 1{,}530$, $P < 0.001$; Table S1.1.2) and BCS ($Z = 1{,}631$, $P < 0.001$; Table S1.1.4) compared to males. Between the three diets, ZIRC-diet fed fish had the highest mean BCS compared to fish fed Gemma- ($Z = 150$, $P < 0.001$; Figure 2B) and Watts-diet ($Z = 197$, $P < 0.001$; Table S1.1.3). Gemma- and Watts-diet fed fish did not significantly differ from one another in terms of weight and BCS. These results indicate that ZIRC-diet contributes to heavier fish compared to Gemma- and Watts-diet fed fish.

We next built generalized linear models (GLM) to determine if diet associated with variation in one of three measures of microbiome alpha-diversity: richness, Simpson's Index, and Shannon Entropy. An ANOVA test of these GLMs revealed that alpha-diversity varies as a function of diet for all three measures of diversity we assessed ($P < 0.05$; Figure 2C; Table S1.2.1). A post hoc Tukey test clarified that ZIRC- and Watts-diet fed fish exhibited significant differences in alpha-diversity as measured by richness and Shannon Entropy ($P < 0.001$, Table S1.2.2). Moreover, we observed significant differences in diversity between Gemma- and Watts-diet fed fish in terms of richness ($P < 0.001$; Table S1.2.2), and between Gemma- and ZIRC-diet fed fish when considering the Simpson's Index ($P < 0.001$; Table S1.2.2). These results indicate

that diet associates with fish gut microbiome diversity, and that diet may differentially impact rare and abundant microbial members of the gut.

To evaluate how diet associates with microbiome community composition, we quantified the Bray-Curtis, Canberra and Sørensen dissimilarity amongst all samples. We detected a significant clustering of microbial gut community composition based on diet as measured by all beta-diversity metrics (PERMANOVA, $P < 0.05$; Figure 2D, Table S1.3.1). These results indicate that microbial communities of fish fed the same diet are more consistent in composition to one another than to fish fed other diets. Additionally, we assessed beta-dispersion, a measure of variance, in the gut microbiome community compositions for each diet group. We find the beta-dispersion levels were significantly different between the diet groups as measured by Bray-Curtis and Canberra metrics ($P < 0.05$; Table S1.4.1). Beta-dispersion levels were significantly reduced in Gemma-diet fed fish compared to Watts-diet fed fish when measured by Bray-Curtis metric, as well as significantly reduced compared to Watts- and ZIRC-diet fed fish when measured by Canberra metric (Table S1.4.1). These results indicate that Gemma-diet fed fish are more consistent in community composition than Watts- and ZIRC-diet fed fish at 129 dpf. Collectively, these results indicate that 129 dpf fish gut microbiome communities stratify by diet, but the composition of these microbial communities differ in consistency depending on diet.

Finally, to better understand the interactions between the diet and the members of the gut microbiome community, we quantified differential abundance using ANCOM-BC2. We observed 24 significantly abundant taxa at the genus level in at least one of the three diets (Table S1.5.1). Gemma-diet fed fish were enriched for *Chitinibacter* and were depleted of *Aeromonas* and *Flavobacterium*. Watts-diet fed fish enriched for *Flavobacterium*, *ZOR0006*, *Peptostreptococcus*, *Cetobacterium*, *Tabrizicola*, *Cellvibrio*, and unnamed genera of

Microscillaceae and *Chitinibacteraceae*, and depleted of *Crenobacter* and a *Sutterellaceae* genus. ZIRC-diet fed fish enriched for *Cloacibacterium* and *Acinetobacter*, and depleted of *Fluviicola*. Many of these taxa are identified as common members of the zebrafish gut microbiome[144,145]. These results indicate that diet differentially supports particular members of the zebrafish microbiome community.

Diet impacts the successional development of the zebrafish gut microbiome

To determine how maintaining fish on different diets impacts the development of the gut microbiome, we continued to grow fish from the same diet cohorts until 214 days post fertilization (dpf; Figure 1). Microbiome samples were collected from cohort members prior to quantification of fish weight and body condition score. To determine the effect of diet on the body condition score and the gut microbiome of 214 dpf fish, we conducted the same analyses as we applied to the 129 dpf fish. At 214 dpf, we find body condition score is significantly associated with diet ($P < 0.05$; Table S2.1.3.1). Additionally, linear regression analyses revealed statistically significant main effects of diet on gut microbiome alpha- and beta-diversity for all metrics we considered ($P < 0.05$; Figure 3A & B, Table S2.1.3.2-3). Furthermore, an ANOVA test of beta dispersion found significant levels of dispersion as measured by the Canberra metric ($P < 0.05$; Table S2.1.3.4), but the Bray-Curtis and Sørensen metrics did not reach our threshold for significance ($P > 0.05$; Table S2.1.3.4). These results demonstrate that diet impacts the physiology and gut microbiome of 214 dpf fish.

Next, we compared our results between the 129- and 214 dpf fish to determine how diet impacts the successional development of the gut microbiome. Linear regression revealed microbial gut

alpha-diversity was significantly associated with the main effect of time ($P < 0.05$; Table S2.2.1) for each diversity metric. However, we did not find a diet dependent effect on time for any alpha-diversity metric we assessed ($P > 0.05$; Table S2.2.1). A post hoc Tukey test clarified that microbiome diversity was significantly different between 129- and 214 dpf Gemma- and ZIRC-diet fed fish as measured by the Shannon and Simpson's alpha-diversity metrics ($P < 0.05$; Figure 3C, Table S2.2.2), but we did not find a statistically significant association between 129- and 214 dpf Watts-diet fed fish with any alpha-diversity metric ($P > 0.05$; Table S2.2.2). These results indicate that the alpha-diversity of the gut microbiome of Watts-diet fed fish were temporally stable, while Gemma- and ZIRC-diet fed fish diversified over time in diet-consistent ways.

A PERMANOVA test of the 129- and 214 dpf samples using the Bray-Curtis dissimilarity metric revealed that community composition was best explained by diet ($P < 0.05$; Figure 3D, Table S2.3.1), but an analysis using the Canberra measure found that variation in microbiome composition was best explained by time ($P < 0.05$; Figure 3E, Table S2.3.2). Given how these metrics weigh the importance of abundant versus rarer taxa, respectively, these results indicate that abundant members of the microbiome community are more sensitive (i.e., exhibit greater amounts of change) to the effects of diet, while rarer community members are sensitive to the effects of time. Moreover, we found beta-dispersion levels were significantly elevated between 129- and 214 dpf Gemma-diet fish when considering the Bray-Curtis and Sørensen metrics, in Watts-diet fed fish when considering the Canberra and Sørensen metrics, and in ZIRC-diet fed fish across all three beta-diversity metrics ($P < 0.05$; Table S2.4.1-3). These results indicate that abundant and rarer gut microbiome community members were differentially

impacted by the effects of time depending on diet. Collectively, these results indicate that diet can have a substantial impact on how the gut microbiome successionally develops in zebrafish.

Differential abundance analysis revealed taxa that were significantly associated with the effects of time and diet in one of the diet groups (Table S2.5.1). Across all three diets, the taxa that were more abundant included *Fluviicola*, *Macellibacteroides*, *Bacteroides* and an unnamed genus in the *Barnesiellaceae* family were, while taxa that were less abundant included *Phreatobacter* and *Flavobacterium*. These results indicate that irrespective of diet, the abundances of taxa change over the course of zebrafish development. We also measured how taxon abundance changed over time within each diet (Figure S2.5.2-46.2.5). The Gemma-diet fed fish uniquely enriched for *Exiguobacterium* (Table S2.5.2). *Exiguobacterium* are gram-positive facultative anaerobes in the phylum Bacillota, and are linked to fatty acid metabolism in zebrafish [187,188]. The Watts-diet fed fish were uniquely depleted of *Gemmobacter* (Table S2.5.3). Previous work has found that *Gemmobacter* has a positive association with parasite exposure in infected zebrafish[141,189]. The ZIRC-diet fed fish were uniquely enriched for *Pseudomonas* and *Haliscomenobacter* (Table S2.5.4). *Pseudomonas* is a common member of the gut microbiome and associated with fatty acid metabolism in zebrafish[187]. Less is known about the *Haliscomenobacter* genus, but an analysis of its genome revealed it is an aerobic chemoorganotroph found in aquatic systems [190]. Together, these results indicate that particular members of the gut microbiome associate with diet and zebrafish development.

To determine if fish size associated with diet across zebrafish development, we used Wilcoxon Signed-Ranks Tests to identify parameters that best explained the variation in body condition score (BCS) between 129- and 214 dpf fish. At 129 dpf, the BCS significantly differed between fish fed different diets ($P < 0.05$; Table S2.1.1). However, we did not find that BCS of

fish were impacted by time ($P > 0.05$; Table S2.1.1). These results indicate that while fish differ in BCS between diets at 214 dpf, their weight and length grow proportionally at a similar rate from 129- and 214 dpf. Interestingly, we observed a significant negative association of BCS and microbial gut diversity uniquely in fish fed the ZIRC diet as measured by Shannon Entropy and Simpson's Index ($P < 0.05$; Figure 3F, Table S2.1.2.1). This result indicates that fish gut microbiomes with higher body condition scores are lower in diversity compared to fish with lower body condition scores. For Canberra and Sørensen beta-diversity metrics, there were significant main effects of body condition score, and significant interaction effects between BCS and diet ($P < 0.05$; Table S2.1.2.2). However, the model coefficient for the effect of body condition score and its interaction with diet is far smaller than the coefficient for the effect of diet (Table S2.1.2.2). We did not find a significant association between BCS and specific taxon abundance (Table S2.1.2.2). Collectively, these results indicate that while the gut microbiome's composition associates with BCS, the effect of diet on the gut microbiome is much stronger.

Fish fed different diets are differentially infected by *Mycobacterium chelonae*

Next, we sought to determine how zebrafish respond to the common pathogen of zebrafish, *Mycobacterium chelonae*. Mycobacteria has been reported in zebrafish from about 40% of research facilities and is a major driver of mortality across research facilities [155]. *Mycobacterium chelonae* infection is usually only diagnosed by histology, and hence is only diagnosed to the genus level based on the presence of acid-fast bacteria. When species identifications are made using molecular methods, the identification is most frequently *M. chelonae*[191]. It is hypothesized to be introduced through diet early in life[155,163,192]. *M. chelonae* forms granulomas coelomic organs, swim bladder and kidney, and in many cases, it

ultimately causes death. Despite the extensive research into the pathogenesis of *M. chelonae*, very little is known about the factors that determine infection outcomes. Diet has been hypothesized to influence infections and may be a currently cryptic determinant of *M. chelonae* infection[163]. To clarify whether diet affects *M. chelonae* infection, we injected *M. chelonae* into the coelomic cavities of fish from each diet cohort at 129 dpf following fecal collection. These *M. chelonae* injected fish comprised the pathogen exposure cohort for this experiment, which we compared to the remaining, unexposed cohort of fish in our subsequent microbiome analyses. At 214 dpf, we performed a histopathological analysis of intestinal tissue to assess infection rate, and measured body condition score. These 214 dpf fish that were exposed to *M. chelonae* were utilized to determine if infection rates differ across fish as a function of diet.

We first evaluated whether diet impacted infection outcomes, as determined by histological confirmation of infection 3.5 months following pathogen injection. We conducted a Chi-Square test to compare the infection count between fish fed the three diets. The results showed that there was a statistically significant difference in infection rates between the groups ($X2 = 11.519$, $df = 2$, $N = 66$, $P < 0.05$; Table S3.1.1). Across all three diets, all females had infected ovaries (Figure 4C), indicating there is no diet-driven difference in infection rates for female fish. As a result, we verified that the differential infection rates across diet groups was driven by male fish in a follow-up Chi-Square test using only male fish. This analysis confirms that infection rates are statistically different between male fish fed the ZIRC diet as compared to male fish fed either the Gemma or Watts diets ($X2 = 11.556$, $df = 2$, $N = 53$, $P < 0.05$; Figure 4D, Table S3.1.2). Because we obtained infection data from all injected fish and corresponding controls reared in our study, whereas we produced microbiome data from only a subset of these fish, we also determined whether this pattern holds across the subset of male fish for which we

also have microbiome data. This Chi-Square test finds no significant effect, ($X2 = 4.069$, $df = 2$, $N = 44$, $P > 0.05$; Table S3.1.3.1-2), likely due to being underpowered to detect infection rate differences on this relatively small subset of the data. Infections in males included the testis (Figure 4B), coelomic cavity, swim bladder and kidney. We observed colonization of the intestinal lumen by acid fast bacteria across all three diets in both male and female fish (Figure 4A). A linear regression did not find evidence of an association between extraintestinal infection of zebrafish and body condition score across all fish ($P > 0.05$; Table S3.1.4.1), even when considering sex ($P > 0.05$; Table S3.1.4.2). Taken together, these results indicate that the diet impacts *M. chelonae* infection outcomes in zebrafish, but not in a way that manifests as differences in body condition score (i.e., fish size).

Diet influences gut microbiome's sensitivity to pathogen exposure

Lastly, we sought to determine whether the gut microbiome changes in response to *Mycobacterium chelonae* infection. *M. chelonae* infections can introduce inconsistencies in study outcomes, but the impacts on the gut microbiome are not known[155]. Prior to pathogen infection at 129 dpf, we collected fecal samples of a subset of fish for microbiome analysis. At 214 dpf, we collected fecal samples from control and pathogen exposed fish. The 129- and 214 dpf fecal samples were then measured for microbial gut diversity, composition, and taxon abundance. We next built generalized linear models (GLM) to determine if extraintestinal infection as a function of diet associated with microbial diversity and composition measures. We did not observe any significant associations between extraintestinal infection and any of the gut microbiome diversity and composition measures ($P > 0.05$; Table S3.1.5-6), likely because we

were under powered to detect a difference. While not all fish that were injected with *M. chelonae* manifested evidence of an infection at 214 pdf, all of these fish were exposed to the pathogen. Since gut microbiomes in zebrafish diversify in response to pathogen exposure, we next asked how exposure to *M. chelonae* affects the zebrafish gut microbiome and whether these effects differ across diets[141]. Comparing exposed to unexposed fish found that microbial gut diversity significantly differs between exposure groups as measured by richness and Shannon Entropy alpha-diversity metrics ($P < 0.05$; Figure 5A, Table S3.2.1). That said, based on linear regression, the impact of exposure on the gut microbiome alpha-diversity does not appear to differ as a function of diet, as the interaction term for these covariates did not yield a significant effect ($P > 0.05$; Table S3.2.1). Furthermore, we used a post hoc Tukey test to clarify whether microbial gut diversity of fish differed between exposure groups by diet. Unique to ZIRC-diet fed fish, we observed microbiome diversity differed in unexposed controls compared to exposed fish as measured by all alpha-diversity metrics ($P < 0.05$; Figure 5B, Table S3.2.2). Watts-diet fed fish differed in unexposed controls compared to exposed fish in terms of richness ($P < 0.05$, Table S3.2.2). These results suggest that the gut microbiome diversity of ZIRC-diet fed fish, and to some extent Watts-diet fed fish, are more impacted by the effects of *M. chelonae* exposure, but Gemma-diet fed fish are less impacted by pathogen exposure. While the gut microbiomes are impacted by the effects of pathogen exposure, we find the statistical effect of diet shaping the gut microbiome is an order of magnitude greater across all alpha-diversity metrics ($P < 0.05$, Table S3.2.1). Collectively, these results indicate that gut microbiome diversity is sensitive to *M. chelonae* exposure, but diet is the primary driver of gut microbiome diversity.

Next, we evaluated how pathogen exposure influenced microbial community composition across fish fed each diet. For each beta-diversity metric considered, PERMANOVA tests found

that the main effects of diet and pathogen exposure significantly explained the variation in microbiome composition, but that the main effect of diet was consistently larger than the effect of exposure ($P < 0.05$; Figure 5C, Table S3.3.1). Furthermore, a PERMANOVA test found that the model coefficient effect for the interaction of diet and pathogen exposure was statistically significant when considering Canberra and Sørensen beta-diversity metrics, however this effect was marginal as compared to the aforementioned main effects. Moreover, a pairwise analysis of beta-dispersion did not find significant levels of dispersion between exposed and unexposed fish within each diet ($P > 0.05$; Table S3.4.1-3). These results indicate that exposure to *M. chelonae* did not affect dispersion of the gut microbiome communities. Collectively, these results indicate that the gut microbiome is impacted by pathogen exposure, but that dietary effects tend to overwhelm the effects of pathogen exposure.

We also observed several microbiota that stratified exposed and unexposed groups of fish in both diet-robust and diet-dependent manners. Unexposed Gemma-diet fed fish were enriched for *Macellibacteroides* and *Aurantisolimonas* (Table S3.5.2), unexposed Watts-diet fed fish were enriched for an unnamed genus of *Barnesiellaceae*, *Fluviicola*, *Paucibacter*, and *Brevibacterium* (Table S3.5.3), and unexposed ZIRC-diet fed fish were enriched for *Macellibacteroides*, *Bacteroides*, *Mycobacterium* and unnamed genera of *Barnesiellaceae* and *Sutterelaceae* (Table S3.5.4). Across all the diets, the taxa that were more abundant in unexposed, control fish included *Macellibecateroides*, *Fluviicola*, *Bacteroides*, *Aurantisolimonas*, *Cerasicoccus*, and three unnamed genera of *Barnesiellaceae*, *Commonadaceae*, and *Sutterellaceae*. *Plesiomonas* were more abundant in exposed fish compared to controls (Table S3.5.1). These results indicate that pathogen exposure impacts the abundance of certain taxa within and across the diets. Next, to see if *Mycobacterium* species abundance differed from background, pre-exposure levels we

compared *Mycobacterium* abundance between pre-exposure and unexposed control fish to that of exposed fish within each diet. Unexposed Gemma- and ZIRC-diet fed fish had significantly higher abundances of *Mycobacterium* to exposed fish ($Q < 0.05$; Figure 5D, Table S3.5.5). Pre-exposed Watts-diet fed fish had significantly more *Mycobacterium* compared to pre-exposed Watts-diet fish, but they did not differ significantly from unexposed Watt-diet control fish. These results indicate that the abundance of taxa from the genus *Mycobacterium* changes in response to exposure to a pathogenic species in a diet-dependent manner.

Discussion

Zebrafish are an important emerging model organism for understanding the microbiome. Yet, there is little consistency across studies in terms of the husbandry practices used to conduct zebrafish microbiome experiments, especially in terms of diet. This lack of consistency likely stems from a dearth of knowledge about how different standard zebrafish diets impact study outcomes, both in terms of the gut microbiome's composition and the physiological endpoints of the host. Our study offers critical insight into how three standard zebrafish dietary formulations impacts these outcomes, finding that the zebrafish gut microbiome's development and response to pathogen exposure is more impacted by diet. These observations help clarify inconsistencies across studies, underscore the importance of considering diet when integrating data across investigations, and inform on efforts to develop standard approaches in zebrafish microbiome research.

We found that diet had a substantial impact on the structure of the gut microbiome in adult zebrafish. Previous research has found that diets with varying compositions of key macronutrients (e.g., protein, lipids, and fiber content) impacts zebrafish physiology and the gut

microbiome[94,95,142,166,184–186,193]. Moreover, diet's effect on restructuring the host's gut microbiome has been observed across an evolutionarily diverse array of vertebrate and invertebrate animal hosts[69,179–182]. However, the nutritional compositions used in these prior studies tend to vary considerably. In particular, the feeds our study considered are far more consistent in their composition than the diets that are typically included in studies of the effect of diet on the gut microbiome (e.g., high-fat v. low-fat diets). Moreover, a unique strength of our study is that fish were fed the same diets over the vast majority of their lifespan (30 to 214 dpf), which is more consistent with a standard husbandry approach that maintains fish on a specific diet than the relatively short-term exposures to different types of diet that are typically employed in related research. Because of these features of our experimental design, our work provides important clarity into how seemingly subtle differences in husbandry practice can result in substantial differences in the composition of the adult zebrafish gut microbiome.

We also found that diet impacts the developmental variation in the gut microbiome. Prior work investigating the successional development of the zebrafish gut microbiome has had inconsistent results; our efforts indicate that these inconsistencies may be attributable to the different diets utilized in these prior studies [95,142,186,193,193]. For instance, Stephens *et al*. used a variety of live and dry food diets and found that juvenile zebrafish gut microbiomes were highly diverse but declined with age[142], while Wong *et al*. found opposite results for juvenile zebrafish that were fed defined diets[186]. Furthermore, prior work indicates that early life variability of the gut microbiome could be a result of husbandry choices involving diet[94,95,193]. Despite differences in study duration, we find congruent trends in gut microbiome diversity to these previous studies when comparing sampling time points within similar developmental periods. However, comparing our results to prior studies is challenging

because of differences in sampling time points, varied diets used, and undisclosed diet information. It is worth nothing that while our fish were fed the same diet from 30 days onward, at 114 dpf fish in our study were switched from a juvenile formulation to an adult formulation of their respective diets. These formulations differed slightly in some diets (e.g., Gemma and Watts), but in others more substantially (e.g., ZIRC). These differences in formulation may contribute to the variability we observed in the gut microbiome between diets across zebrafish development. Despite these limitations, we found adult zebrafish fed diets of similar nutritional composition manifest distinct gut microbiome successional patterns in community compositions across adulthood. Future work should seek consistency in diet formulations and increase sampling time points throughout zebrafish development to further clarify the successional development of zebrafish gut microbiomes.

Finally, we observed that the gut microbiome of zebrafish were sensitive to pathogen exposure, but diet was the main driver of gut microbiome structure. We ensured all fish were exposed to the pathogen by injecting *Mycobacterium chelonae* into the coelomic cavities of the fish at 129 dpf. We found that presence of infection was not sufficient to explain associations with microbiome diversity or community composition, which is likely due to being under powered to detect them. Additionally, we did not observe an association between infection outcomes and body condition score (i.e., fish size), which aligns with prior work that did not observe effects of *M. chelonae* infection on fish size in a larger cohort of zebrafish[194]. Furthermore, we found infection by diet interactions on a larger number of individuals who were assessed for histopathology, but not with the subset of fish sampled for microbiome analysis. This indicates that having a sufficiently large sample size is important for observing infection effects on the gut microbiome. However, we found that gut microbiome diversification did not

change after exposure to *M. chelonae*, except in ZIRC-diet fed fish relative to their unexposed controls. We did find that fish fed different diets show differential infection outcomes and microbiome sensitivities to pathogen exposure, which may indicate that the diet driven microbiome differences are a defining factor in infection outcomes. Alternatively, certain diet-driven microbiome compositions may be more susceptible to perturbation, and thus may be more likely to yield dysbiosis following pathogen exposure compared to fish fed other diets. For husbandry purposes, these observations are important considerations regarding long-term health management of fish, especially given that mycobacteriosis is the second most common infection in zebrafish research facilities (over 35% of all facilities). Additionally, *M. chelonae* is thought to drive non-protocol induced variation in zebrafish studies possibly as a result of dysbiosis, which can undercut experimental conclusions[153].

Our results contrast our prior work that found exposure to an intestinal helminth was associated with an increase in microbiome diversity[141]. One possible explanation for this discrepancy is our prior study investigated an intestinal helminth which may have different impacts on the gut microbiome associated with differences in intestinal lesion to that of a pathogenic bacterial species. For example, the nematode *Pseudocapillaria tomentosa* penetrates the intestinal epithelium and causes profound pathologic changes[141], whereas disease caused by *Mycobacterium* species in zebrafish are characterized by extraintestinal infections and lesions [155]. *Mycobacterium* spp. in zebrafish are hypothesized to be introduced early in life through ingestion, including diet[163,195], while fish in our study were exposed by injection into their coelomic cavities at adulthood when their gut microbiomes have been firmly established. Priority effects may have hindered the injected species of *Mycobacterium* from more substantially altering the gut microbiome at adulthood than if it had been introduced through a natural route

during early life microbiome assembly [28, 36]. Future work should consider using a natural mode of infection and exposing fish to a variety of pathogens to elucidate the gut microbiome's role in mediating pathogen exposure. Furthermore, because we found that the effect of diet was far greater than pathogen exposure on shaping the gut microbiome, future studies must consider diet effects, as they may overwhelm infection effects.

In conclusion, we found diet is one of the most important factors driving variation in the zebrafish gut microbiome. Unlike prior studies, including the extensive research conducted in mammalian models, that have evaluated dietary effects on the gut microbiome using diets that fundamentally differ in macronutrient composition, our work reveals that even relatively consistent diets that are commonly selected as normal husbandry practices elicit these large impacts on microbiome composition. While the zebrafish gut microbiome differs taxonomically from other animal systems, there is a substantial amount of shared functional capacity between zebrafish and mammalian gut microbiomes[148]. Consequently, the taxa-specific associations we found here may not directly translate to other animal systems, but the interactions between the microbiome, diet and pathogen exposure may be similar. Notably, our work used fecal samples, which may not appropriately reflect all members of the zebrafish gut microbiome, in particular mucosa associated taxa. Therefore, mucosal populations of microbiota may manifest different patterns with response to diet compared to taxa we observed in the fecal microbiota[196]. Further complicating investigations of diet's effect on the gut microbiome are inconsistencies within diets introduced through the manufacturing process that vary in ingredient sourcing and nutrient profile between batches [167]. Future work should illuminate the underlying mechanisms of the diet's influence on zebrafish development, gut microbiome structure and the microbiome's sensitivity to pathogen exposure. Collectively, our study

demonstrates that investigators should carefully consider the role of diet in their microbiome-targeted zebrafish investigations, especially when integrating results across studies that vary by diet.

Conclusions

Collectively, our study demonstrates the effect of commonly used laboratory diets on the gut microbiome of zebrafish. We reared zebrafish across their lifespan on three commonly used diets and analyzed the gut microbiome of juvenile and adult fish. Our findings demonstrate that diet impacts the developmental trajectories of the zebrafish gut microbiome, even with similar nutritional compositions. Additionally, diets were found to differentially sensitize the gut microbiome to pathogen exposure, and in the case of male fish result in different rates of infection. These results have important implications for the practice of zebrafish husbandry and the selection of diets in microbiome studies. Our findings will also contribute to ongoing discussions about standardizing husbandry practices, including diet, in the zebrafish research community.

Methods

Fish Husbandry

A total of 270 30 days post fertilization (dpf) AB line zebrafish were randomly divided into eighteen 2.8 L tanks (15 fish/tank) on a single pass flow-system tanks (15 fish/tank). During the experiment, temperature was recorded daily and ranged from 25.5-28.3°C, with the exception of two isolated overnight temperature drops below that range due to two separate power loss events that affected the source water sump heater. All other water conditions were monitored

weekly, pH ranged from 7.0-7.6, total ammonia ranged from 0-0.25 ppm (measured with pH and ammonia API test kits; Mars Fishcare North America Inc. Chalfont, PA), and conductivity ranged from 109 −166 microsiemens. Light in the vivarium was provided for 14 hours/day. One plastic aquatic plant piece approximately 6 inch in length was added to each tank for enrichment when fish were 129 dpf. A stock of similarly aged Casper line fish were maintained for the duration of the experiment, with a third of the stock being maintained on each of the diet regimens matching the AB line zebrafish. These fish served as filler fish and were added to the tanks after each histological sampling time point to maintain the 15 fish/tank ratio required to maintain the prescribed food-to-fish density per feeding as well as mitigate social stress effects on the fish. Casper fish were not sampled for microbiome or infection analyses.

Diets

Fish were all fed the same nursery diet until 30 dpf, a combination of paramecia, brine shrimp, and the ZIRC Nursery Mix: Zeigler AP Larval Diet (Ziegler Bros Inc., Gardners, PA) and freeze dried rotifers. Fish were then transferred to the OSU facility and assigned randomly to one of three juvenile diets: Gemma Micro 150/300 (Skretting, Fontaine-les-Vervins, France), Watts High-Fat Juvenile Mix, or ZIRC Juvenile Mix, twice daily (9 AM and 3 PM local time) until 60 dpf. From 60 dpf onward, OSU fish were not fed on weekends and 1-day holidays as per the facility institutional animal care and use protocol. The total quantity fed daily was 3% fish body weight. This continued until fish were 114 dpf and then they were transitioned to the adult version of their previously assigned juvenile diet: Gemma Micro 500 (Skretting, Fontaine-les-Vervins, France), Watts Low-Fat Adult Mix, or ZIRC Adult Mix, twice daily (9 AM and 3 PM local time), except weekends and 1-day holidays. The total quantity fed daily was 3% fish body

weight. The prescribed amounts of each diet regiment, for both the juvenile and adult diets were delivered by 3D printed spoons specific to the diet and stage of life. These spoons were paired with conical tubes retrofitted with leveling wires to ensure consistent feeding volumes as prescribed. All fish were only fed once, in the afternoons, on sampling days.

Diet and Pathogen Exposure

Each of the eighteen tanks was assigned one of the three diet regimens: Gemma, Watts, or ZIRC. There were three tank replicates per diet regimens for a total of nine tanks that were exposed to *M. chelonae* via intraperitoneal injection (3 tanks/diet with 15 fish/tank). The remaining nine tanks were similarly assigned to diet regimens and were exposed to a sterile 1X-phosphate buffered saline (PBS) solution via intraperitoneal injection. Each fish was injected with 10 µLof either the *M. chelonae* inoculum or saline solution. The injections were completed over the course of two days and the *M. chelonae* inoculum was prepared as a 0.5 McFarland each day with a target dose/fish of 5 X 10^4 viable bacteria/fish. This target dose was chosen as we have found that it induces a higher prevalence of *M. chelonae* in zebrafish with minimal mortality[154,194,197].

Day 1 *M. chelonae* inoculum was afterwards determined by plating to be 3.1x10^3 dose per fish, while Day 2 *M. chelonae* inoculum was determined by plating to be 1.0x10^5 dose per fish. For ZIRC and Gemma, two tanks for ZIRC fish were injected on Day 1, and 1 tank on Day 2. For Watts, one tank was injected on Day 1 (low dose) and 2 tanks were injected on Day 2 (high dose). No significant difference was observed in prevalence, so further analyses treated the exposed fish with in each diet group together.

Low and high dose across tanks:

- Gemma:
    - Low: Tank 14 and 35
    - High: Tank 26
- Watts
    - Low: Tank 6
    - High: Tank 12 and 33
- ZIRC
    - Low: Tank 7 and 10
    - High: Tank 4

Growth Parameters and Sex Determination

Growth and sex parameters were collected when fish were 129-130 and 213-214 dpf for interfacility comparison. Sex was determined by gross differences in morphology and confirmed by histology for all samples collected for disease severity evaluation. Following overnight fecal collection, individual fish would be placed in a pre-anesthetic solution of 50 ppm MS-222 prepared with Tricaine-S (Western Chemical Inc., Ferndale, WA; a subsidiary of Aquatic Life Sciences Inc.) briefly before being transferred to a 150 ppm MS-222 anesthetic solution in a Petri dish on centimeter grid paper to be photographed. Fish were photographed when immobile but still upright. Standard length and width were evaluated via photographs taken with an iPhone (Apple Inc., Cupertino, CA) and analyzed with ImageJ software (https://imagej.net). Weight was obtained while the fish was still under the effects of anesthesia by transferring them from the photography Petri dish to a Petri dish on a scale with a volume of tared fish water, with excess

water was removed. Body condition score is a length normalized metric of weight (for equation, see Methods) and serves as a general indicator of health in zebrafish and was calculated using the following equation:

- BCS = Weight (mg)/Length $(mm)^3$ x 100

Histopathology

Fish were euthanized by hypothermia preserved in Dietrich's solution, processed, and slides stained with Kinyoun's acid-fast[198]. Fish were processed into mid-sagittal sections as previously described[199]. Infection in fish were scored as positive when acid fast bacilli were observed in extra-intestinal organs[199]. A Chi-square test was used to compare positive and negative infections between fish fed each diet.

Fecal Collection

Five fish from each tank at 129- and 214-days post fertilization sampling time points were randomly selected for fecal sampling. Fecal material was collected from individual fish at the same sample intervals as outlined for the growth parameters. Fecal collection was set up the day before growth parameter sampling. Fish were transferred to 1.4 L tanks (1 fish/tank) containing ~0.4 L of fish water at least 30 minutes after the last feeding of the day. Fish were left to defecate overnight and all fecal material was collected from each tank the following morning in a 1.5ml microcentrifuge tube. Fecal samples were immediately spun at 10k rpm for 2 minutes, excess tank water was removed, and samples were snap frozen on dry ice and stored at -80 ˚C until processing.

16S Sequencing

Microbial DNA was extracted from zebrafish fecal samples and 16S rRNA gene sequence libraries were produced and analyzed following established approaches[200]. Briefly, the DNeasy PowerSoil Pro DNA kits (Qiagen) were used to extract and purify DNA. The V4 region of the 16S rRNA gene was PCR amplified using the Earth Microbiome Project 16S index primers and protocols (Walters et al., 2016). PCR products were visualized on a 1.5% agarose gel and quantified on a Qubit 2.0 (Thermofisher Scientific) using the Qubit dsDNA HS Assay. One hundred nanograms of PCR product for each DNA sample was pooled and cleaned using the QIAquick PCR Purification Kit (Qiagen). The quality of the pooled library was verified on the Agilent TapeStation 4200. The prepared library was submitted to the Oregon State University Center for Quantitative Life Sciences (CQLS) for 300 bp paired-end sequencing on an Illumina MiSeq System (RRID:SCR_016379).

Statistical Analysis

All microbiome DNA sequence analyses and visualizations were conducted in R (v 4.2.1)[201]. Fastq files were processed in using the DADA2 R package (v 1.18.0)[202]. Briefly, forward and reverse reads were trimmed at 250 and 225 bp, respectively, subsequently merged into contigs, and subject to amplicon sequence variant (ASV) identification. ASVs unannotated at the Phylum level were removed to result in 2029 remaining detected ASVs. We used Wilcoxon Signed-Ranks Tests to identify parameters that best explained the variation in weight and body condition scores. Alpha-diversity was calculated using the estimate_richness function (Phyloseq v 1.38.0) and transformed using Tukey's Ladder of Powers using methods described previously[200]. After transformation, scores were normalized from 0 to 1 by dividing each

score by the maximum value, which allowed us to compare results across alpha-diversity metrics using general linear models (GLMs). Post hoc Tukey Tests evaluated pairwise comparisons of models using multcomp (v1.4-2) glht function[203]. We corrected for multiple tests using Benjamini-Hochberg correction[204]. Two-way ANOVA was used to determine if the expanded models of these GLMs significantly improved the response variable relative to the null model. Beta-diversity models were generated using methods described previously[200]. Briefly, we evaluated three beta-diversity metrics—Bray-Curtis, Canberra, and Sørensen and resolved the relationship between experimental parameters and beta-diversity by applying a step-wise model selection approach as implemented in the capscale function (vegan package v 2.5)[205]. Optimal models were subsequently subject to PERMANOVA analysis to determine if the selected model parameters significantly explained the variation in microbiome composition across samples. Differential abundance was measured using ANCOM-BC (v 2.0.1)[206].

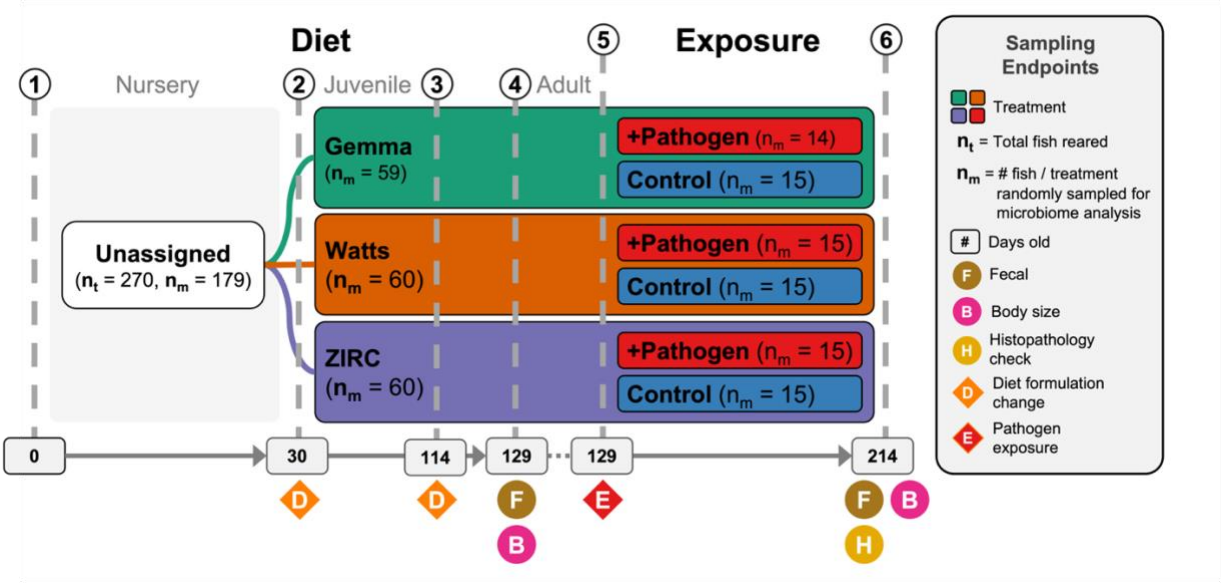


Figure 1: Experimental design showing treatments and husbandry events during the course of the study.

Symbols indicate when an event occurred. **1)** 270 fish were reared from 0 to 30 days post fertilization (dpf) on a nursery diet across 18 tanks (15 fish per tank). **2)** At 30 dpf, fish were assigned one of three diets (e.g., Gemma, Watts, or ZIRC), and fed a juvenile formulation until 114 dpf. **3)** At 114 dpf, fish were switched to an adult formulation of their respective diets. **4)** At 129 dpf, body size measurements were conducted on all fish and fecal samples were collected from a random selection of five fish per tank (n = 90). **5)** Afterwards, a cohort of fish from each diet were exposed to *Mycobacterium chelonae*. **6)** Three months later when fish were 214 dpf, body size measurements were conducted on all fish and fecal samples were collected from a random selection of five fish per tank (n = 89). Histopathology check was conducted to assess infection burden on all fish.

Figure 2: Effects of 129 days post fertilization (dpf) fish fed one of three diets (Gemma, Watts, or ZIRC) on physiology and microbiomes of zebrafish.

(A) Weight of ZIRC-diet fed fish significantly differs from Watts- and Gemma-diet fed fish. Gemma- and Watts-diet fed fish do not differ from each other. (B) Body condition score is a length normalized measure of weight. Fish fed the ZIRC diet have significantly higher body condition scores from fish. fed the Gemma and Watts diets. (C) Shannon Entropy of diversity shows that gut microbiome diversity significantly differs between Gemma- and Watts-diet fed fish, ZIRC- and Watts-diet fed fish, but not between Gemma- and ZIRC-diet fed fish. (D) Capscale ordination based on the Bray-Curtis dissimilarity of gut microbiome composition. The analysis shows that physiology and gut microbiome composition significantly differs between the diets. “ns” indicates not significantly different, *, **, *** indicates significant differences below the 0.05, 0.01, and 0.001 levels, respectively.

Figure 2: Effects of 129 days post fertilization (dpf) fish fed one of three diets (Gemma, Watts, or ZIRC) on physiology and microbiomes of zebrafish (continued)

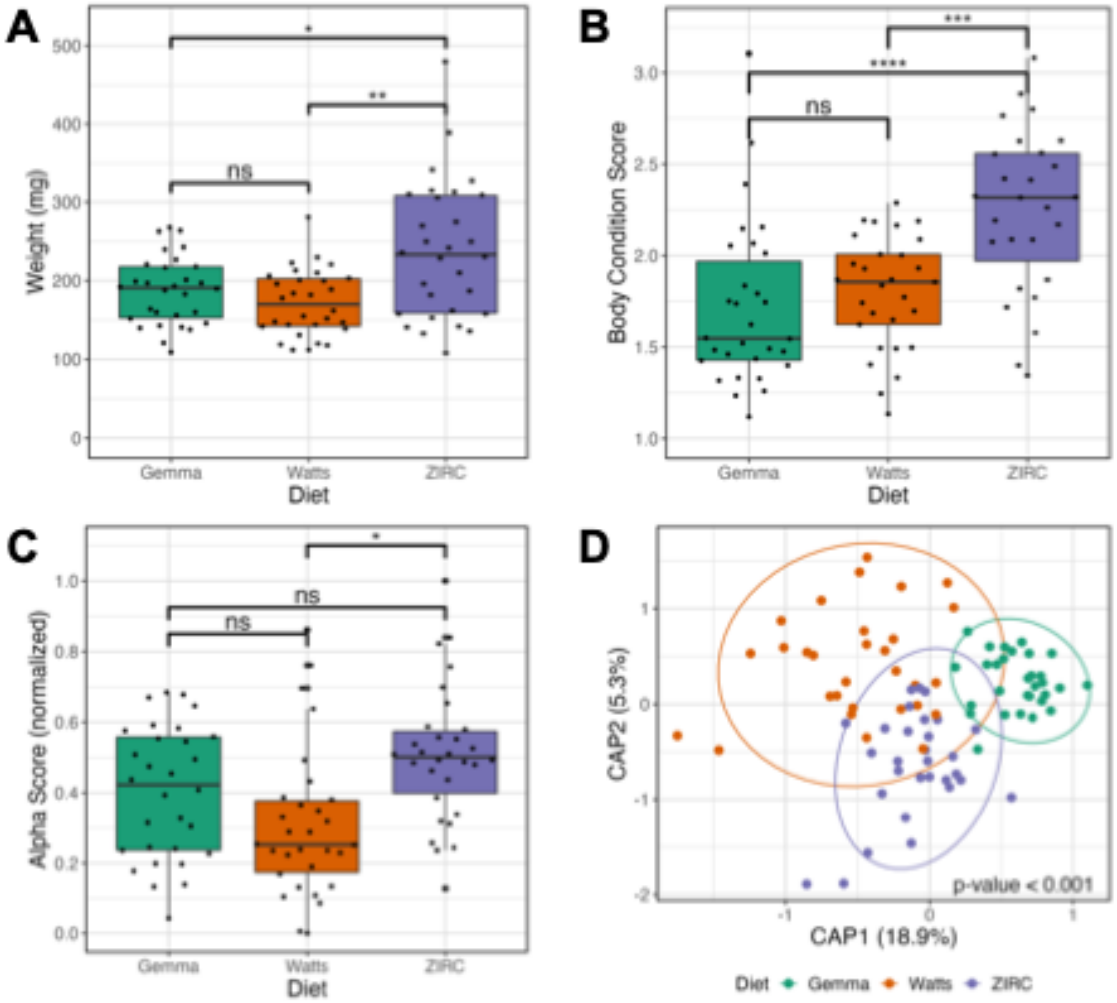
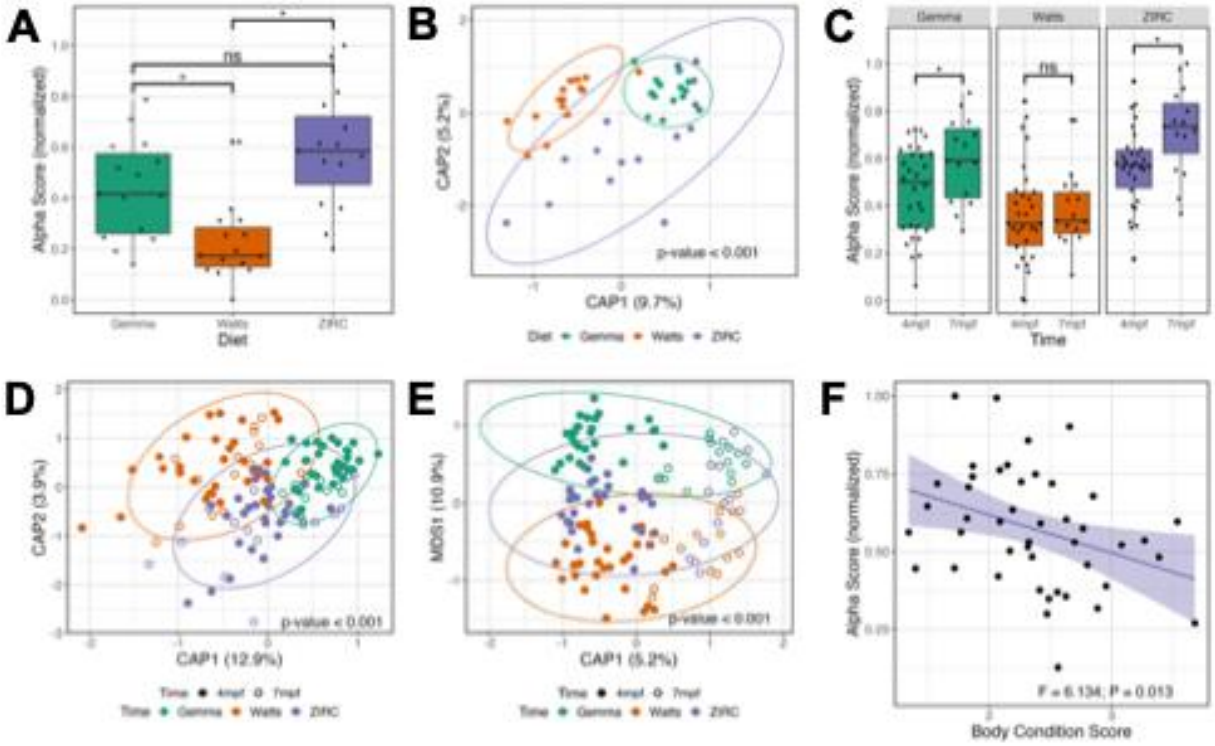


Figure 3: Development is associated with altered microbiome composition.

(A) Shannon Entropy of diversity shows that gut microbiome diversity significantly differs between Watts-diet fed fish to fish fed the Gemma- and ZIRC-diets in 214 days post fertilization (dpf) zebrafish. **(B)** Capscale ordination based on the Bray-Curtis dissimilarity of gut microbiome composition in 214 dpf zebrafish. **(C)** Shannon Entropy for diversity shows microbial gut diversity increases with development in 129- to 214 dpf zebrafish fed the Gemma- and ZIRC-diets, but not Watts-diet fed fish. Capscale ordination of gut microbiome composition based on the **(D)** Bray-Curtis dissimilarity by diet and **(E)** Canberra measure by time. **(F)** Body condition score negatively associates with gut microbiome diversity as measured by Simpson's Index across 129- and 214 dpf zebrafish fed the ZIRC diet. The analysis shows that fish size and gut microbiome composition significantly differs between the diets across development, and there may be diet-dependent link with physiology. A "ns" indicates not significantly different, "*" indicates significant differences below the 0.05 level.

Figure 3: Development is associated with altered microbiome composition (continued)



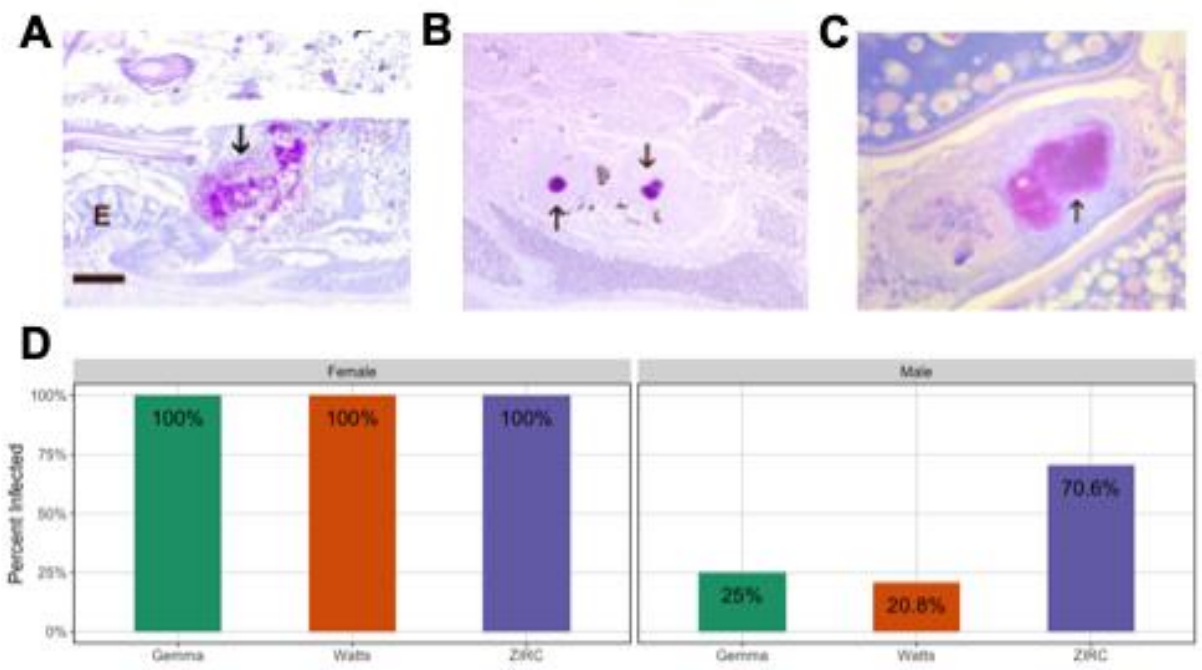


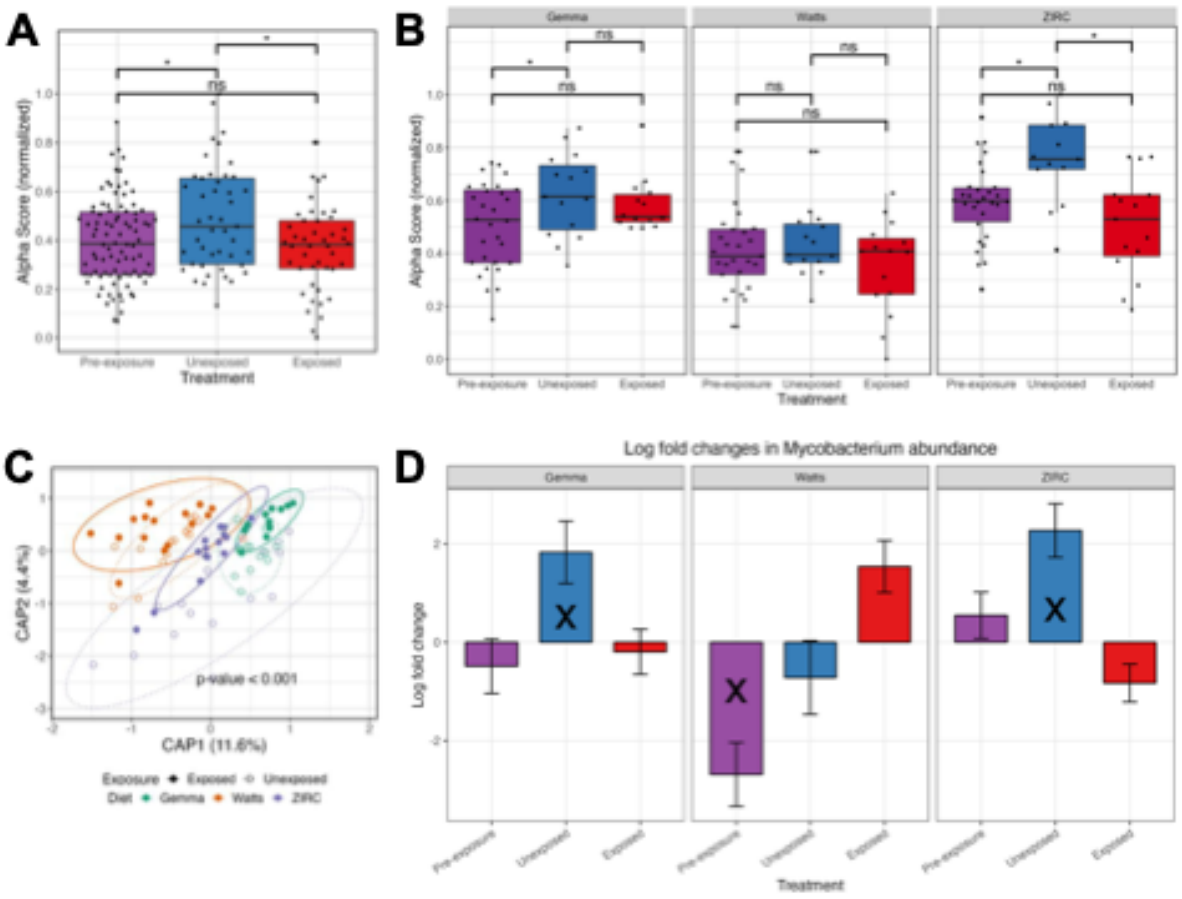
Figure 4: Histologic sections stained with Kinyon's acid fast stain in zebrafish exposed to *Mycobacterium chelonae* examined at 15 week post exposure.

Arrow = acid fast bacteria. **(A)** Bacteria in intestinal lumen (E = intestinal epithelium, Bar = 25 µm). **(B)** Testis with two granulomas with acid fast bacteria. **(C)** Ovary with two granulomas, one containing abundant acid fast bacteria. **(D)** Infection outcome analysis of male and female fish injected with *M. chelonae* (n = 66). All exposed female fish were positive for infections, but male fish differed in infection outcomes depending on diet.

Figure 5: Exposure to *Mycobacterium chelonae* inhibits diversification of gut microbiome.

(A) Shannon Index for diversity of pre-exposed 129 days post fertilization (dpf), 214 dpf exposed and unexposed fish, and **(B)** for exposure groups within each diet. Capscale ordination based on the Bray-Curtis dissimilarity of gut microbiome composition of fish by **(C)** diet. **(D)** Log fold change of *Mycobacterium* of pre-exposed, exposed and unexposed fish within each diet as calculated by ANCOM-BC. Values are in reference to exposed fish within each diet. The analysis shows gut microbiome's sensitivity to pathogen exposure is linked to diet, but *Mycobacterium's* abundance is diet-dependent. A "ns" indicates not significantly different, and * indicates significant differences below the 0.05. An "X" indicates a group is significantly differentially abundant compared to the exposed treatment reference group.

Figure 5: Exposure to Mycobacterium chelonae inhibits diversification of gut microbiome (continued)



CHAPTER 3

MODELING THE ZEBRAFISH GUT MICROBIOME'S RESISTANCE AND SENSITIVITY TO CLIMATE CHANGE AND PARASITE INFECTION

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Abstract

As climate change increases global water temperatures, ecologists expect intestinal helminth infection ranges to expand and increase the health burden on aquatic organisms. However, the gut microbiome can interact with these parasites to influence infection outcomes, raising the possibility that its response to increasing temperatures may help buffer against increased infection burden or worsen infection outcomes (e.g., inflammatory bowel disease). To evaluate this hypothesis, we sought to determine if the microbiome is resistant or resilient to the stressors of increased water temperature, helminth exposure, and their combination, and whether this variation linked to infection outcomes. We leveraged the zebrafish (Danio rerio) model organism to measure how these variables relate to the temporal dynamics of the gut microbiome. In particular, we exposed adult zebrafish to Pseudocapillaria tomentosa, parasitic capillarid with a direct life cycle, across three different water temperatures (28°C, 32°C, 35°C), and analyzed fecal microbiome samples at five time points across 42 days. Our findings show that parasite exposure and water temperature independently alter gut-microbiome diversity. Moreover, water temperature moderates the association between parasite infection and the gut microbiome. Consistent with this observation, yet counter to prevailing expectations, we find that increasing water temperature reduces P. tomentosa infection worm development and overall abundance in zebrafish. The decline in worm burden at 35°C may be due to either direct thermal inhibition of P. tomentosa development or temperature-mediated interactions with the host microbiome and immune response. Overall, our results indicate that water temperature alters the contextual landscape of the gut microbiome and shapes its response to an intestinal parasite in zebrafish. To our knowledge, this represents the first report of elevated temperature constraining nematode development in a fish host, underscoring that climate change may impose unanticipated, context-dependent impacts on vertebrate gut microbiomes and health outcomes.

Introduction

The steady increase in global temperatures due to climate change challenges vertebrate health [207]. These threats to vertebrate health take on many forms, including the expected expansion of infectious agents [172,208]. Of particular concern are the increased infection burdens faced by aquatic organisms experiencing increasing water temperatures [209]. Due in part to the varied coincident effects of climate, the impacts of a warming climate on aquatic organisms are anticipated to be nonuniform in effect [209,210] and vary biogeographically [209,211], which in turn complicates harm mitigation and conservation efforts [212]. Consequently, there's an urgent need to better understand climate change's contextual impacts on organisms depending on the unique environmental conditions of the ecosystems they inhabit.

In recent years researchers have considered that climate change may also elicit harm to vertebrates by disrupting the composition of their gut microbiome [27]. While prior work has shown that varying temperatures impacts gut microbiome composition across a variety of vertebrate host species [213], less is known about how coincident variables, such as parasite or pathogen exposure, collide with temperature to drive variation in the gut microbiome. Recent work in fish underscores that intestinal parasites alone can restructure community composition and host physiology, even without a thermal component [214–216]. Yet, to our knowledge, no study has investigated how rising temperature and intestinal parasite exposure together shape both gut-microbiome dynamics and infection outcomes in a vertebrate host. Whether warming amplifies parasite-induced dysbiosis, buffers the host via microbiome-mediated resistance, or simply constrains the parasite itself remains unknown. Filling this gap is critical for forecasting disease risk under climate change and for pinpointing microbiome traits that promote host resilience. These potential interaction effects are important to quantify, because it may be that they elicit even greater effects on the gut microbiome than anticipated by investigations of

temperature alone, and could possibly result in increased frequency of dysbiotic disorders. It's important to elucidate these interactions because increasing work points to the gut microbiome as a key determinant of whether vertebrate physiology is able to buffer against stress [213,217], and whether temperature induced perturbations to the gut microbiome may sensitize individuals to subsequent stressors [72].

To answer these questions, we evaluated the gut microbiome's temporal response to an exogenous stressor across a gradient of environmental conditions. To do so, we levered the zebrafish (*Danio rerio*) model organism to measure how gut microbiomes differ across fish reared to adulthood at one of three water temperature conditions (28°C, 32°C, or 35°C; Fig. 6). Zebrafish are highly thermal tolerant, capable of existing across a wide spectrum of temperature ranges from 4°C to 40°C [218], but living outside their thermal optimum can come at a physiological and microbial cost [218,219]. While much is known about the thermal range of zebrafish, the effects of altered water temperature on their gut microbiome structure has not been elucidated. We also sought to determine if water temperature affected how the microbiome and host responds to exposure to and infection by intestinal nematode *Pseudocapillaria tomentosa*, a common source of disease in aquariums, specifically zebrafish facilities [157,220–222]. *P. tomentosa* is known to cause high mortality and disrupt the gut microbiome [157,160]. Yet, it remains unclear whether and how water temperature mediates interactions between the host-microbiome system and *P. tomentosa* [222]. A key advantage *of P. tomentosa* is its direct life cycle capability, in which infective eggs larvate in ambient water and can be acquired orally by the host, without requiring an intermediate or paratenic host [157,223]. Although, *P. tomentosa* may use paratenic hosts (e.g., oligochaete worms) in natural settings, these are not required in the controlled laboratory conditions used here [157,222,223]. This feature enables us to disentangle

temperature effects on host-microbiome-parasite interactions from confounding mechanisms such as the temperature-sensitive loss of intermediate hosts that commonly constrain parasites with indirect life cycles [222,224]. Overall, our study sought to clarify the environmentally dependent context of a gut microbiome's resistance and sensitivity to climate change-relevant stressors.

Results

Water temperature shapes gut microbiome structure

To determine how zebrafish reared across a gradient of increasing water temperatures impacts the structure of the gut microbiome, we reared 260 zebrafish across one of three water temperatures (28°C, 32°C or 35°C) until 206 days-post fertilization (dpf; Fig. 6). Additionally, within each temperature cohort, fish were evenly divided into two additional treatment groups: either unexposed or exposed to the intestinal helminthic parasite *Pseudocapillaria tomentosa*. Microbiome samples were collected at five time points between 164 and 206 dpf. In the parasite exposed cohort, fish were exposed to *P. tomentosa* following microbiome sampling at 164 dpf, or 0 days post exposure (dpe). Four subsequent microbiome samples were collected at 14 dpe (178 dpf), 21 dpe (185 dpf), 28 dpe (192 dpf), and 42 dpe (206 dpf). Within the parasite unexposed fish cohort, we built generalized linear models (GLM) to determine if water temperature associated with variation in one of four measures of alpha-diversity: Simpson's Index, Shannon Entropy, richness, and phylogenetic diversity (Table S2A.1). An ANOVA test of these GLMs revealed that alpha-diversity varied as a function of temperature for all measures ($P<0.05$; Fig. 7A; Table S2A.2), except Shannon Entropy ($P>0.05$; Table S2A.2). A *post hoc* Tukey test clarified that alpha-diversity scores did not significantly differ between 28°C and

32°C water temperature reared fish for each diversity metric we measured ($P>0.05$; Table S2A.3). However, we observed significant differences in diversity between 28°C and 35°C water temperature reared fish across Simpson's Index, richness and phylogenetic alpha-diversity measures ($P<0.05$; Table S2A.2), and between 32°C and 35°C water temperature reared fish as measured by richness and phylogenetic diversity metrics ($P<0.05$; Table S2A.2). These results indicate that water temperature associates with fish gut microbiome diversity, and that water temperature may differentially impact particular microbial clades of the gut.

To evaluate how temperature associates with microbiome composition in parasite unexposed fish, we quantified dissimilarity amongst all samples and generated distance matrices using the Bray-Curtis, Canberra and half-weighted UniFrac distance metrics. Using permutational multivariate analysis of variance (PERMANOVA), we assessed whether increasing water temperatures explained variance in gut microbial community composition. A PERMANOVA test indicated that microbial communities were significantly stratified by water temperature as measured by all beta-diversity metrics (PERMANOVA, $P<0.05$; Fig. 7B; Table S2B.1). These results indicate that microbial communities of fish reared at the same water temperature are more consistent in composition to one another than fish reared at different water temperatures. Additionally, we assessed beta-dispersion, a measure of variance, in the gut microbiome community compositions for each water temperature group. We find the beta-dispersion levels did not significantly differ between the water temperature groups ($P>0.05$; Table S2B.2). These results indicate that fish reared at different water temperatures are consistent in community composition.

Next, we compared our results across five time points between 0- and 42 dpe to determine how water temperature impacts the successional trajectories of gut microbiome

diversity and composition. Linear regression revealed gut microbial alpha-diversity was significantly associated with the main effect of time for each alpha-diversity metric we assessed ($P<0.05$; Fig. 7C; Table S2C.1-2). Moreover, we found a temperature dependent effect on time as measured by richness and phylogenetic diversity metrics ($P<0.05$; Table S2C.1-2). A *post hoc* Tukey test clarified that microbiome diversity significantly differed between 0- and 42 dpe fish reared at 28°C as measured by richness and phylogenetic diversity ($P<0.05$; Table S2C.3), between 0- and 42 dpe fish reared at 32°C as measured by all alpha-diversity metrics ($P<0.05$; Table S2C.3), and between 0- and 42 dpe fish reared at 35°C as measured by Shannon Entropy and Simpson's Index ($P<0.05$; Table S2C.3). These results indicate that gut microbial alpha-diversity increases over time irrespective of water temperature.

A PERMANOVA test detected significant clustering of microbial gut community composition based on the interaction of water temperature and time as measured by all beta-diversity metrics (PERMANOVA, $P<0.05$; Fig. 7D; Table S2D.1). These results indicate that microbial communities of fish reared at the same water temperature are more consistent in composition to one another across time than fish reared at different water temperatures. Moreover, a pairwise analysis of beta-dispersion found significantly elevated levels of dispersion between fish reared across different temperatures and time as measured by all beta-diversity metrics ($P<0.05$; Table S2D.2). These results indicate that gut microbial community composition varies inconsistently between water temperature groups in a time-dependent manner. Collectively, these results indicate that zebrafish gut microbiomes communities stratify by temperature, and the trajectory of gut microbiome successional development varies depending on water temperature.

Infection burden is highest in fish reared at lower water temperatures

Next, we evaluated infection outcomes of zebrafish reared at different water temperatures and exposed to the intestinal helminthic parasite *Pseudocapillaria tomentosa*. To determine whether water temperature affects infection burden, we exposed zebrafish to 50 *P. tomentosa* eggs per liter of tank water at 164 days post-fertilization (dpf). Infection outcomes were assessed using wet mount and histological evaluation at 0, 14, 21, 28, and 42 days post-exposure (dpe). We built a negative binomial general linear model to compare infection burden (total worm counts) between fish reared at different water temperatures (Table S3B.1). The regression analysis found a statistically significant effect of temperature on infection burden ($P < 0.05$; Fig. 8A; Table S3A.2). However, we did not find a statistically significant interaction effect between water temperature and time on infection burden ($P > 0.05$; Table S3B.3).

Across time points, fish reared at 28°C exhibited the highest mean infection burden (4.86 worms per fish), followed by those at 32°C (3.6 worms per fish). Notably, at 14 dpe, fish at 32°C had a slightly higher infection burden (3.3 worms per fish) than those at 28°C (2.3 worms per fish). Tukey's *post hoc* test revealed that infection burden was significantly higher in fish reared at 28°C and 32°C compared to those at 35°C ($P < 0.05$; Fig. 8A; Table S3B.3). Only a single larval worm was detected by wet mount in two fish from the 35°C group, while histological examination revealed a slightly higher prevalence, with larval worms observed in 9 out of 32 fish at this temperature (Fig. 8B; Table S3B.1). These results indicate that infection burden is highest at lower water temperatures. Because *P. tomentosa* completes a direct life cycle with no intermediate host, this steep decline at 35 °C suggests a direct upper thermal limit on egg hatching or early larval survival in addition to. Alternatively, this could be driven by temperature-mediated changes in host immune response or microbiome resistance. We also

examined the development of mature female worms across temperature conditions. At 28°C, mature female worms were first detected at 28 dpe in 7 fish, whereas at 32°C, mature females were only observed in 4 fish. Interestingly, at 14 dpe, a single mature female was identified in a fish reared at 32°C, marking the earliest recorded instance of worm maturation at this temperature.

Additionally, we compared the sensitivity of infection detection between histology and wet mount methods on a subset of fish selected for microbiome analysis (n = 120; Fig. S3C; Table S3C.1). McNemar's test revealed significant differences in detection sensitivity under specific conditions. At 35°C and 21 dpe, histology identified significantly more infections than wet mount ($\chi^2 = 4.17$, $P < 0.05$; Fig. S3C; Table S3C.3), with 6 samples testing positive by histology alone compared to 0 by wet mount alone. No statistically significant differences were observed at other temperature and dpe combinations ($P > 0.05$; Table S3C.3). In cases where all samples were concordant (e.g., 28°C at 28 dpe and 35°C at 28 dpe), McNemar's test could not be computed due to the absence of discordant pairs. These findings suggest that histological methods may be more sensitive than wet mounts, particularly at higher temperatures and intermediate time points. Collectively, these results suggest that higher water temperatures may have a protective effect against infection burden, limiting worm establishment and development in zebrafish.

Gut microbiome response to parasite exposure varies across water temperature

To investigate how parasite exposure affects the gut microbiome under varying water temperatures, we analyzed fecal samples from exposed and control fish at multiple time points. *P. tomentosa* is known to alter the zebrafish gut microbiome [160], but it remains unclear how

increasing water temperatures affect this response. We collected fecal samples for microbiome analysis of fish in the parasite exposed cohort at 14-, 21-, 28-, and 42 dpe. Similar to our parasite unexposed fish microbiome analyses, we built generalized linear models (GLM) to determine if temperature, time or their combination associated with variation in measures of microbial diversity and composition of parasite exposed fish (Table S4A.1). An ANOVA test of these GLMs revealed that alpha-diversity varied as a function of temperature for all measures ($P<0.05$; Fig. 9A; Table S4A.2). A *post hoc* Tukey test clarified that gut microbial diversity between 28°C and 32°C water temperature reared fish significantly differed for all alpha-diversity metrics ($P<0.05$; Table S4A.3), and gut microbial diversity differed between 28°C and 35°C water temperature reared fish as measured by Simpson's Index. However, we did not find significant differences in gut microbial diversity between 32°C and 35°C water temperature reared fish for all alpha-diversity metrics, or between 28°C and 35°C water temperature reared fish as measured by Shannon Entropy, richness and phylogenetic diversity metrics. These results indicate that moderate increases in water temperature promotes gut microbial diversification in parasite exposed fish, but diversification of gut microbes plateaus in parasite exposed fish reared at higher water temperatures.

For each beta-diversity metrics we considered, PERMANOVA tests found that temperature significantly explained the variation in microbiome composition in parasite exposed fish (PERMANOVA, $P<0.05$; Fig. 9B; Table S4B.1). These results indicate that gut microbiome communities of parasite exposed fish reared at the same water temperature are more similar to each other than fish reared at different water temperatures. Additionally, we found beta-dispersion levels were significantly elevated between water temperature groups ($P<0.05$; Table S4B.2). A *post hoc* Tukey test clarified that beta-diversity dispersion levels were highest in fish

reared at 28°C, followed by fish reared at 32°C and 35°C water temperatures ($P<0.05$; Table S4B.3). These results indicate that that gut microbiome communities of parasite exposed fish reared at lower water temperatures are more inconsistent in composition than parasite exposed fish reared at higher water temperatures.

Next, we compared our results across five time points to evaluate how parasite exposure and water temperature impacted gut microbiome diversity and composition. An ANOVA test did not find significant main effects of time as measured by Shannon Entropy and Simpson's Index ($P>0.05$; Table S4C.2), but found marginally significant main effects of time as measured by richness and phylogenetic diversity ($P=0.064$ and $P=0.078$; Table S4C.2). Moreover, linear regression did not reveal significant interaction effects between temperature and time across all alpha-diversity metrics ($P>0.05$; Fig. 9C; Table S4C.2). These results indicate increasing water temperatures generally do not consistently impact microbial gut diversification over time in parasite exposed fish, and particular microbial clades appear more sensitive to the effects of time depending on temperature.

PERMANOVA tests found that community composition was best explained by the interaction effects between temperature and time using the Canberra beta-diversity metric (PERMANOVA, $P<0.05$; Fig. 9D; Table S4D.1), but a significant interaction effect was not observed using the Bray-Curtis and half-weighted UniFrac dissimilarity metrics ($P>0.05$; Table S4D.1). Given how these metrics weigh the importance of rarer (e.g., Canberra) versus abundant (e.g., Bray-Curtis) microbial community members, these results indicate that abundant members of the microbiome community are more robust to the effects of temperature across time in parasite exposed fish, while rarer taxa are more sensitive to the effects of time depending on temperature. Moreover, a pairwise analysis of beta-dispersion found significantly elevated levels

of dispersion between fish reared across different temperatures and time as measured by all beta-diversity metrics ($P<0.05$; Table S4D.2). These results indicate that parasite exposure inconsistently impacts gut microbial community composition across time depending on temperature ($P<0.05$; Table S4D.2). Collectively, these results indicate that parasite exposure can impact gut microbiome diversity and composition, and these impacts are greatest at lower temperatures.

Gut microbiome response has a non-linear relationship with infection burden

Given the differences we observed in gut microbiome diversity and composition across parasite exposed fish reared at different water temperatures, we further investigated whether gut microbiomes of parasite exposed fish vary depending on presence of infection and infection burden. Linear regression did not find significant main effects of presence of infection or significant interaction effects between presence of infection and temperature on gut microbial alpha-diversity for all metrics we measured ($P>0.05$; Fig 10A; Table S5A.1-2). These results indicate that gut microbial diversity did not differ as a function of presence of infection. Moreover, a PERMANOVA analysis found microbial community composition was best explained by presence of infection as measured by Canberra (PERMANOVA, $P<0.05$; Fig. 10B; Table S5B.1), but a significant result was not observed by the other beta-diversity metrics we measured. Additionally, we did not find statistically significant results between the interaction effects of water temperature and presence of infection on gut microbial community composition. These results indicate that rarer members of microbial communities of parasite exposed fish vary by presence or absence of infection, but abundant microbes do not. However, we did detect elevated levels of beta-dispersion across fish reared at different water temperatures depending on

presence of infection ($P<0.05$; Table S5B.2). These results indicate that gut microbiome composition inconsistently varies between fish depending on presence of infection and water temperature.

Next, we investigated how infection burden (i.e., number of intestinal worms detected) impacted parasite exposed fish gut microbiome diversity and composition. We used GLMs to determine if infection burden associated with variation in gut microbial alpha-diversity (Table S5C.1). An ANOVA test of these GLMs revealed that alpha-diversity varied as a function of infection burden as measured by Shannon Entropy and Simpson's Index ($P<0.05$; Table S5C.2.2), but the interaction effects between infection burden and water temperature did not significantly explain variation in alpha-diversity across all measures ($P>0.05$; Table S5C.2.2). These results indicate that gut microbial diversity varies as a function of parasitic worm count. A PERMANOVA analysis found microbial community composition was best explained by infection burden as measured by all beta-diversity metrics (PERMANOVA, $P<0.05$; Table S5C.2.1), but the interaction effect between infection burden and temperature was not significant ($P>0.05$; Table S5C.2.1). These results indicate that higher infection burden drives increased inconsistency in gut microbial composition, regardless of water temperature.

Upon closer inspection of our infection burden results, we observed a non-linear relationship between infection burden and alpha-diversity scores, where highest infection burden associated with either highest or lowest alpha-diversity scores. To further explore this non-linear relationship between gut microbial diversity and infection burden, we grouped parasite exposed fish samples based on their alpha-diversity scores and infection burden. Parasite exposed fish samples with at least one intestinal worm detected were classified as "Low" or "High" if their alpha-diversity score was less than or greater than the median alpha-diversity score, respectively.

Fish with zero detected worms were classified as “Other”. When grouping samples either Low or High based on alpha-diversity scores as measured by the Simpson’s index, we find that the samples in the Low group are composed of 67% 28°C and 33% 32°C water temperature reared fish, samples in the High group are composed of 33% 28°C and 67% 32°C water temperature reared fish, and samples in the Other group are composed of 14% 28°C, 27% 32°C, and 59% 35°C water temperature reared fish (Table S5C1.0). These results indicate that group membership trends with water temperature. To test this supposition, we used GLMs to determine if infection burden associated with variation in alpha-diversity score grouping (Table S5C.1.1). An ANOVA test of these GLMs revealed significant main effects of group for all alpha-diversity measures ($P<0.05$; Fig 10C; Table S5C.2.1), and significant interaction effects between group and alpha-diversity score. Notably, fish in the Low group had a significant negative slope and fish in the High group had a significant positive slope between alpha-diversity and infection burden as measured by Shannon Entropy and Simpson’s Index. These results indicate that parasite exposed fish have diverging gut microbial alpha-diversity responses to high infection burden.

Additionally, we find that these groups of samples - based on high versus low alpha-diversity scores of parasite exposed fish - also formed two distinct clusters in beta-diversity space. A PERMANOVA analysis detected significant clustering between Low, High, and Other groups across each measure of beta-diversity (PERMANOVA, $P<0.05$; Fig. 10D; Table S5D.1.1). However, this effect was weakest when considering the Canberra metric. Furthermore, a pairwise analysis of beta-dispersion finds significantly elevated dispersion levels between group membership as measured by Canberra metric, but not the other beta-diversity metrics (Table S5D.2). Given that the Canberra metric gives rarer taxa greater importance in its beta-

diversity calculations than the other metrics we evaluated, these results suggest there is more consistency in microbial composition among abundant taxa within samples that share Low or High group membership, but not among more rarer taxa. A *post-hoc* Tukey test also clarified that beta-dispersion levels are significantly different between fish in the High and Other groups compared to fish in the Low group as measured by the Canberra metric (Table S5D.3). Together, these results indicate that rarer members of the gut microbiome are less consistently represented across fish in the Low cluster group as compared to fish in the High and Other cluster groups. Collectively, these results indicate that the microbiome response of fish with heaviest infection burden diverge into two distinct trajectories, which may be influenced by water temperature.

Parasite exposure exacerbates water temperature differences in gut microbiome structure

Next, we sought to determine whether the gut microbiomes of zebrafish exposed to the parasite *Pseudocapillaria tomentosa* respond differentially compared to parasite unexposed control fish across increasing water temperatures. Prior to the parasite exposure at 164 dpf (or 0 dpe), we collected fecal samples from both cohorts of control and parasite exposed fish. Following fecal sample collection, fish in the parasite exposure group were exposed to *P. tomentosa*. We collected subsequent fecal samples at 14-, 21-, 28- and 42 dpe. Fecal samples were then measured for gut microbial diversity and composition and compared between parasite unexposed and exposed fish. We built generalized linear models (GLM) to determine if parasite exposure as a function of water temperature associated with microbial diversity and composition measures (Table S6A.1). Within pre-exposed (i.e., 0 dpe) samples, we did not observe any significant associations between the interaction effect of parasite exposure and water temperature across any of the alpha-diversity measures ($P>0.05$; Fig. 11A; Table S6A.2). These results

indicate that at 0 dpe prior to parasite exposure, gut microbial diversity measures of fish reared at the same water temperature are not different from one another. Furthermore, PERMANOVA tests revealed significant differences in microbiome composition between control and pre-exposed fish across all beta diversity metrics. Homogeneity of dispersion tests revealed a significant difference in group variability for Bray-Curtis ($P<0.05$; Fig. 11B; Table S6B.2), but not for Canberra or Generalized UniFrac. *Post hoc* Tukey tests indicated no significant pairwise differences in dispersion for any metric (Table S6B.3), suggesting that the observed dispersion effect in Bray-Curtis was not driven by specific group outliers. To assess whether these baseline differences in community variation were structured by rearing tank, we tested whether tank explained variation in microbial community composition within each temperature prior to parasite exposure. PERMANOVA tests revealed that tank effects were strong at 32°C across all distance metrics ($P < 0.05$; Fig. S6B.1.1). Furthermore, homogeneity of dispersion tests found that tanks did not differ within temperature groups prior to parasite exposure metric combination ($P > 0.05$; Table S6B.1.2), confirming that the significant tank effects at 32°C reflect shifts in community centroids rather than unequal variances. Given that temperature alone consistently explained the largest share of variation, followed by treatment and the more context-dependent tank effects, these results indicate that before parasite exposure microbial communities differ primarily by water temperature, with additional variability introduced by stochastic differences among tanks.

We next compared our results between control and exposed fish across each water temperature to determine how parasite exposure impacts gut microbiome diversity and composition. Linear regression revealed microbial gut alpha-diversity was significantly associated with the interaction effect between temperature and treatment for any alpha-diversity

metric we assessed ($P<0.05$; Fig. 11C; Table S6C.1-2). A *post hoc* Tukey test clarified that microbiome diversity was significantly different between exposure groups of fish reared at 28°C water temperature as measured by Simpson's Index ($P<0.05$; Table S6C.3), at 32°C water temperature as measured by all alpha-diversity metrics ($P<0.05$; Table S6C.3), and at 35°C as measured by richness and phylogenetic diversity ($P<0.05$; Table S6C.3). These results indicate that gut microbial diversity differs between unexposed and exposed fish depending on water temperature, and parasite exposure uniquely impacts particular microbial clades, rare and abundant taxa depending on water temperature. Additionally, PERMANOVA tests found that microbiome composition differed between control and exposed fish reared at all water temperatures as measured by all beta-diversity metrics ($P<0.05$; Table S6D.1). These results suggest that the gut microbiomes compositions between control and parasite exposed differed in microbiome community composition regardless of water temperature. Moreover, a pairwise analysis of beta-dispersion found elevated levels of dispersion across all beta-diversity metrics measured, and dispersion levels were highest among parasite exposed fish reared at lower water temperatures ($P<0.05$; Table S6D.2). These results suggest that gut microbiome community composition is less consistent between parasite unexposed and exposed fish reared at lower water temperatures. Collectively, these results demonstrate that water temperature dictates how exposure to parasites alters the temporal trajectory of the gut microbiome.

Gut microbial relative abundance significantly associates with environmental conditions and stressors

Finally, to evaluate how gut microbial abundance is influenced by environmental conditions and stressors (e.g., worm infections), we quantified differential abundance using

MaAsLin2. Our analysis revealed 277 unique taxa at the genus taxonomic level with at least one significant associations between taxon abundance and a covariate (FDR <0.05, Fig. 12; Table S7A.1). We observed several taxa were significantly associated with the effect of water temperature. Fish reared at 35°C water temperature were enriched for 37 taxa, and depleted of 54 taxa relative to fish reared at 28°C water temperature. Fish reared at 32°C were enriched for 42 taxa, and depleted of 47 taxa relative to fish reared at 28°C water temperature (Fig. 12). Notably, *Aeromonas and Pseudomonas* bacterial abundance significantly associated negatively and positively with the effects of water temperature, respectively. *Aeromonas and Pseudomonas* are common members of the zebrafish gut microbiome [145,187], and these genera's bacterial abundance has previously been observed to associate with the effects of water temperature in zebrafish [219]. These results indicate that gut microbes are differentially selected for across varying water temperatures and time. We also measured how taxon abundance change over time regardless of water temperature. Over the course of 42 days, fish were enriched for 73 taxa and depleted of 36 taxa (Fig. 12). Notably, *Bosea* and *Cloacibacterium* bacterial abundance were negatively associated with the effects of time. *Bosea* and *Cloacibacterium* are common members of the zebrafish gut microbiome [139,145,187], and were also previously identified as having negative associations with the effects of time in zebrafish [160]. These results indicate that particular members of the gut microbiome associate with time irrespective of water temperature.

Next, we sought to determine how parasite exposure impacted gut microbial abundance in fish. Fish exposed to *P. tomentosa* were significantly enriched for 74 taxa, and depleted of 35 taxa relative to parasite unexposed fish (Fig. 12). Notably, we find *Aeromonas*, *Chitinibacter*, and *Flavobacterium* are positively associated with parasite exposure, while *Plesiomonas, Phreatobacter* and *Cetobacterium* are negatively associated with parasite exposure. With the

exception of *Phreatobacter* and *Cetobacterium*, these data are consistent with our prior work that found *P. tomentosa* exposure associated with altered bacterial abundance of these members of the zebrafish gut microbiome [160]. We further investigated the effects of parasite exposure and measured how infection burden or presence of infection impacted gut microbial abundance. Fish with higher infection burden (i.e., number of parasitic worms present) enriched for 49 taxa and were depleted of 13 taxa, while fish that were positively infected enriched for 117 taxa and were depleted of 5 (Fig. 12). Notably, we find abundance of members of *Cetobacterium, Shewanella, Vibrio* and *Zooglogea* are negatively associated with infection burden. These taxa are identified as common members of the zebrafish gut microbiome [142]. Because infection burden varied widely at 28°C and 32°C, we ran temperature-stratified MaAsLin2 models to link genus-level abundance to worm count (FDR < 0.05; Table S7A.2.1-2). At 28°C, 55 genera were significantly associated with burden, whereas 45 genera were significant at 32°C (FDR < 0.05; Table S7A.2.3). Eight genera showed concordant responses across the two temperatures and only *Novispirillum* displayed an opposite trend, indicating strong directional consistency. Shared positive correlates included *Bryobacter, Vampirovibrio and Inhella*, while *Cetobacterium* and *Shewanella* were consistently depleted in heavily infected fish. Temperature-specific effects were evident: 46 genera were unique to 28°C, led by *Paraclostridium* and *Rubrivivax*, whereas 36 genera were unique to 32°C, with *Novispirillum* exhibiting the most significant coefficient (FDR < 0.05; Table S7A.2.3). The ten taxa with the smallest q-values further emphasize that nine of the strongest signals arise at 28°C, underscoring the broader taxonomic shift that accompanies high parasite burden at the lower temperature (FDR < 0.05; Table S7A.2.4). Together, these data show that parasite burden has a pronounced yet partly temperature-dependent influence on zebrafish gut microbial abundance.

To gauge how strongly the microbiome, temperature, and time predict infection severity, we trained a random-forest regression model using the MaAsLin2-identified genera plus water temperature and days post exposure (DPE). Ten-fold cross-validation showed that the model reduced prediction error by twenty-five per cent compared with a mean-only baseline (RMSE = 2.684 worms versus 3.554; Table S7B.1.4-5) and explained thirty-two per cent of the variance in worm burden ($R^2 = 0.321$; Table S7B.1.4). Permutation importance and a one-hundred-run stability screen highlighted a compact predictive core: *Plesiomonas*, *ZOR0006*, *Cetobacterium*, *Bryobacter*, and *Rhizobacter* appeared in the top ten predictors in at least eighty-seven per cent of runs (Table S7B.1.3). In contrast, temperature and DPE entered the top ten in fewer than two per cent of runs, indicating that infection intensity is encoded primarily in the abundance patterns of these key genera rather than the measured environmental covariates alone. Together, these findings identify a concise set of microbiome members that both respond to parasite exposure and collectively capture a substantial share of the variation in worm burden.

To deepen our analysis of parasite exposure on the zebrafish gut microbiome, we investigated how taxon relative abundance associated with gut microbiome diversity and composition. Previously, we found that parasite exposed fish reared at 28°C and 32°C water temperatures clustered into two distinct groups of community composition, which associated with high infection burden and either high or low alpha diversity scores. This observation led us to investigate which gut microbiota might be driving the clustering of the gut microbiomes of heavily infection burdened fish. We did not find significantly abundant taxa in the High group. We detected 1 taxon that was significantly enriched and 192 taxa that were significantly depleted among fish in the Low group (Fig. 12). Notably, we find *Aeromonas* was enriched, while *Mycobacterium* were depleted in the Low group fish. Some species of *Mycobacterium* are

common pathogens in zebrafish facilities [158]. These results indicate that the gut microbiome communities of parasite exposed fish experiencing heavy infection burden stratify into two distinct groups represented by the unique depletion of particular members of the gut microbiome. Collectively, these results indicate that environmental conditions associate with altered gut microbial abundance, and the response of specific members of the gut microbiome to environmental stressors varies depending on environmental conditions.

Discussion

The zebrafish is an important model organism for understanding how environmental stressors impact the microbiome [139,145]. Our work capitalized on the experimental control and scale afforded by the zebrafish model system to investigate how temperature and parasite exposure interact to influence infection and microbiome outcomes. While previous research has investigated how water temperature [219] and parasite exposure [160] independently impact the zebrafish gut microbiome, no studies in any *in vivo* experimental system, to our knowledge, have examined the microbiome's temporal response to the combined effects of increasing water temperature and parasite exposure. Overall, we found that water temperature serves as a key contextual variable that dictates the severity of infection**,** the developmental state of worms, the composition of the gut microbiome in unexposed fish, and how the gut microbiome responds to parasite exposure and infection. These results underscore that the gut microbiome's response to, and potentially its ability to buffer against, intestinal parasitic infection is influenced by other exogenous factors, in this case, water temperature. Furthermore, our findings challenge current expectations of climate change's anticipated impact on aquatic organismal parasite burden [172,208]. Consequently, it is important that we consider going forward how stacking multiple

stressors, an experience inherent to life in the Anthropocene, may accelerate the arrival of dysbioses.

We found that parasitic infection burden was highest among zebrafish reared at ambient water temperatures. Given that *P. tomentosa* has a direct life cycle with no intermediate or paratenic host under laboratory settings, the temperature-linked drop in worm burden and development at 35°C could stem from direct thermal inhibition of egg hatching, initial, or larval development, rather than microbiome-mediated resistance alone. While prior field studies have documented arrested development of *P. tomentosa* in colder conditions [221,222,225], to our knowledge, this study provides the first controlled laboratory evidence that elevated temperatures can suppress parasite development in a fish host. Consistent with our prior work, temporal trends in *P. tomentosa* infection burden were similar for fish at ambient temperatures of 28°C [160]. However, contrary to expectations that elevated temperatures increase infection burden, we observed the opposite outcome: fish reared at the highest temperatures of 35°C exhibited the lowest infection burden, with only a few larval-stage worms detected. Because parasite eggs were larvated at ambient temperature before being transferred to warmer tanks, we hypothesize that elevated temperatures may have impaired hatching once the eggs were ingested, reducing overall abundance of worms. Nevertheless, at 35°C, worms that did establish infections persisted but remained in an arrested state out to 28- and 42 days post-exposure (dpe), whereas at 28°C and 32°C, worms completed development and mated within 3 to 4 weeks, consistent with previous observations [157]. Such arrested developmental stages are characteristic of nematodes approaching their upper thermal limit (e.g*., Wuchereria bancrofti* larvae fail to develop above 31°C in mosquitoes), reinforcing the hypothesis that elevated temperature acts directly on the parasite [226]. Although Kent et al. 2019 demonstrated that *P. tomentosa* egg larvation is

inhibited at temperatures exceeding 40°C, some eggs still larvated following brief exposures to 40°C for 1 or 8 hours [223]. These findings suggest that egg hatching may be sensitive to thermal stress but not completely abrogated at extreme temperatures. However, no studies have yet examined how larvation and development respond across a more ecologically relevant thermal range. Controlled in vitro hatching assays across 28-35°C will therefore be essential to disentangle parasite-specific constraints from potential host- or microbiome-mediated effects.

These findings are particularly notable given the broad geographic and thermal distribution of *P. tomentosa* in natural and captive settings. *P. tomentosa* is a remarkably cosmopolitan parasite, with natural infections reported in a wide variety of freshwater fish species from Europe, the Middle East, and North and Central America [222]. The type locality is France, and it is widespread throughout central Europe [220]. There are also reports of natural infections in subtropical climates, including southern Mexico. In temperate regions, *P. tomentosa* infects hosts living in environments where winter water temperatures drop below 10°C and summer temperatures exceed 30°C. It has also been found in freshwater aquarium fishes, which are typically maintained at 20-28°C [220,221]. These reports suggest a broad thermal range for infection under field conditions. Notably, these observations assume that the many global records of *P. tomentosa* represent a single species, rather than a complex of cryptic, morphologically indistinguishable species. One relevant field study by Moravec et al. 1983 found that parasite development was seasonally arrested at temperatures below 25°C in the Czech Republic [225], but no prior work has evaluated outcomes at the warmer limits we tested. Our findings begin to define *P. tomentosa's* upper thermal boundaries under controlled conditions.

In a broader context, many fish pathogens exhibit upper thermal limits to development and infectivity. For example, the ciliate *Ichthyophthirius multifiliis,* a common aquacultural

pathogen, fails to develop above 30°C [227]. Comparable upper-temperature ceilings have not yet been documented in fish nematodes, but terrestrial filarial worms offer a parallel example of larval development in mosquito vectors halts once temperatures exceed 31°C [226]. In parasites with indirect or complex life cycles, warming often suppresses prevalence simply by eliminating intermediate or paratenic hosts [208,223]. As noted previously, *P. tomentosa's* direct life cycle rules out temperature-sensitive effects on intermediate hosts, reinforcing that observed patterns likely stem from direct thermal impacts. These results highlight how climate change may suppress, rather than exacerbate, certain infections and challenging expectations in aquatic disease ecology and emphasizing the need to test thermal constraints across a range of pathogens [152].

Future research should investigate whether arrested development in *P. tomentosa* reflects direct thermal limits or host-mediated processes. Beyond direct effects on parasite development, poikilothermic (i.e., animals with variable body temperature and the inability to regulate it) hosts may gain protections against infection through temperature-dependent immune responses or gene expression changes. While studies on zebrafish immunity under elevated temperatures are limited, prior research in teleosts indicates that immune responses are host- and environment-specific, varying with the direction and duration of temperature shifts [228,229]. For example, Dittmar et al. found that immune activity was highest at thermal limits and inversely related to acute temperature shifts in three-spine sticklebacks [230]**,** whereas Bailey et al. observed suppressed immunity and increased parasite burden in rainbow trout exposed to chronic upper optimal thermal ranges [231]. Although these studies differ in exposure regimes to ours, they highlight that colonization resistance may be influenced by temperature-sensitive immune responses and gene expression. Future research integrating immune function, gene expression,

and histopathological assessments will be crucial to disentangling the host's role in colonization resistance under chronic parasite exposure and elevated temperatures. Notably, controlled temperature manipulation is already used to mitigate certain aquaculture pathogens, such as *Ichthyophthirius multifiliis [227]***,** where increasing tank temperature to 30°C can eliminate infections in susceptible fish. Our findings suggest that similar interventions may help mitigate or delay parasite infection in aquaculture settings.

We also found that zebrafish gut microbiome structure stratified depending on the environmental conditions of increasing water temperature. Our results are congruent with previous research that found increased water temperatures altered zebrafish gut microbial diversity and composition [219]. Moreover, Wang et al. observed that zebrafish reared at different water temperatures manifested distinct liver carbohydrate metabolism profiles and temperature-dependent sensitivity to irradiation. A unique aspect of our study considered how the gut microbiome temporally varies as a function of water temperature. We found that water temperature acts as a filter on initial zebrafish gut microbiome assembly, and these initial differences in assembly between water temperature remained stable across time. Beyond zebrafish, analogous investigations have investigated how temperature variation shapes gut microbiome composition and function in mammals, fish, and other animal species [82,232]. In particular, a recent meta-analysis of aquatic organisms' response to temperature found similar, but inconsistent results to our study, wherein increasing water temperature is associated with both increases and decreases to gut microbial diversity, differences in gut microbiota community composition, and altered gut taxon abundance [232]. Inconsistencies between prior work and ours could be driven by differences in magnitude of the stressor (i.e., press vs pulse; [70]), host species [233], facility or habitat effects [144,232,234], or diet [158]. Despite these differences,

the results of prior studies in conjunction with ours are consistent with the concept of environmental conditions acting as an abiotic filter to shape initial gut microbiome assembly [235] and illicit environmentally dependent responses to biotic exogenous stressors.

Finally, we observed a nonlinear relationship between gut-microbiome diversity and infection outcomes, with water temperature moderating these dynamics. Consistent with our prior research on zebrafish infected by *P. tomentosa* [160], heavily infected fish displayed dysbiotic microbiomes that matched the Anna Karenina Principle (AKP) expectation of elevated dispersion [31]. However, once water temperature was included in our model, the AKP signal weakened and at least two alternative stable states emerged. Viewing these patterns through the lens of community-assembly theory helps reconcile this apparent contradiction [235]. Temperature acts as a selective abiotic filter that deterministically favors taxa possessing traits that confer thermal tolerance, whereas parasite exposure behaves as a largely neutral process, introducing stochastic variation by differentially perturbing communities without a strong trait-based direction. When the selective (e.g., temperature) and neutral (e.g., parasite) forces interact, they generate divergent assembly trajectories that resemble multiple stable states rather than a single AKP-like dysbiosis. This interpretation is supported by the contrasting dispersion trends in unexposed fish versus exposed fish and by the dispersion results. These findings indicate that deterministic (e.g., selective) and stochastic (e.g., neutral) processes jointly shape the assembly of the zebrafish gut microbiome under combined thermal and parasitic stress. Furthermore, our findings underscore the need to consider both individuals' temporal and spatial contexts and the balance of neutral and selective drivers when assessing microbiome stability [109]. Moreover, current homeostatic definitions of stability may be insufficient to describe such dynamic shifts [72,79,98]. Rather, a homeorhetic framework [113,114], which conceptualizes stability as a

change along a stable trajectory rather than a fixed state, may better capture how microbiomes respond to exogenous stressors across environmental gradients, and could reconcile discrepancies in AKP detection across studies.

In conclusion, we found that water temperature alters the contextual landscape of the microbiome to impact its response to an exogenous stressor of an intestinal parasite. Our work revealed that differences in environmental conditions of water temperature were sufficient to temporally change the gut microbiome's response to parasitic exposure and impact infection outcomes in zebrafish. While the zebrafish gut microbiome differs taxonomically from other animal-microbiome systems, a considerable amount of functional capacity is shared between animals [236]. Thus, zebrafish serve as a powerful model for investigating how environmental changes and stressor exposures influence microbiomes and host health. Our findings have important implications for microbiome research in the context of climate change, demonstrating that rising temperatures may have unexpected effects on gut microbiomes and infection outcomes. Future work should further clarify how gut microbiomes and host responses buffer against combined environmental stressors, ultimately shaping health outcomes in vertebrates.

Methods

Fish husbandry

5D strain zebrafish embryos were obtained from the Sinnhuber Aquatic Resource Center at Oregon State University, and reared in our vivarium at Nash Hall (Corvallis, OR, USA). This facility is specific pathogen-free (SPF) and has no known history of *Pseudocapillaria tomentosa* or other intestinal parasitic infections [237]. The vivarium is a single pass flow through, using dechlorinated city water. Fish were then randomly divided into twelve 2.8 L tanks. The

temperature was recorded daily and the ambient temperature ranged from 27 to 28°C. All other water conditions were monitored weekly, pH was maintained at 7.6, total ammonia was not detected, and conductivity ranged from 102 to 122. Light in the vivarium was provided for 14 hours/day. Fish were fed Gemma Micro 300 (Skretting; Fontaine-les-Vervins, France) at 1.5% body weight twice daily, except on weekends or during exposure to parasitic eggs. One plastic aquatic plant piece, approximately six inches in length, was added to each tank for enrichment. The use of zebrafish in this study was approved by the Institutional Animal Care and Use Committee (IACUC) at Oregon State University (permit number: 5151).

Temperature exposure

At 5 months old, or 206 days post-fertilization (dpf), corresponding to early adulthood in zebrafish, fish were randomly divided into 12 9.5-L tanks (approximately 25 fish/tank). Each tank was outfitted with a 50W (28°C treatment only) or 100W HG-802 Hygger titanium aquarium heater (Hygger, Shenzhen Mago Co., Ltd., Shenzhen City, Guangdong Province, China). Four of the twelve tanks were assigned to each of the temperature treatments: 28°C, 32°C, or 35°C. These temperatures were selected to simulate baseline (28°C), 32°C reflects near-future warming scenarios (+4°C)[171], and upper sublethal thermal limits (35°C) for zebrafish physiology [238]. Two tanks for each temperature were held as pathogen negative controls and two tanks were exposed to *Pseudocapillaria tomentosa* as described below. Fish were acclimated to the prescribed temperature treatments by increasing the heater thermostat settings by 1°C every two days until the final prescribed temperature was achieved. Two temperature logging thermometers, one for the six pathogen negative control tanks and one for the six *P. tomentosa* exposed tanks, were rotated through the tanks every two days on weekdays to monitor

temperature at each temperature treatment. The average range recorded for the water temperature treatments was +/- 1°C.

Pseudocapillaria tomentosa exposure

Pseudocapillaria tomentosa is monoxenous; no invertebrate or vertebrate intermediate host is required for laboratory transmission. We maintain a laboratory population of infected zebrafish at 26-28°C, from which all *P. tomentosa* eggs used in this study were obtained (Kent lab, OSU; see Martins et al. 2017) [239]. Eggs were allowed to larvate for 6 days at 28°C, and fish were exposed at 25 larvated eggs/fish. Water flow was turned off for 36h to enhance exposures, while an airstone was provided to each tank to maintain adequate oxygen levels. This was a lower exposure dose than many of our previous studies [157]. Therefore, we enhanced exposure adding 1 L of water from a stock tank holding infected fish twice a day during the 36 h hour post exposure period. This additional water supplement was created by siphoning water from the bottom of the exposed stock fish tank because the infectious stage is a larvated egg, which sinks in water.

Infection assessment

Exposed and control fish were collected and examined for worm prevalence, abundance and state of development using wet mounts of whole intestines as described in Schuster et al. 2023 [240]. After recording observations in wet mounts, the individual intestine was preserved in Dietrich's solution and intestines of 95 fish were processed for histology prepared as described in Gaulke et al. 2019 [160]. Here we focused on selected samples from fish from the 35°C group as

very few worms were detected by wet mounts in this group. Two stepwise sections, 50 um apart, were obtained from each block to enhance the possibility of larval worms

Fecal collection

Five fish from each tank were randomly selected for fecal sampling at 0 dpe (n=60; 5 samples/tank), prior to parasite exposure. Subsequent fecal sampling took place at 14- (n = 54), 21- (n = 48), 28- (n = 47), and 42 (n = 51) dpe to parasites. Fecal material was collected as previously described [158]. In brief, fish were transferred to 1.4 L tanks (1 fish/tank) containing ~ 0.4 L of fish water at least 30 min after the last feeding of the day. Fish were left to defecate overnight and all fecal material was collected from each tank the following morning in a 1.5ml microcentrifuge tube. Fecal samples were immediately spun at 10k rpm for 2 min, excess tank water was removed, and samples were snap frozen on dry ice and stored at -80 ˚C until processing. However, not all fish produced a fecal sample for a variety of reasons. For instance, experiments involving fish have expected mortality, and fish which died prematurely did not produce fecal samples. Additionally, infection conditions may have prevented infected fish from producing a fecal sample. Instances where fish failed to produce a fecal sample are noted in the metadata sheet.

Microbial 16S rRNA library preparation and sequencing

Microbial DNA was extracted from zebrafish fecal samples and 16S rRNA gene sequence libraries were produced and analyzed following previously described methods [241]. DNA was isolated from fecal samples using the DNeasy 96 PowerSoil Pro DNA kits (Qiagen, Hilden, Germany), in accordance with the manufacturer's directions. In brief, samples were

subjected to bead beating for 10 minutes using the Qiagen TissueLyser II, spun a max speed in the centrifuge, supernatant was process using 96 well columns, and DNA was eluted with 100µl Tris buffer. The V4 region of the 16S rRNA gene was PCR amplified using dual-index 16S primers and protocols [242]. PCR was performed using 1 µl of purified DNA, 2µl of a 5µM mix of the forward and reverse dual-index primers, 5µl of Platinum II Hot-Start PCR Master Mix (Thermo Fisher), Carlsbad, CA), and 2µl water with the following conditions, 94˚C, 3m; (94˚C, 30s; 50˚C, 30s; 68˚C, 1m)x 35; 68˚C 10m. PCR products were visualized on a 1.5% agarose gel and quantified on the BioTek Synergy H1 Hybrid Multi-Mode Plate Reader using the Quand-iT 1X dsDNA HS Assy kit (Thermo Fisher, Carlsbad, CA, USA). A 100ng aliquot of DNA was selected from each of the 300 samples, the pooled DNA was cleaned using the QIAGEN QIAquick PCR purification kit, and quantified using Qubit HS kit (Carlsbad, CA). The quality of the pooled library was verified on the Agilent TapeStation 4200. The prepared library was submitted to the Oregon State University Center for Quantitative Life Sciences (CQLS) for paired end 2x300 bp read sequencing on an Illumina MiSeq System

Bioinformatic processing

All microbiome DNA sequence analyses and visualization were conducted in R (v 4.3.3)[201]. Raw reads were filtered for quality, merged, and assigned using the DADA2 R package (v 1.26.0) as previously described [124]. In brief, forward and reverse reads were trimmed at 250 and 225 bp, respectively, subsequently merged into contigs, and subject to amplicon sequence variant (ASV) identification. ASVs unannotated at the Phylum level or identified as non-bacterial were removed, which resulted in 674 remaining detected ASVs. Samples containing reads below the minimum required read count (<5000) were dropped from

downstream analysis. The final sample number for microbiome analysis was 260. Phylogenetic analysis was conducted using MOTHUR (v 1.46.1)[243] with default parameters as previously described [145]. Phylogeny was inferred using FastTree2 [244], an approximately-maximum-likelihood method. Microbiome and sample data were contained in a Phyloseq object using the Phyloseq R package [245], and the tidyverse (v 2.0.0)[246] and microViz (v 0.12.1) R packages were used for downstream data processing, analyzing, and visualization [247]. Code for bioinformatic processing are available at https://github.com/sielerjm/Sieler2025__ZF_Temperature_Parasite/.

Microbiome diversity metrics

All microbiome analyses were conducted at the genera level unless otherwise noted. We estimated four alpha-diversity metrics for each microbiome fecal sample: Simpson [248], Shannon [249], phylogenetic diversity (Faith's PD [250]; ASVs), and richness. We also estimated beta-diversity between each pair of microbiome fecal samples using three metrics. These included Bray-Curtis [251], Canberra [252], and half-weighted generalized UniFrac [253].

Statistical Analyses

All statistical analyses were conducted in R (v 4.3.3)[201] with a significance level of $\alpha = 0.05$, and randomization procedures employed a fixed seed (42) to ensure reproducibility. Code for statistical analyses are available at https://github.com/sielerjm/Sieler2025__ZF_Temperature_Parasite/.

Using methods previously described [200], we assessed normality of alpha-diversity scores using Shapiro-Wilk test [201,254], transformed non-normal scores using Tukey's Ladder

of Powers [200,255] and normalized from 0 to 1 [158] before incorporation into linear models. We used generalized linear models (GLM), we assessed the relationship between alpha-diversity score and experimental parameters. *Post hoc* Tukey Tests evaluated pairwise comparisons of models using the multcomp R package (v 1.4-25)[203]. We corrected for multiple tests using Benjamini-Hochberg correction [204]. Two-way ANOVA was used to determine if the expanded models of these GLMs significantly improved the response variable relative to the null model.

Beta-diversity models were generated using methods described previously [158]. In brief, we assessed the relationship between experimental parameters and beta-diversity by applying a step-wise model selection approach as implemented in the capscale function (vegan R package v 2.6-4)[205]. Beta diversity was measured using Bray-Curtis, Canberra, and UniFrac distance measures [251,256–258]. Optimal models were subsequently subject to permutation analysis of variance (PERMANOVA) with anova.cca using the vegan R package to determine if the selected model parameters significantly explained the variation in microbiome composition across samples [205,259]. Differential abundance was measured using MaAsLin2 [260]. We assessed beta-diversity dispersion within groups with betadisper using the vegan R package [205,261,262].

To assess the relationship between parasite infection outcomes and experimental parameters, we used negative binomial generalized linear models (GLM) with the glm.nb function from the MASS R package (v 7.3-60.0.1)[263] and used the negative binomial distribution. We used the negative binomial distribution to account for overdispersion in the count data, a common characteristic of parasite infection data [157,241,264]. Significance of main effects and interactions was assessed using two-way ANOVA implemented through the Anova function with the Car R package (v 3.1-2)[265]. Post-hoc comparisons were conducted

using Tukey's HSD tests via the emmeans package, where we estimated marginal means and performed pairwise contrasts with p-value adjustment using the Tukey method [266]. Detection method comparisons were analyzed on a subset of samples used for microbiome analysis (120 samples, 20 samples/tank, ~10 samples/time point; Table S3C.1). To compare detection methods between wet mount and histology, we used McNemar's test [267], with discordant pairs (wet only vs histology only) examined at each temperature and DPE combination through 2×2 contingency tables Table S3C.2-3). Using similar methods as described above, we assessed the relationship between infection outcomes and microbiome diversity using GLMs (Table S3B.1).

Random Forest Analysis

To identify microbial features associated with worm burden, we employed a random forest regression approach using the ranger package in R (v 0.17.0) (89). The analysis was restricted to exposed fish samples and utilized only microbial genera that were previously identified as significantly associated with worm burden through MaAsLin2 analysis ($q < 0.1$). Prior to model training, the microbiome data underwent compositional normalization through total sum scaling followed by log2 transformation with half-minimum replacement for zero values. The dataset was randomly split into training (80%) and testing (20%) sets, with the random forest model trained using 1000 trees and permutation importance. To ensure robust feature selection, we performed a stability analysis by repeating the model training 100 times with different random seeds, tracking the frequency of genera appearing in the top 10 most important features across iterations. Model performance was evaluated through 10-fold cross-validation, comparing the random forest predictions against a null model that predicted the mean worm burden. Performance metrics included root mean squared error (RMSE) and R^2 values,

with variable importance assessed through the percentage increase in mean squared error when each feature was permuted.

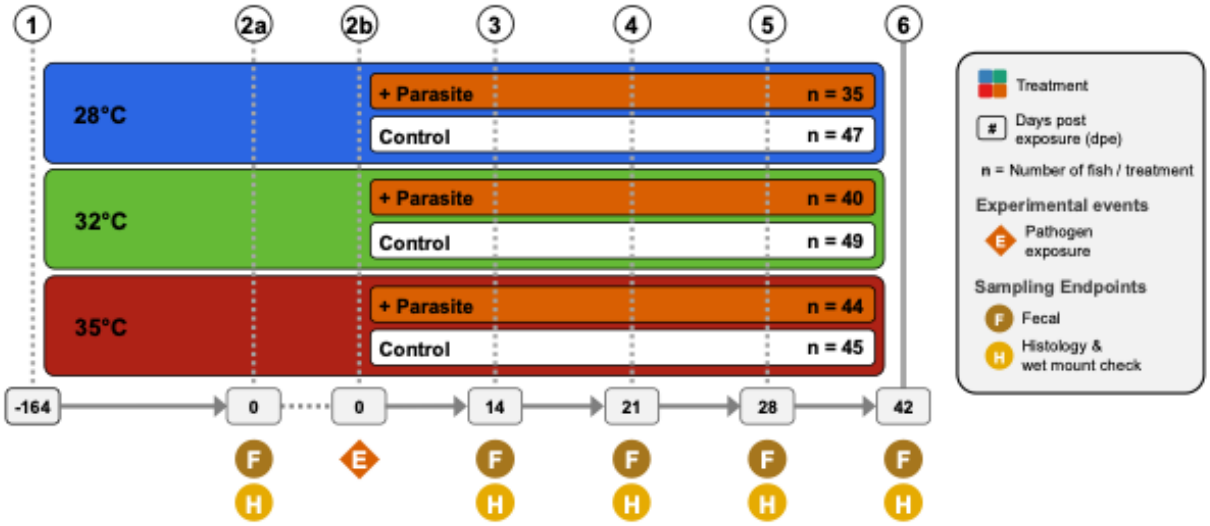


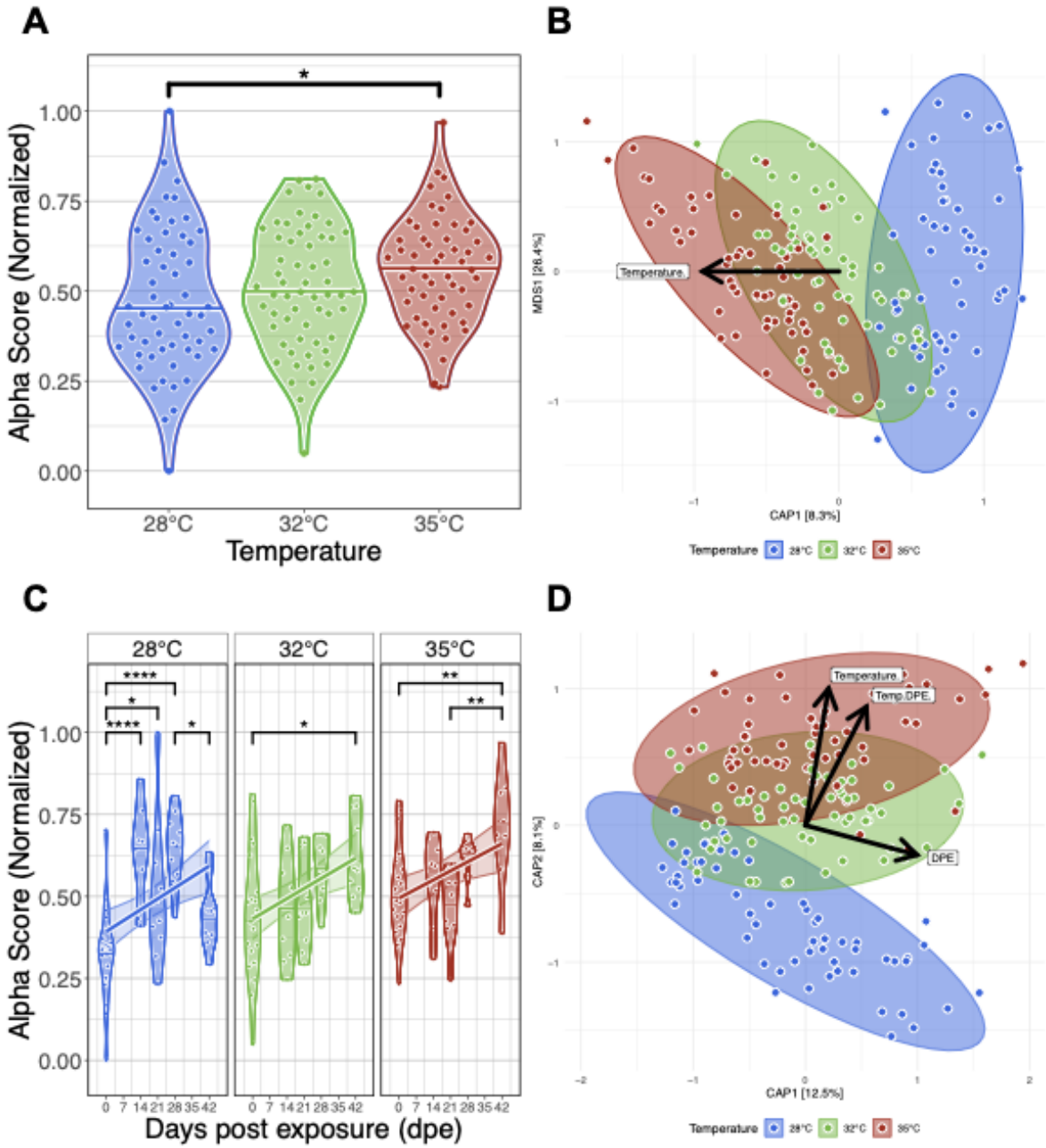
Figure 6: Experimental design showing treatments and husbandry events during the course of the study.

Symbols indicate when an experimental event occurred at each time point **(1)**. 260 zebrafish were assigned and acclimated to one of three water temperature groups (e.g., 28°C, 32°C, or 35°C) and reared from 0 to 164 days post fertilization (dpf). **(2a)** At 164 dpf (or 0 days post exposure; dpe), fecal collections were collected from a random selection of five fish per tank (n = 60). Additionally, histological and wet mount assessments were conducted on selected fish to assess presence of infection and infection burden. **(2b)** Afterwards, a cohort of fish from each water temperature group were exposed to the nematode Pseudocapillaria tomentosa **(4–6)**. Subsequent fecal samples were collected and histopathological assessments were conducted at 14 dpe (n = 54) **(4)**, 21 dpe (n = 48) **(5)**, 28 dpe (n = 47), and **(6)** 42 dpe (n = 51).

Figure 7: Effects of water temperature on zebrafish gut microbiomes.

(A) Simpson's Index of diversity shows that gut microbiome diversity significantly differs between fish reared at 28°C and 35°C water temperatures. **(B)** Capscale ordination based on the Bray-Curtis dissimilarity of gut microbiome composition constrained on the main effect of temperature. The analysis shows that gut microbiome composition significantly differs between fish reared at different water temperatures. **(C)** Simpson's Index of diversity shows microbial gut diversity increases with time from 0 days post exposure (dpe) to 42 dpe, irrespective of water temperature. **(D)** Capscale ordination of gut microbiome composition based on the Bray-Curtis dissimilarity constrained on the main effects of water temperature and time (days post exposure, dpe), and their interaction. The analysis shows that shows that gut microbiome composition differs between fish across time depending on water temperature. Ribbons and ellipses indicate 95% confidence interval. Only statistically significant relationships are shown. A "*" indicates statistical significance below the "0.05" level. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. Black arrows indicate direction of greatest change in the indicated by covariates.

Figure 7: Effects of water temperature on zebrafish gut microbiomes (continued)



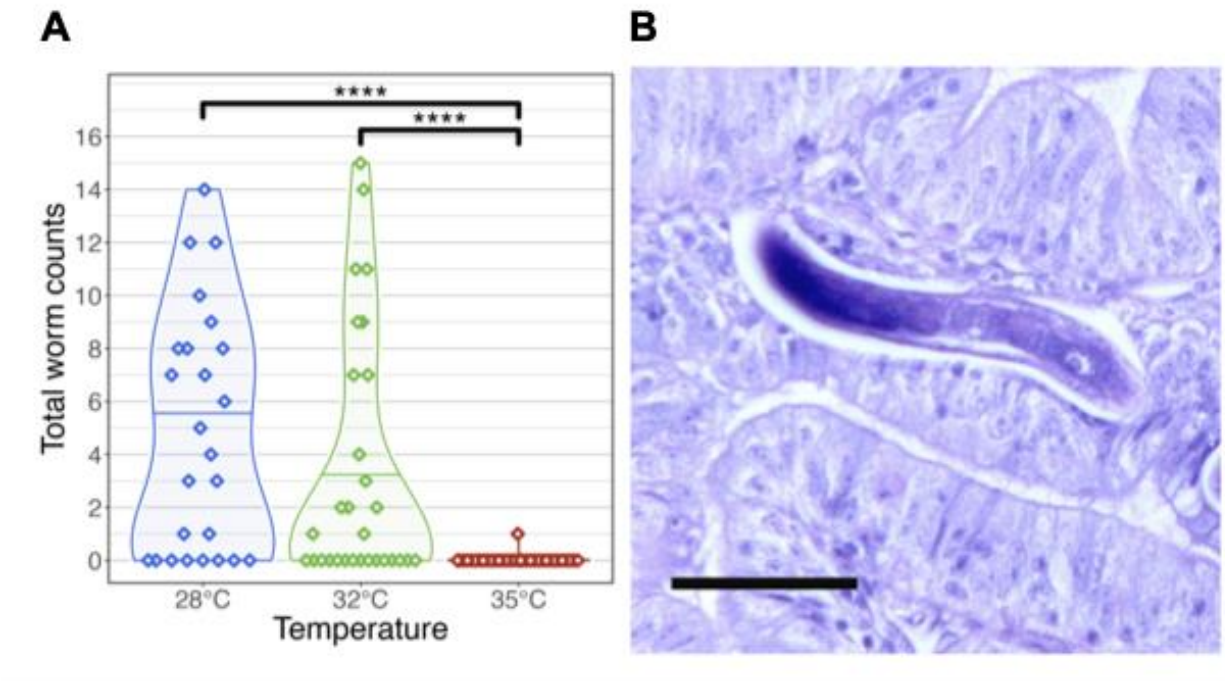


Figure 8: Infection outcomes in zebrafish exposed to *Pseudocapillaria tomentosa*.

Infection outcomes in zebrafish exposed to Pseudocapillaria tomentosa. **(A)** Infection outcome analysis of fish exposed to P. tomentosa (n = 89) by temperature. Fish reared at 28°C and 32°C water temperatures had significantly different infection burden to fish reared at 35°C water temperature. Only one fish in our microbiome analysis reared at 35°C was identified as being positively infected by wet mount. Only statistically significant relationships are shown. ****p < 0.0001. **(B)** Histological sections stained with H&E stain in zebrafish exposed to P. tomentosa examined at 35°C at 21 days post exposure. Arrow = larval worms, sagittal and cross sections. Bar = 50 µm.

Figure 9: Effects of *Pseudocapillaria tomentosa* exposure on zebrafish gut microbiomes reared at different water temperatures.

(A) Simpson's Index of diversity shows that gut microbiome diversity significantly differs between fish reared at 28°C water temperature to fish reared at 32°C and 35°C water temperatures. **(B)** Capscale ordination based on the Bray-Curtis dissimilarity of gut microbiome composition constrained on the main effect of temperature. The analysis shows that gut microbiome composition significantly differs between parasite exposed fish reared at different water temperatures. **(C)** Simpson's Index of diversity shows microbial gut diversity decreases with time from 0 days post exposure (dpe) to 42 dpe in parasite exposed fish reared at 28°C water temperature. **(D)** Capscale ordination of gut microbiome composition based on the Canberra dissimilarity constrained on the main effects of water temperature and time (days post exposure, dpe), and their interaction. The analysis shows that shows that gut microbiome composition differs between parasite exposed fish across time depending on water temperature. Ribbons and ellipses indicate 95% confidence interval. Only statistically significant relationships are shown. A "*" indicates statistical significance below the "0.05" level. Black arrows indicate direction of greatest change in the indicated covariates. A "*" indicates statistical significance below the "0.05" level. *$p < 0.05$, **$p < 0.01$.

Figure 9: Effects of Pseudocapillaria tomentosa exposure on zebrafish gut microbiomes reared at different water temperatures (continued)

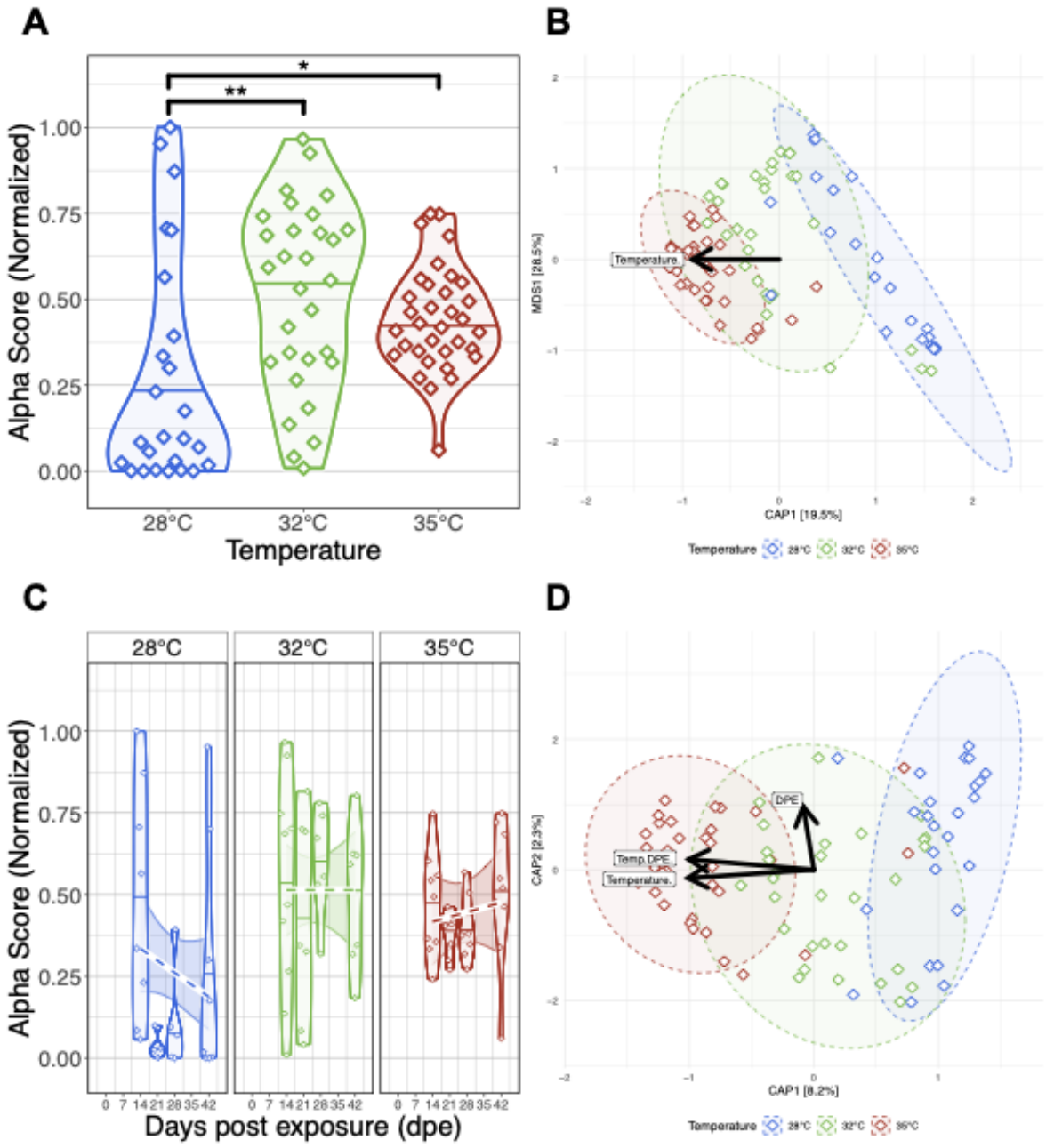


Figure 10: The impacts of presence of infection and infection burden on the gut microbiomes of *Pseudocapillaria tomentosa* exposed zebrafish.

(A) Simpson's Index for diversity of parasite exposed fish. Gut microbial alpha-diversity does not significantly differ between fish reared at the same water temperature depending on presence of infection. **(B)** Capscale ordination based on the Canberra dissimilarity of gut microbiome composition of parasite exposed fish constrained on the main effects of temperature and pathology result. The analysis shows that gut microbiome composition significantly differs between positively infected fish reared at different water temperatures. **(C)** Infection burden (total worm counts) is positively correlated with lowest or highest alpha diversity scores in positively infected fish. **(D)** Capscale ordination based on the Bray-Curtis dissimilarity of gut microbiome composition constrained on the main effects of water temperature and infection burden. The analysis shows that gut microbiome composition significantly differs between clusters of Low, High and Other fish. Samples points are colored by water temperature, and filled by "Cluster" grouping. Samples with at least one detectable worm and an alpha-diversity score less than 0.5 are categorized as Low (orange fill), samples with at least one detectable worm and an alpha-diversity score greater than 0.5 are categorized as High (purple fill), and samples with no observable infection are categorized as Other (white and transparent fill). Ribbons and ellipses indicate 95% confidence interval. Only statistically significant relationships are shown. Black arrows indicate statistically significant covariates and direction of greatest change in the indicated covariates.

Figure 10: The impacts of presence of infection and infection burden on the gut microbiomes of *Pseudocapillaria tomentosa* exposed zebrafish (continued)

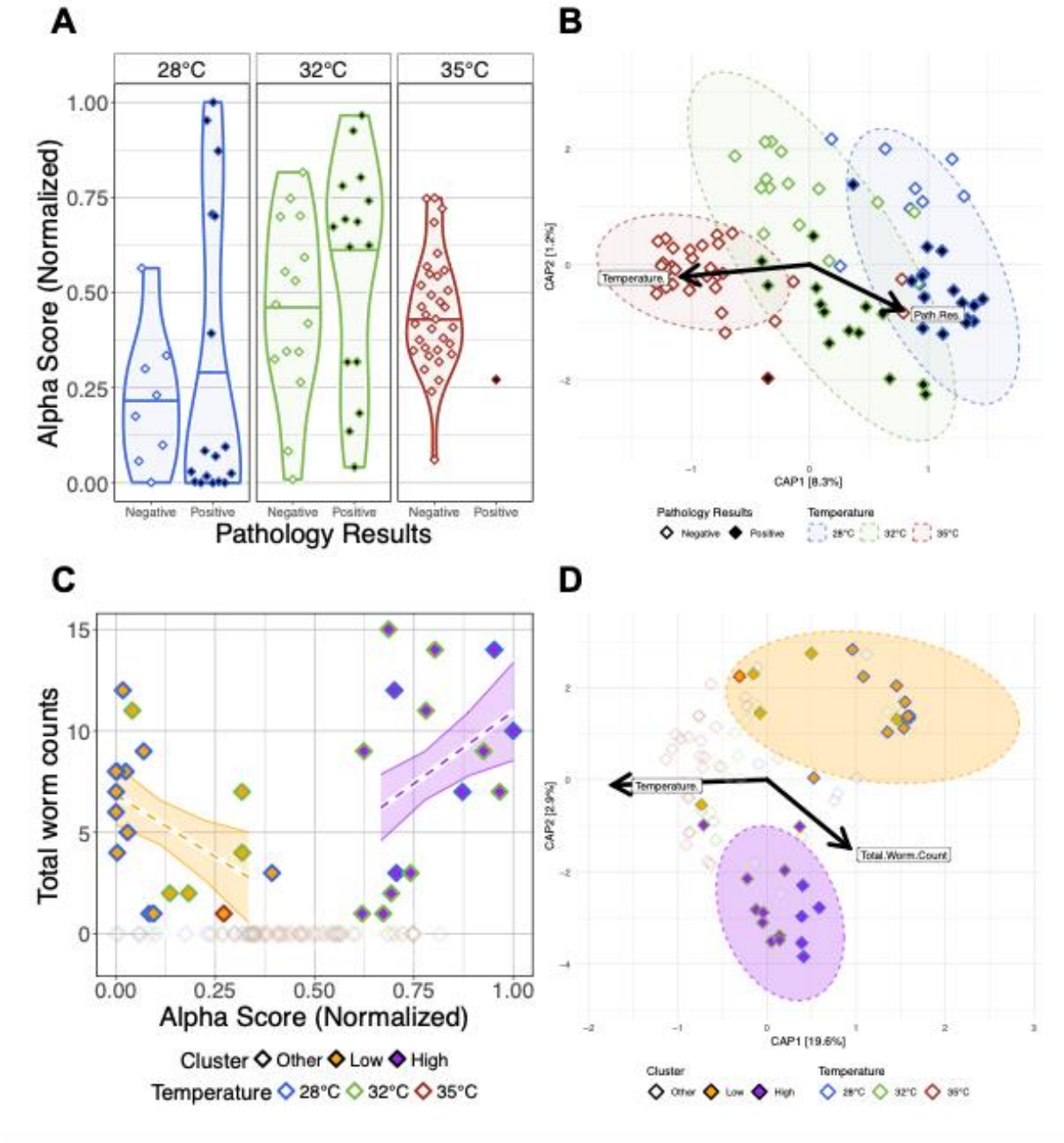


Figure 11: Comparison of the effects of water temperature on the gut microbiome between parasite exposed fish and parasite unexposed fish.

(A) Simpson's Index for diversity of parasite unexposed and pre-exposed fish at 0 days post exposure (dpe). Prior to parasite exposure gut microbial alpha-diversity does not significantly differ between fish reared at the same water temperature. **(B)** Capscale ordinations based on the Bray-Curtis dissimilarity of gut microbiome composition constrained on the main and interaction effects of temperature and parasite exposure (treatment) of pre-exposure samples at 0 dpe. **(C)** Simpson's Index for diversity of parasite unexposed and exposed fish. Gut microbial alpha-diversity significantly differs between parasite exposed fish reared at 28°C and 32°C water temperature relative to unexposed control fish, but gut microbial alpha-diversity did not differ between parasite unexposed and exposed fish reared at 35°C water temperature. **(D)** Capscale ordinations based on the Bray-Curtis dissimilarity of gut microbiome composition constrained on the main and interaction effects of temperature and parasite exposure (treatment) of post-exposure samples after 0 dpe. The analysis shows gut microbiome composition differs between fish reared at different water temperatures prior to parasite exposure, and parasite exposure further drives these temperature associated differences in microbiome community composition. Ribbons and ellipses indicate 95% confidence interval. Ribbons and ellipses indicate 95% confidence interval. Only statistically significant relationships are shown. A "*" indicates statistical significance below the "0.05" level. Black arrows indicate statistically significant covariates and direction of greatest change in the indicated covariates.

Figure 11: Comparison of the effects of water temperature on the gut microbiome between parasite exposed fish and parasite unexposed fish (continued)

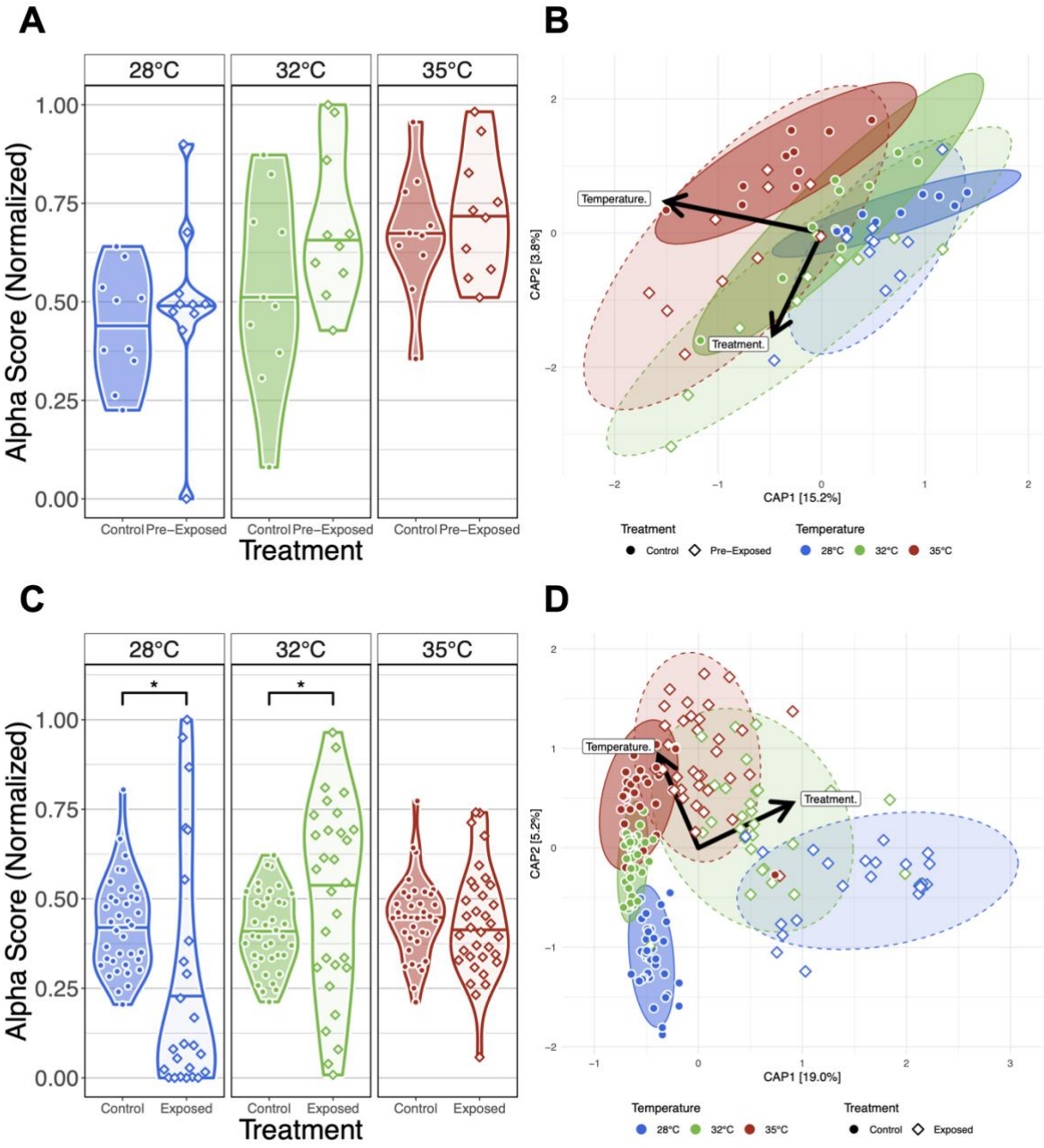
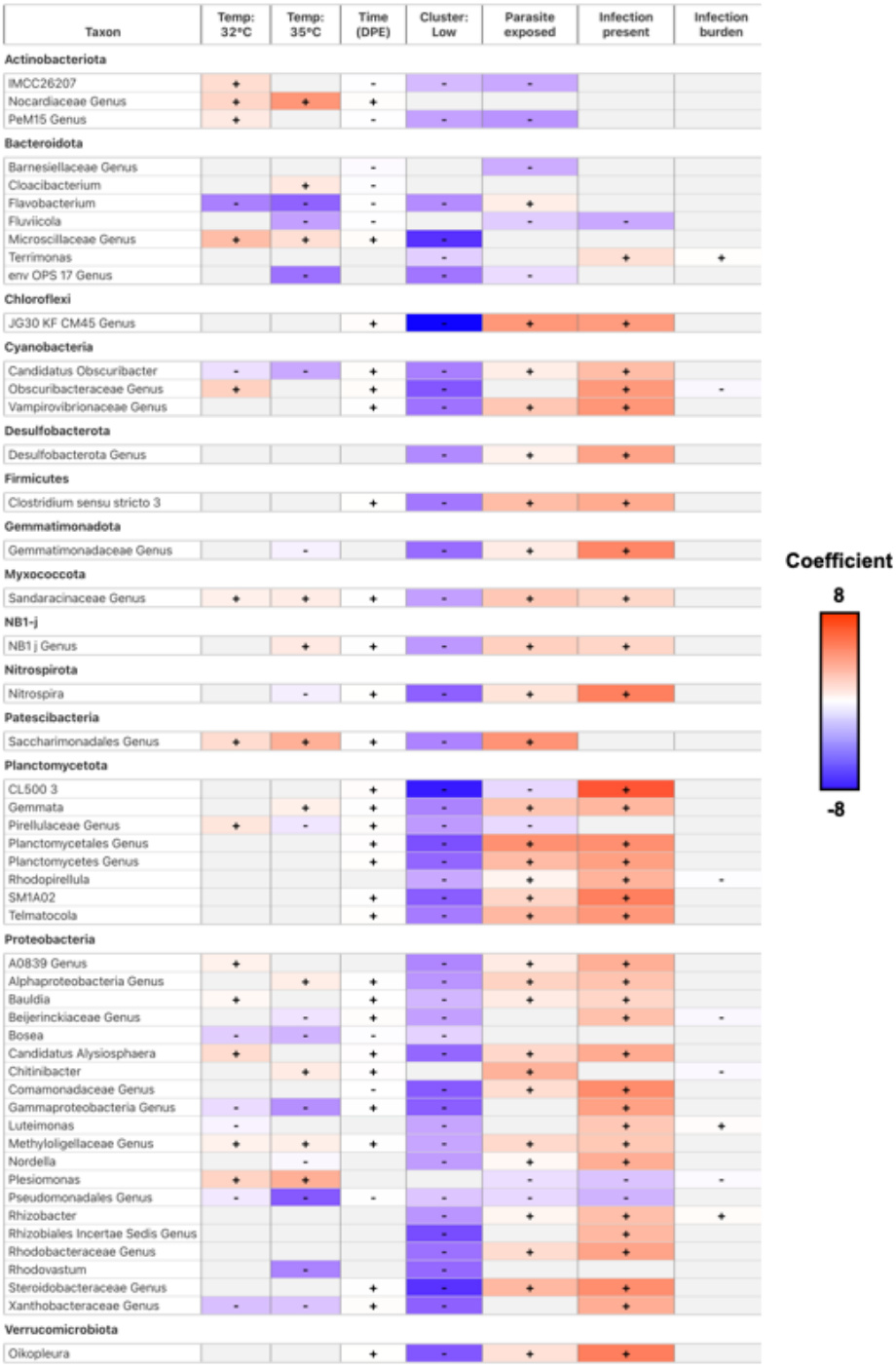


Figure 12: A heatmap of model coefficient values of the top 50 statistically significant abundant gut microbial taxa identified by MaAsLin2.

The color of each cell represents the coefficient value and direction (red is positive, blue is negative). A “+” or “-” indicates a statistically significant association was observed between taxon abundance and a covariate. Gray colored cells indicate a significant effect was not observed.

A heatmap of model coefficient values of the top 50 statistically significant abundant gut microbial taxa identified by MaAsLin2 (continued)



CHAPTER 4

EVALUATING WHETHER STRESS HISTORY IMPACTS ZEBRAFISH GUT MICROBIOME'S RESISTANCE AND RESILIENCY TO ENVIRONMENTAL STRESSORS

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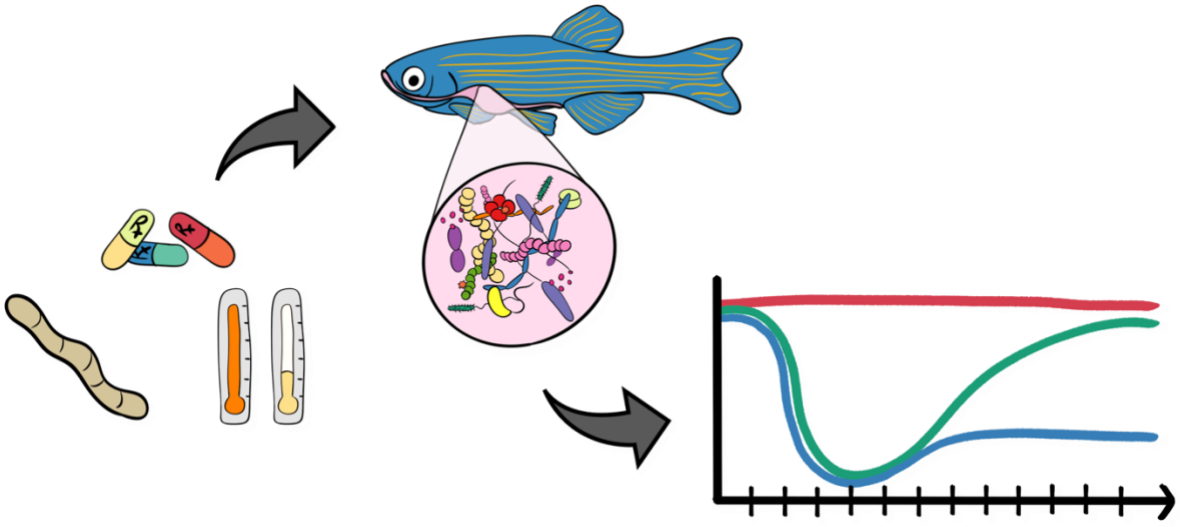


Abstract

Anthropogenic stressors are reshaping ecosystems through intersecting anthropogenic forces that may exceed the adaptive capacities of vertebrate holobionts. While gut microbiomes are key mediators of host health, little is known about how mounting environmental pressures affect host-microbiome system responses to future perturbations. Historical contingency – where past events shape future community assembly – has been studied in microbial ecosystems, but its role in defining host-microbiome responses to perturbations remains unclear. We used zebrafish (*Danio rerio*) to investigate whether host-microbiome system responses to parasite exposure and recovery from prior stressors are contingent upon historical stressors. We sequentially exposed 720 adult zebrafish to antibiotics (mixture of streptomycin, ciprofloxacin, and ampicillin), elevated temperature (32°C), and/or the intestinal parasitic helminth (*Pseudocapillaria tomentosa*) across eight unique exposure regimes, then quantified host health outcomes, gut microbiome diversity and composition, and host intestinal gene expression using integrative multi-omic analysis. We found that cumulative stress exposure produced dose-dependent increases in host mortality, with mortality rising from 9.4% in unstressed fish to 27.8% after two stressors, regardless of parasite exposure. Prior antibiotic treatment paradoxically reduced parasite burden but increased mortality, indicating stress history can uncouple pathogen load and survival. Gut microbiome responses were stress-specific: antibiotics amplified parasite-associated changes, heat stress produced minimal compositional shifts, and combined stressors created unique microbial assemblages that persisted beyond stress cessation. Host transcriptional responses showed similar historical contingency, with distinct unique expression states that persisted into recovery phases. Most importantly, host-microbiome response itself was historically contingent, where prior stressors enhanced, redirected, or disrupted coupling between host transcriptional programs and microbial community dynamics. Our integrative analysis

identified *Culicoidibacter* as a putative health indicator (negatively correlated with mortality) and *Tundrisphaera* and *Cloacibacterium* as disease risk indicators. Together, these findings demonstrate that historical contingency operates across multiple levels within holobionts, fundamentally influencing both resistance to future perturbations and resilience during recovery phases, with profound implications for understanding organismal responses to the sequential stressors characteristic of the Anthropocene.

Introduction

Anthropogenic stressors are reshaping ecosystems at an unprecedented pace and scale, driven by intersecting forces such as agricultural nutrient runoff, rapid climate change, and the expansion of novel infectious diseases [4,6]. Ecologists worry that these converging pressures exceed the ecological and evolutionary adaptive capacities of vertebrates to resist or recover, which are resulting in biodiversity loss, turnover in community composition, and disruption of their functions that sustain ecosystem stability [7,8]. Recent research is illuminating how wildlife respond to ecological disruption [15,207], however, this work has largely neglected an integral component to animal health: their microbial symbionts [268]. Increasing evidence implicates the gut microbiome, the community of microorganisms and their genetic material, as a key mediator of host health and homeostasis [34,269]. Yet, little is known about how the mounting pressures of the Anthropocene will affect gut microbiomes to influence host health.

Understanding how gut microbial communities respond to environmental stressors is particularly important given the gut microbiome's central role in metabolizing nutrients, fighting pathogens, and cross-organ communication [48]. Prior work has investigated the effects of one or more stressors on the microbiome and its host, but few studies have examined how past

exposure to multiple stressors affects vertebrate host-microbiome systems [110]. Past events that affect community assembly are classified as historically contingent events, which can be either biotic (e.g., parasite exposure) or abiotic (e.g., antibiotic exposure, heat stress) and can inhibit or facilitate community restructuring through alterations of ecosystem niches [16]. While historical contingency has been studied in microbial ecosystems to some degree (e.g., priority effects, competitive exclusion)[175], much less is known about whether the host-associated microbial communities' response to a future perturbation is historically contingent upon past exposure to stressors [14,174,270]. More specifically, whether the gut microbiome's ability to maintain host health and homeostasis in the face of a perturbation is historically contingent. Therefore, determining whether host-microbiome system responses to future perturbations are historically contingent is crucial for predicting the resiliency of host-associated microbial ecosystems to increasing anthropogenic stressors.

To address these gaps, we used the aquatic vertebrate model system zebrafish (*Danio rerio*) to interrogate how exposure to multiple historical environmental stressors affects the host and its gut microbiome’s response to a future perturbation. To do so, we sequentially exposed 720 adult 5D strain zebrafish to one of eight pairwise combinations of antibiotics (mixture of streptomycin, ciprofloxacin, and ampicillin) and elevated temperature (32°C), as well as the intestinal helminth, *Pseudocapillaria tomentosa* (Fig. 13). We quantified baseline host-microbiome responses to parasite exposure; then evaluated how prior antibiotic or heat stress, alone or in combination, modified those responses; and finally assessed host-microbiome resiliency in the absence of parasite exposure. We further integrated microbiome and host gene expression to evaluate how host-microbiome association varies as a function of historical

exposure regimes. In so doing, our work reveals that historical exposures dictates how holobionts respond to new stressors.

Results

Host health outcomes vary with parasite exposure and prior stress history

To determine whether the host-microbiome's response a perturbation is contingent upon historical exposure to environmental stressors we reared 720 adult 5D strain zebrafish, and exposed fish to one of eight pairwise combinations of exposure regimes, including antibiotics, a temperature increase, and/or an intestinal helminthic parasite Pseudocapillaria tomentosa (Fig. 14; Table 1). Fish within the antibiotic group were exposed to antibiotics (A+) or controls (A-) from days 0 to 14, fish within the temperature group were exposed to heat stress (T+) or ambient temperatures (T-) from days 15 to 29, and fish in the parasite group were exposed to parasites (P+) or no parasites (P-) from days 30 to 60. We first evaluated host physiological response to parasite exposure by calculating integrated final percent mortality. To do so, we subtract the total number of starting fish by the final total number of samples we collected by the conclusion of the experiment. When comparing parasite exposure on mortality outcomes between fish with similar stress histories (e.g., A- T- P- vs A- T- P+, A+ T+ P- vs A+ T+ P+), pairwise Chi-Square tests did not find any significant differences in mortality ($P>0.05$; Fig. 14A; Table S2.1.3). These results indicate that after 30 days of parasite exposure, fish mortality did not differ between control and parasite exposed fish with similar stress histories.

Next, we assessed percent mortality across cumulative stress histories. A Chi-square test detected a significant association between the number of prior stressors and mortality ($\chi^2 = 22.54$, $df = 2$, $P < 0.05$; Fig. 14; Table S2.2.2). Percent mortality increased from 9.4 percent in

fish with no stress history (17 of 180 dead) to 15.6 percent after one stressor (56 of 360 dead) and 27.8 percent after two stressors (50 of 180 dead). Pairwise tests showed that the two-stressor cohort died more frequently than the zero-stressor ($P < 0.05$; Table S2.2.3) and one-stressor cohorts ($P < 0.05$; Table S2.2.3), whereas the difference between zero and one stressor was not significant ($P < 0.05$; Table S2.2.3). To assess whether there was a systematic increase or decrease in mortality across stressor histories, we conducted a Cochran-Armitage trend test between groups with zero, one, and two past stressor histories. A trend test confirmed an increase in mortality with each added stressor ($P < 0.05$; Table S2.2.4). Stratifying by parasite exposure did not alter this pattern, where history and pathogen interaction remained significant ($\chi^2 = 18.39$, $df = 2$, $P < 0.05$; Table S2.2.6), and within each stress history the presence of the parasite failed to change mortality ($P > 0.05$). These results indicate that after 30 days of parasite exposure, cumulative environmental stress, rather than parasite exposure alone, is the main driver of zebrafish mortality.

Finally, we sought to determine whether exposure to historical stressors impacted infection outcomes. From day 30 until the conclusion of the experiment at day 60, Pseudocapillaria tomentosa eggs were continuously exposed to fish assigned to the parasite exposure groups. On day 60, fish were histologically assessed for infection, and the total number of intestinal worms was counted. We compared infection outcomes between parasite-exposed groups by assessing infection prevalence and mean worm burden (average number of worms of only infection fish). A Chi-square test showed no overall difference in infection prevalence among parasite-exposed groups ($P > 0.05$), although pairwise comparison revealed lower prevalence in the dual-stressor group (A+ T+ P+; 21.4% or 15/70 positively infected fish) compared with the parasite-only group ($P < 0.05$; 37.8% or 31/82 positively infected fish; Table

S2.3.3). Consistent with these results, a negative binomial GLM followed by ANOVA showed that mean worm burden differed among groups, with a post-hoc Tukey test confirming fewer worms in the antibiotic and parasite exposure group (A+ T- P+) relative to the parasite-only group (A- T- P+) ($P < 0.05$; Table S2.4.3). In contrast, a one-way ANOVA on mean worm burden found no significant differences across treatments ($P > 0.05$; Table S2.4.2), and a *post*-hoc Tukey test corroborated this non-significance (all $P > 0.05$; Table S2.4.3). These results indicate that, among infected fish, individuals host similar numbers of worms regardless of prior stress history. Together, prior stressors do not alter parasite burden within already-infected hosts, but prior antibiotic exposure alone appears to limit infection burden. Collectively, these results indicate that zebrafish health outcomes worsen with increasing exposure to past stressors regardless of parasite exposure, but infection outcomes do not strongly associate with number of stressors.

Gut microbiome diversity and composition diverge according to stress history

To evaluate how the gut microbiome responds to parasite exposure, we built generalized linear models (GLM) to determine whether parasite exposure is associated with variation in displacement scores by the final time point at day 60 across two measures of alpha-diversity: Simpson's Index and Shannon Entropy (Table S3.1.1). Displacement is a measure of change from a reference point, in this case from mean initial baseline alpha diversity measures collected at day 0 for each unique exposure regime and tank. An ANOVA test of these GLMs revealed that alpha-diversity displacement did not differ as a function of parasite exposure by day 60. These results indicate that parasite exposure does not impact gut microbial diversity after 30 days of parasite exposure. Next, we evaluated how parasite exposure associates with gut microbial

community composition. To do so, we quantified dissimilarity amongst all samples and generated distance matrices using the Bray-Curtis and Canberra dissimilarity metrics. Using beta diversity distance matrices, we calculated displacement of personalization scores, a measure of how unique a sample's gut microbial community composition is relative to all other samples. An ANOVA test of GLMs assessing whether parasite exposure is associated with displacement of gut microbial communities revealed that displacement varied as a function of parasite exposure ($P<0.05$; Fig. 15B; Table S3.2.2). These results indicate that by day 60, gut microbial communities exposed to parasites become more unique relative to parasite unexposed gut microbial communities. Using permutational multivariate analysis of variance (PERMANOVA), we tested whether parasite exposure explained variation in gut microbial community composition. Microbial communities were significantly stratified by parasite exposure across both beta diversity metrics ($P<0.05$; Fig. 15C&D; Table S3.2.1). Additionally, we did not observe elevated beta-dispersion ($P>0.05$; Table S3.2.2), indicating that differences in gut microbial composition were not due to increased variability among individuals but rather reflected consistent shifts associated with parasite exposure. Beta-dispersion measures within-group variability, whereas personalization scores capture consistent, individual-specific changes in composition over time, which explains why these results are not always congruent. These results indicate that the differences in gut microbial community composition are driven by the effects of parasite exposure. Next, quantified differential abundance using MaAsLin2 to assess whether relative taxon abundance varies in parasite exposed fish. Our analysis revealed that 30 unique taxa at the Genus taxonomic level significantly differed ($P < 0.05$; Table S3.3.1.1). Of these, 16 genera were significantly enriched and 14 were significantly depleted relative to controls. Notably, *Vogesella*, *Polymorphobacter*, and *Pseudomonas* were enriched, while

Plesiomonas, *Culicoidibacter*, *Paenirhodobacter* were depleted in relative abundance. These results indicate that gut microbes may be differentially selected for in response to parasite exposure, or gut microbes could be driving infection outcomes..

We then evaluated how the microbiome responds to exposure to one or more historical stressors. We built GLMs to determine if parasite exposure was associated with displacement scores at day 60 as measured by gut microbial alpha (Table S3.1.1) and beta diversity (Table S3.2.2). An ANOVA detected no effect of parasite exposure on alpha-diversity displacement at day 60, indicating that gut microbial diversity returned to or differed from baseline regardless of historical stress. In contrast, Bray-Curtis displacement of beta diversity scores differed among treatments, whereas Canberra displacement did not (Table S3.2.2). Pairwise contrasts showed that every combination of parasite-exposed groups diverged from one another ($P < 0.05$; Table S3.1.3) except the comparison between A+ T+ P+ and A+ T− P+. These results suggest that prior antibiotic exposure, with or without preceding heat stress, produces comparable compositional inconsistency, while heat stress alone moderates compositional consistency. A PERMANOVA test confirmed that community composition itself varied with historical stress across both distance metrics ($P < 0.05$; Table S3.2.1), pointing to distinct endpoint assemblages rather than parallel trajectories converging on a common state. Finally, a homogeneity test of dispersion indicated significantly elevated within-group dispersion after parasite exposure (Table S3.2.2). These results indicate possible loss of deterministic assembly and an increased influence of stochastic processes when multiple stressors precede parasite exposure.

Finally, we assessed whether differential relative abundance of gut microbial members differed as a function of historical stress exposure in parasite exposed groups. Differential abundance analysis resolved 41 genus-level taxa whose relative abundance responded to the

combination of prior stress and parasite exposure ($Q < 0.05$; Table S3.3.2.1). The antibiotics-only history (A+ T- P+) produced the largest shift, with 23 significant taxa – 19 depleted and 4 enriched – highlighted by marked reductions in *Gemmataceae*, *Mycobacterium*, and *Rhodovarius* ($Q \leq 0.05$; Table S3.3.2.2), and a compensatory rise of *Uliginosibacterium*. Among parasite exposed fish with prior heat stress alone (A- T+ P+) affected only three genera, suggesting that experiencing heat stress beforehand reduces the extent of parasite-driven taxonomic changes observed in the antibiotic only exposure group. Among fish exposed to the dual stressors of antibiotics and heat stress (A+ T+ P+), 15 taxa changed – 14 enriched and 1 depleted – notably, increases in the genera *Pararhodobacter*, *Pseudoxanthomonas*, and *Rhodococcus*. Only two genera were shared between any two treatments, and directionally consistent overlap was rare (≤ 2 genera per comparison; Table S3.3.2.3), underscoring the treatment-specific nature of parasite-induced selection. Together, these results indicate that the gut microbiome's taxonomic response to parasite exposure is highly contingent on the host's stress history, where antibiotics amplify parasite-associated depletions, heat alone induces minimal change, and the dual stressor regime favors a distinct set of opportunistic taxa.

Finally, we compared the microbiome recovery outcomes of parasite unexposed fish with varying historical stressor exposure to assess how prior stress impacted microbiome recovery when no new stressor is introduced. Using GLMs we evaluated the displacement of alpha diversity scores at day 60 (Table S3.1.1). An ANOVA test found that microbiomes significantly differed in displacement of alpha diversity scores across the exposure regimes at day 60 across all alpha diversity metrics ($P < 0.05$; Table S3.1.2). A post-hoc Tukey test revealed that gut microbial diversity differed between A+ T+ P-and A- T- P- as measured by Shannon Entropy, between A+ T+ P- and A- T- P- , A- T+ P- and A+ T- P-, and A+ T+ P- and A- T+ P- as

measured by the Simpson's Index, and between A+ T+ P- and A- T+ P- as measured by observed richness ($P < 0.05$; Table S3.1.3). These results indicate that prior exposure to both antibiotics and heat leaves the microbiome furthest from its baseline diversity, reflecting compounded disruptions from multiple stressors. In contrast, antibiotic-only and heat-only histories drive distinct, intermediate recovery trajectories, suggesting that each stressor leaves a unique imprint on the microbiome that shapes how it recovers in the absence of further perturbation. This pattern highlights that the legacy of past stress exposure not only delays recovery but also alters its trajectory, implying that different stress histories may lead to divergent, alternative stable states rather than a uniform return to baseline. We then evaluated the displacement of beta diversity scores between parasite unexposed exposure regimes using GLMs (Table S3.2.2). An ANOVA test found that displacement of gut microbial community compositions significantly differed among samples as measured by Canberra metric ($P < 0.05$; Table S3.2.2), but not Bray-Curtis. A post-hoc Tukey test revealed that beta diversity displacement scores differed between A- T+ P- and A+ T- P-, and between A+ T+ P- and A+ T- P- as measured by Canberra metric. These results indicate that antibiotic and heat exposures generate divergent gut microbial community composition trajectories, with the dual-stressor history producing the greatest compositional difference from controls ($P < 0.05$; Table S3.2.3). A PERMANOVA test found significant differences in community centroids for both beta-diversity metrics ($P < 0.05$; Table S3.2.1). Additionally, a homogeneity of dispersion found elevated dispersion among gut microbial communities as measured by both beta-diversity metrics ($P < 0.05$; Table S3.2.2). A post-hoc Tukey test revealed that differences in dispersion between A+ T+ P- and A- T- P- as measured by Bray-Curtis, and differences in dispersion between A+ T- P- and A- T- P- as well as A- T+ P- and A+ T- P- as measured by Canberra metric ($P < 0.05$; Table S3.2.3). These beta-

diversity results indicate that prior antibiotics expand community heterogeneity, heat stress alone moderately perturbs composition, and their combination amplifies gut community compositional variation during recovery.

To identify specific taxa that may be impacted by or driving these recovery patterns, we quantified differential abundance among parasite unexposed exposure regimes. Differential abundance testing highlighted 73 genus-level taxa whose relative abundance shifted as a function of historical stress in parasite unexposed fish. The antibiotics-only group (A+ T- P-) showed the largest reconfiguration, with 27 significant genera: nine enriched, led by a > 4.5-fold rise in an unnamed *Gemmataceae* genus, and 18 depleted, including *Agromyces*, *Flavobacterium*, and unnamed *Cytophagales* genus ($Q \leq 0.001$). Heat stress alone (A- T+ P-) produced 23 changes, 20 of which were depletions. Notably, a 5.7-fold loss of *Culicoidibacter* and marked reductions in *Legionella* and *Ensifer*, suggesting that elevated temperature suppresses several common gut residents. The dual antibiotic + heat treatment (A+ T+ P-) also yielded 23 significant taxa, but with the opposite balance: 17 enriched and six depleted. This profile featured strong enrichment of an unnamed *Vampirovibrionaceae* genus, *Neochlamydia*, and several *Chlamydiales* lineages, alongside a pronounced depletion of *Flavobacterium* ($\log_2FC \approx -5.0$). Taxonomic overlap among treatments was limited (≤ 7 shared genera per pairwise comparison), and concordant directionality was observed for only three to five genera at most, underscoring that each stressor combination selects a largely distinct subset of gut microbes. These patterns indicate that antibiotic exposure favors opportunistic and *Gemmataceae*-related taxa, heat alone depletes multiple taxa, and the dual stressor reshapes the microbiome toward a chlamydial- and *Vampirovibrionaceae*-enriched state while exacerbating loss of key *Bacteroidota* members. Together, these results indicate that recovery of the gut microbiome to a stressor is historically

contingent, where prior stressors drives a distinct shift in diversity, composition, and key taxa leading to exposure-specific divergences from the baseline trajectory. Collectively, these results demonstrate that the gut microbiome's recovery is historically contingent, with prior stressors shaping diversity, composition, and taxon-specific responses in ways that drive distinct, exposure-specific trajectories away from the baseline state.

Host intestinal gene expression varies across exposure regimes

We sought to determine host response to parasite exposure by measuring differentially expressed genes host intestinal samples. We profiled 20,648 intestinal transcripts and identified 3,753 genes that differed between parasite-exposed and control fish ($P < 0.05$; Table S4.1.1.1), with 1,994 up-regulated and 1,759 down-regulated (Fig. 16A; Table S4.1.1.2). Strongly expressed genes included the antiviral GTPases gbp1 and gbp2, the transcription factor batf2, and proliferation markers pcna, ercc6l, and smc2, whereas notable repressed transcripts comprised the lysosomal enzyme acp2 and calcium-binding protein pvalb9. Gene-ontology (GO) enrichment of the up-regulated set highlighted chromosome segregation, DNA replication, and mitotic cell-cycle process ($P < 0.05$), while down-regulated genes were associated with lipid transport, xenobiotic metabolic process, and response to estrogen stimulus ($P < 0.05$; Table S4.2.1.1). Concordantly, KEGG analysis found up-regulated genes in Cell cycle, DNA replication, and Mismatch repair pathways and down-regulated genes in PPAR signaling, FoxO signaling, and arachidonic-acid metabolism ($P < 0.05$; Table S4.2.1.2). Collectively, these data reveal that *P. tomentosa* triggers a vigorous host response of cell proliferation, DNA repair, and innate immunity while suppressing lipid-handling and detoxification pathways, indicating a shift toward epithelial renewal and immune defense at the expense of metabolic homeostasis.

Pairwise DESeq2 contrasts ($P < 0.05$; Fig. 16B; Table S4.1.3.1) against the parasite-only baseline (A- T- P+) revealed that prior heat stress (A- T+ P+) elicited the largest intestinal response, with 137 differentially expressed genes (DEGs; 93 up-, 44 down-regulated); enrichment analysis linked these genes to actomyosin-structure organization, reactive-oxygen-species metabolism, and one-carbon compound transport, consistent with cytoskeletal remodeling and redox defense during infection. Antibiotic history (A+ T- P+) produced a moderate shift of 49 DEGs (26 up, 23 down), dominated by strong repression of xenobiotic-responsive transcripts such as pvalb9 and zmp:0000000629 and KEGG over-representation of stress response profiles of drug metabolism-cytochrome P450 and related xenobiotic pathways, indicating a detoxification response carried into the infection phase. We did not find any significantly enriched GO or KEGG pathways associated with heat shock genes or proteins. In contrast, the dual antibiotic-plus-heat history (A+ T+ P+) yielded only 18 DEGs (5 up, 13 down); these clustered in GO terms associated with acrosome reaction and cell-cell adhesion, and in KEGG pathways for arginine and proline metabolism and cytoskeletal regulation in muscle cells, pointing to a narrowly focused adjustment rather than broad activation. Together, these data show that prior heat stress amplifies, antibiotics redirect toward xenobiotic handling, and combined stressors markedly dampen the host transcriptional reaction to *P. tomentosa*, demonstrating that both the magnitude and functional orientation of the parasite exposure response are historically contingent on preceding environmental stress.

Next, we next asked whether prior stress history leaves a lasting transcriptional imprint on recovery once all perturbations cease. Using DESeq2 contrasts ($P < 0.05$; Fig. 16C; Table S4.1.4.1) against the unstressed control (A- T- P-), we detected 35 differentially expressed genes (DEGs) after antibiotics alone (A+ T- P-; 8 up- and 27 down-regulated), 47 DEGs after heat

alone (A- T+ P-; 6 up, 41 down), and 117 DEGs after the combined stressors (A+ T+ P-; 18 up, 99 down) (Table S4.1.4.2). Antibiotic pre-exposure was marked by strong repression of xenobiotic-responsive transcripts (LOC100147849, si:ch211-125e6.14, pla2g3; $\log_2FC \leq -4.8$) alongside induction of the vacuolar proton-pump modulator ncoa7a and the ankyrin-repeat protein ankrd66 ($\log_2FC \geq 3.2$). KEGG enrichment for this comparison converged on drug metabolism-cytochrome P450, metabolism of xenobiotics by P450, and other drug-metabolizing enzymes (FDR < 0.05), indicating stress response.

fHeat history produced a largely suppressive profile dominated by vitellogenin (vtg5/7/8, $\log_2FC \leq -9.4$) and the cysteine protease inhibitor cpdb ($\log_2FC = -18.4$); GO terms enriched among the down-regulated set included actomyosin-structure organization and reactive-oxygen-species metabolic process (FDR < 0.05), suggesting reduced contractile activity and altered redox handling during the recovery phase. The dual antibiotic + heat history yielded the broadest, predominantly repressive response, exemplified by silencing of class II MHC antigen-presentation gene mhc2dgb and interferon effector mxa ($\log_2FC \approx -2.7$) by robust induction of the histone-like repeat LOC137491340 ($\log_2FC \approx 19.5$). Although GO terms were limited, enrichment pointed to acrosome reaction and cell-cell adhesion, while KEGG pathways highlighted arginine and proline metabolism and cytoskeletal regulation in muscle cells (FDR < 0.05). Together, these results indicate that antibiotics and heat stress each down-regulate distinct gene sets – detoxification and cytoskeletal/redox modules, respectively, whereas their combination triggers a broader wave of gene repression – indicating that recovery trajectories of host transcription are strongly contingent on the type and number of previous stressors. Together, these results indicate that host response to parasite exposure or recovery varies depending on exposure to past stressors.

Finally, to assess how prior stress shapes host intestinal transcriptional response to *P. tomentosa* or the recovery phase, we compared every exposure regime history with the control (A- T- P-) at an FDR < 0.05. Parasite infection alone (A- T- P+) perturbed 3,753 genes, 1,994 up-regulated and 1,759 down-regulated, or 18% of the transcriptome (Fig. 16D; Table S4.1.1.1). When infection followed antibiotics (A+ T- P+) or heat (A- T+ P+), the response nearly doubled to 6,473 (3,453 up, 3,020 down; 31%) and 6,196 (3,235 up, 2,961 down; 30%) genes, respectively, whereas the dual antibiotic-plus-heat history (A+ T+ P+) shifted 5,017 genes (2,603 up, 2,414 down; 24%). Across all parasite-bearing groups, strongly expressed transcripts included the antiviral GTPases gbp1/gbp2, the transcription factor batf2, and proliferation markers pcna, ercc6l, and smc2, while repressed transcripts featured the lysosomal enzyme acp2 and calcium-binding protein pvalb9. GO enrichment of the up-regulated sets consistently highlighted DNA replication, chromosome segregation, and mitotic cell-cycle processes ($P < 0.05$; Table S4.2.1.1), and KEGG analysis pointed to Cell cycle, DNA replication, and Mismatch repair pathways ($P < 0.05$; Table S4.2.1.2). Conversely, down-regulated genes were enriched for lipid transport, xenobiotic metabolic process, and response to estrogen stimulus, with KEGG over-representation of PPAR and FoxO signaling and arachidonic-acid metabolism ($P < 0.05$; Table S4.2.1.2).

Recovery cohorts without parasites displayed far fewer changes: antibiotics alone (A+ T- P-) yielded 35 DEGs (8 up, 27 down), heat alone (A- T+ P-) 47 DEGs (6 up, 41 down), and the combined stressors (A+ T+ P-) 117 DEGs (18 up, 99 down), each affecting < 0.6% of the transcriptome (Fig. 16; Table S4.1.4.1). The antibiotic legacy was dominated by repression of xenobiotic-responsive genes such as LOC100147849 and pla2g3 with KEGG enrichment for cytochrome P450 pathways ($P < 0.05$; Table S4.2.4.2), whereas the heat imprint suppressed

vitellogenins (vtg5/7/8) and the cysteine protease inhibitor cpdb, enriching GO terms linked to actomyosin organization and reactive-oxygen-species metabolism ($P < 0.05$; Table S4.2.4.1). Dual stressors produced the broadest, largely repressive profile, silencing antigen-presentation (mhc2dgb) and interferon-effector (mxa) genes yet inducing the histone-like repeat LOC137491340 ($\log_2FC \approx 19.5$), with enrichment for arginine-proline metabolism and cell-cell adhesion pathways ($P < 0.05$; Table S4.2.4.2). Collectively, these data show that P. tomentosa exposure is the principal catalyst of the exposures we tested host intestinal transcriptional remodeling, but its magnitude and functional orientation are strongly contingent on prior environmental stress, where antibiotics and heat amplify a proliferation-and-repair response, while their absence manifests a metabolic realignment towards baseline.

Multi-omic integration reveals stress-specific taxon-gene coupling

To deepen our analysis of how host-microbiome association varies as a function of historical exposures, we integrated the results from the differential abundance and differential gene expression analyses to evaluate how gut taxon abundance associates with host intestinal gene expression. First, we evaluated whether the 3,753 parasite-responsive DEGs associated with the 30 parasite-responsive DATs and recovered 11,681 gene-taxon correlations at $P < 0.05$ (Table S5.1.2). When we recomputed these associations as partial correlations while adjusting for worm burden, 80 remained significant at $FDR < 0.1$ (Table S5.1.3). The pattern was strongly skewed toward inverse relationships (73 of 80 edges), indicating that parasite-induced gene activation generally paralleled depletion of particular microbes. The most extreme negative edge joined hephl1b, a predicted ferroxidase, with *Paucibacter* ($\rho = -0.93$; Table S5.1.2), whereas the top positive edge connected the uncharacterised transcript LOC101884777 with *Vogesella* ($\rho =$

0.88). Two genera, *Culicoidibacter* and *Paucibacter*, emerged as network hubs, each engaging more than 40 host genes that included cell-cycle regulators (cdk10, srpk1b) and innate-immune modulators (gng10, bbc3). GO enrichment of the *Culicoidibacter*-linked gene set highlighted chromosome segregation and mitotic cell-cycle process ($P < 0.05$; Table S5.6.1), while KEGG analysis of the *Paucibacter* module pointed to Cell cycle and DNA replication pathways ($P < 0.05$; Table S5.6.1), suggesting that these taxa may be tightly coupled to epithelial turnover during infection. Together, the dense, and predominantly negative, gene-microbe connections imply that shifts in a handful of bacterial taxa coincide with broad transcriptional re-programming of the host intestine, supporting the idea that the zebrafish host-microbiome system mounts a coordinated, and partly compensatory, response to *Pseudocapillaria tomentosa*.

To assess whether prior stress exposure influences parasite exposed host-microbe interactions, we merged the 204 stress-responsive DEGs with the 41 DATs obtained for each treatment and recalculated gene-taxon associations while conditioning on worm burden. Across all three contrasts (A+ T- P+, A- T+ P+, A+ T+ P+) only two edges survived (FDR < 0.1; Table S5.3.2). The antibiotic exposed group (A+ T- P+) contributed a single positive link between the splicing-factor transcript zranb2 and the opportunistic genus *Uliginosibacterium* ($\rho = 0.68$), whereas the heat stress group (A- T+ P+) yielded one negative link joining the uncharacterised transcript zmp:0000000629 with *Polymorphobacter* ($\rho = -0.72$). The dual-stressor history (A+ T+ P+) produced no significant edges despite harboring comparable sets of DEGs and DATs, implying that the combined stress dampens host-microbiota coupling. GO enrichment of the two gene cohorts pointed to RNA splicing, mRNA processing, and other nucleus-associated categories for the *Uliginosibacterium* module, and the response to oxidative stress and cellular iron ion homeostasis for the *Polymorphobacter* module ($P < 0.05$; Table S5.6.3); no KEGG

pathway reached significance. Together, these findings indicate that prior stress had minimal impact on gene-taxon associations among parasite-exposed fish. Across all contrasts, only two significant links emerged – one in the heat stress comparison and one in the antibiotic comparison – while the dual stressor produced none. This scarcity suggests that historical stress does not substantially modify host-microbiome response beyond the effects of parasite exposure alone.

To determine whether earlier stressors imprint coordinated host-microbe relationships during the recovery phase, we combined the DEGs and DATs from each stress history with those of the unstressed baseline (A- T- P-) and recalculated gene-taxon partial correlations while conditioning on worm burden (Table S5.4.2). The antibiotic-only group (A+ T- P-) yielded a sparse network of 16 significant edges (FDR < 0.1), all modest and predominantly positive; the strongest linked the wound-repair transcripts adam12b and pla2g3 with increased *Flavobacterium* abundance ($\rho \approx 0.43$). Heat alone (A- T+ P-) produced a denser web of 45 edges, 31 negative and 14 positive, featuring near-perfect positive associations between several uncharacterised LOC transcripts and the opportunist *Pseudoxanthomonas* ($\rho \geq 0.95$), whereas immune regulators such as maml3 were inversely coupled to *Bosea* and *Pirellula*. The combined antibiotic + heat history (A+ T+ P-) generated the largest network, 83 edges with a strong negative bias (46 of 83), typified by inverse links between apoptosis-related genes (tnfa, rnf213a) and *Pirellula*, alongside positive correlations connecting stress-response genes (znf1069, ly6m5) to Legionella and Flavobacterium. Across treatments, *Pseudoxanthomonas* and *Pirellula* emerged as recurrent hubs, each interacting with ≥ 10 host transcripts, suggesting they are pivotal partners in stress-specific recovery response. No GO category passed multiple-testing correction, but KEGG analysis of the dual-stressor module revealed four enriched pathways–

Ribosome, Oxidative phosphorylation, Protein processing in endoplasmic reticulum, and Arginine & proline metabolism ($P < 0.05$; Table S5.6.4)–indicating links between microbial shifts and host translational and energetic rewiring. Together, the step-wise increase in network size and negative associations from antibiotic-only to dual-stressor exposure regimes shows that accumulated stress amplifies and polarizes host-microbiota coupling during recovery, with distinct taxa and gene cohorts orchestrating treatment-specific recovery trajectories.

To evaluate treatment-wide associations between host transcription and microbial shifts, we combined every set of significantly differentially expressed genes (DEGs) and differentially abundant taxa (DATs) and recalculated gene-taxon partial correlations while adjusting for worm burden. This global screen yielded 554 significant edges out of 700 tests at FDR < 0.1, spanning 139 host genes and 47 bacterial taxa (Fig. 17A; Table S5.1.2). Associations were moderate to strong ($|\rho|$ 0.35-0.92) and slightly biased toward positive directionality (59% positive), indicating that increases in many taxa coincided with up-regulation of linked host genes. Of these significant partial correlations, 52.8% of these network edges were captured by ten genera (Table S5.1.3). Notably, these correlations were led by *Culicoidibacter* (65 links, mean $|\rho| = 0.53$) and *Tundrisphaera* (46 links, $|\rho| = 0.58$), followed by *Polymorphobacter*, *Rubrivivax*, and *Bosea* (≥ 21 edges each). Extremes illustrated the range of coupling: the ferroxidase homolog argo1a was inversely paired with Bosea ($\rho = -0.88$; adjusted $P \approx 1.8 \times 10^{-2}$), whereas the mitochondrial enzyme gcdhb showed a strong positive link with *Ensifer* ($\rho = 0.91$; $P < 0.05$; Table S5.1.2). GO enrichment of hub-associated gene sets repeatedly highlighted DNA replication and mitotic cell-cycle processes, and KEGG analysis recovered cell cycle, DNA replication, and mismatch repair pathways ($P < 0.05$; Table S5.6.1). Together, the dense, hub-dominated network suggests that a small set of bacterial taxa are central to host-microbiome response to stress exposure.

Focusing on *Culicoidibacter*, the preeminent hub across all exposure regimes, reveals a nuanced, treatment-specific pattern. Nearly three-quarters of its 65 significant correlations were captured in the comparison between controls and parasite exposed fish without prior stress (47 edges in A- T- P- vs A- T- P+; Table S5.1.3), yet additional links emerged after antibiotics (two edges) and heat stress (eleven edges), indicating the responsiveness of *Culicoidibacter's* association with host response. Most *Culicoidibacter* edges were negative (81%), among the negative correlations, the strongest involving cell adhesion gene, cldn5b ($\rho = -0.64$), and a cancer-implicated gene, wrap53 ($\rho = 0.86$). GO analysis of the Culicoidibacter-linked gene cohort enriched for nucleus organizing, and modification of catabolic and metabolic processes ($P < 0.05$; Table S5.6.1), while KEGG terms centered on proteasome, apoptosis, signaling pathways, folate transport and metabolism, and base excision repair ($P < 0.05$; Table S5.6.1). Moreover, upregulation of *Culicoidibacter*-linked genes for nuclear organization and ubiquitin-mediated protein catabolism, alongside KEGG pathways including proteasome, apoptosis, and base excision repair, suggests associations with processes central to epithelial turnover and quality control. Additional enrichment for NOD-like receptor and C-type lectin signaling pathways points to links with innate immune surveillance, while folate transport and metabolism implicate interactions with host one-carbon metabolism and nucleotide synthesis. These patterns, combined with *Culicoidibacter's* depleted abundance under parasite exposure groups and heat stress, indicate that its abundance is highest when cellular renewal and proteostatic regulation are intact, but declines under conditions that disrupt barrier integrity and metabolic balance. Together, these findings indicate *Culicoidibacter* as a putative keystone taxon whose associations span epithelial maintenance, immune modulation, and host energy allocation,

potentially aligning its ecological success with host resistance or resilience to environmental stressors.

Motivated by the stressor-specific patterns in gene-taxon coupling described above, we correlated tank-level percent mortality with the relative abundance of the ten most strongly differentially abundant taxa across all exposure regimes. This approach allowed us to identify gut microbial taxa whose abundance consistently tracked with zebrafish mortality across the different treatments. Across each of the exposure regimes, Pearson correlations between tank-level mortality and the ten taxa that were most differentially abundant highlighted three genera with statistically supported associations (Fig. 17B; Table S6.1.1). *Culicoidibacter* showed a moderate, negative correlation with percent mortality ($\rho = -0.57$, $n = 24$, $P < 0.05$; Table S6.1.1), indicating that tanks harboring greater relative abundances of this genus tended to lose fewer fish. In contrast, *Tundrisphaera* ($\rho = 0.43$, $P < 0.05$; Table S6.1.1) and *Cloacibacterium* ($\rho = 0.41$, $P = 0.050$; Table S6.1.1) correlated positively with death rates, suggesting that higher levels of these taxa accompany, or possibly contribute to, heightened lethality. The remaining seven genera, including *Vogesella* and *Acinetobacter*, exhibited weaker, non-significant relationships ($P > 0.05$; Table S6.1.1). These findings indicate that *Culicoidibacter* may act as a putative health indicator, while *Tundrisphaera* and *Cloacibacterium* as putative disease risk indicators. Collectively, these results suggest that parasite infection alone triggers strong links between host genes and gut microbes, that prior stress reshapes or even erases these links during infection, and that, during recovery, host-microbiome coupling re-emerges in patterns that depend on the type and number of earlier stressors and associate with the depletion or enrichment of health- or disease-promoting taxa.

Discussion

In this study, we integrated the results of mortality, infection outcomes, gut microbiome analyses, and host intestinal gene expression in order to investigate whether a holobiont's response to a perturbation is historically contingent upon past exposure to stressors. We found that the type and number of prior stress history shapes how a holobiont responds to new stressors and how it recovers from previous stressors. Moreover, our integrative analytical approach identified several taxa that correlate with host intestinal gene expression and mortality outcomes in a stress regime contingent manner, and may act as putative markers of health or disease. Together, our study demonstrates that the host-microbiome system response to perturbation and recovery from stressors is historically contingent, which has important implications for predicting and managing the increasing pressures of anthropogenetic stressors impacting wildlife populations and human health.

Our findings reveal a dose-dependent relationship between cumulative stress exposure and host mortality, with mortality increasing progressively as the number of prior stressors increases. This pattern is consistent with previous work showing that cumulative environmental stress, rather than parasite exposure alone, is the primary driver of negative health outcomes [271–273], as parasite exposure by itself did not significantly increase zebrafish mortality relative to controls. This finding is consistent with our prior work that found that despite severe intestinal pathology, adult 5D strain zebrafish displayed low mortality after 12 weeks post-parasite exposure [141]. Interestingly, infection outcomes in our study showed the opposite pattern, where fish with prior antibiotic exposure had significantly lower total worm burden and infection prevalence compared to parasite-only controls, while mean worm burden did not differ among groups. These findings align with previous studies showing that antibiotic exposure can reduce parasite establishment in some host organisms [274,275], but contrast with other studies

that found the opposite effect [276]. However, in contrast to these studies, our study is unique in that antibiotic exposure preceded parasite exposure by 14 days, so it's unclear if the antibiotics had a direct impact on infection outcomes. Moreover, the observation that parasite exposure alone did not increase mortality, and may even have protective effects in some stress contexts, aligns with the hygiene hypothesis in mammals, which posits that reduced exposure to parasites and microbes can lead to increased vulnerability to subsequent challenges [277]. However, our observation that prior stressors increase mortality despite reducing infection burden contrasts with studies in other systems where reduced parasite load typically correlates with improved host survival [271,278]. Notably, we found parasite unexposed fish with dual stressor history (antibiotics and heat stress) showed higher mortality than their parasite-exposed counterparts, indicating that the presence of parasites may paradoxically reduce mortality in some stress contexts, possibly through immune system activation or other compensatory mechanisms. This paradoxical effect has been observed in some human studies where mild infection (i.e., hygiene hypothesis) can enhance host resistance to subsequent challenges through immune system priming, though the specific mechanisms remain unclear [279]. In the case of our study, one possible explanation, is that while peak infection is typically observed in zebrafish by 30 days post exposure of *P. tomentosa* [141,152,157,280], fish in our study may not have manifested peak negative health consequences, in terms of mortality, from parasite exposure. Another possible explanation is that some fish may have been co-infected with undetected pathogens, which can alter parasite virulence and host outcomes. Co-infection is known to influence pathogen transmission, disease severity, and host survival through complex interactions among pathogens, the host immune system, and environmental conditions [281]. Such interactions could help explain why parasite presence did not consistently translate into higher mortality in our

study, and why parasite-exposed fish sometimes fared better than unexposed fish under stress. Taken together, these results show that increasing stress exposure compromises host physiological outcomes, with antibiotic exposure and heat stress jointly predisposing fish to worse outcomes when faced with a subsequent perturbation of parasite exposure. These findings suggest that wildlife already challenged by a series of human-driven disturbances may be particularly vulnerable to additional stressors, even when each individual stressor might be tolerable on its own.

We found that both microbiome response to perturbation and recovery from past stressors were historically contingent. The gut microbiome's response to parasite exposure was shaped by prior stress history, with antibiotics amplifying parasite-associated taxonomic changes, heat stress alone producing minimal compositional shifts, and combined stressors creating unique microbial assemblages distinct from either stressor alone. These patterns likely reflect stress-induced alterations to host physiology and gut environment that create distinct ecological niches for microbial colonization and persistence. Additionally, recovery trajectories after stress cessation were also historically contingent, as gut microbial diversity and compositional displacement, as well as taxonomic abundance recovery patterns all varied significantly based on the type and number of prior stressors. The increased within-group dispersion seen in fish with a history of prior stressors following parasite exposure suggests a shift from deterministic to more stochastic community assembly, meaning that multiple stressors may make community outcomes less predictable and amplify the role of random colonization events. This finding aligns with ecological theory predicting that environmental stress can disrupt deterministic community assembly by altering niche availability and competitive interactions. Our results also support the concept of alternative stable states, as some

microbial communities did not return to baseline composition after stress cessation, suggesting that prior stressors can push communities across critical thresholds into persistent alternative configurations. Furthermore, these findings align with a homeorhetic framework (Waddington 1942), which conceptualizes stability as change along a stable trajectory rather than a fixed state, and may better capture how microbiomes respond to exogenous stressors across environmental gradients. This homeorhetic perspective, building on our prior work showing that deterministic and stochastic processes jointly shape microbiome assembly under combined stress [280], suggests that historical contingency operates through persistent alterations to the trajectory of community development rather than simple shifts between discrete states.

Consistent with microbiome responses to prior stressors, we found host transcriptional responses to pathogen exposure and recovery from prior stressors were also historically contingent. Parasite exposure alone triggered the largest transcriptional response, affecting over 3,700 genes with strong induction of antiviral GTPases, transcription factors, and proliferation markers, alongside repression of lipid transport and xenobiotic metabolic genes. However, fish with prior heat stress showed nearly doubled transcriptional changes compared to parasite-exposed only controls, while those with dual stressor history exhibited a dampened response. These results indicate that prior heat exposure heightens the host's transcriptional response to infection, perhaps by priming immune pathways. Heat stress history produced a largely suppressive profile dominated by marked downregulation of vitellogenin genes (vtg5/7/8) and the cysteine protease inhibitor cpdb, reflecting altered reproductive and redox pathways. Interestingly, we did not observe strong induction of heat shock proteins, which contrasts with previous reports such as Morgan et al., where zebrafish exposed acutely to elevated temperatures mounted robust hsp responses [282]. A likely explanation is that in our study heat stress occurred

30 days prior to transcriptomic sampling, and transient hsp induction may have already subsided, leaving only longer-term signatures of stress history. Conversely, the combination of stressors either depletes transcriptional reserves or channels expression into pathways specific to the stress encountered. During recovery, each exposure history left a distinct transcriptional signature that persisted after the stress had ended. Antibiotic history was characterized by strong repression of xenobiotic-responsive transcripts such as pvalb9 and pla2g3, alongside induction of vacuolar proton-pump modulators, creating a persistent detoxification signature. Heat stress history produced a largely suppressive profile dominated by marked downregulation of vitellogenin genes (vtg5/7/8) and the cysteine protease inhibitor cpdb, reflecting altered reproductive and redox pathways. Dual stressor history yielded the broadest repressive response, exemplified by silencing of class II MHC antigen-presentation genes and interferon effectors, but with robust induction of histone-like repeat genes, suggesting epigenetic modifications and immune suppression. Together, these findings demonstrate that host transcriptional response varies depending on stress history, with implications for understanding how environmental stress history shapes host resilience to future perturbations.

Recent work has demonstrated that microbiomes can buffer hosts from environmental stress through coordinated host-microbiome responses [213,217]. Using our integrative analysis of differentially abundant taxa and differentially expressed genes, we find evidence in support of a buffering capacity, but also observed that the coupling between host transcription and microbial shifts was historically contingent upon past stress. Parasite exposure alone created a dense network of gene-taxon associations, with over 11,000 significant correlations that were predominantly negative, *indicating* that parasite-induced gene activation generally paralleled depletion of particular microbes. *Culicoidibacter* served as a central node in the network,

displaying significant associations with more than forty host genes, including key regulators of the cell cycle and innate immunity, which implies a close link to epithelial renewal and host defense during infection. However, prior stress history fundamentally altered this coordination, where fish with prior antibiotic exposure showed only a single significant gene-taxon link, while those with prior heat stress exhibited three significant associations, and dual stressor history produced no significant edges despite comparable numbers of differentially expressed genes and abundant taxa. These patterns suggest that prior stress decouples the coordinated relationship between host transcriptional responses and microbial taxa observed in parasite exposed only fish. During recovery phases, host-microbiome coordination re-emerged but in stress-specific patterns, with network size and complexity increasing with accumulated stress history. These findings demonstrate that historical contingency operates not only at the level of individual host and microbiome responses, but also at the level of their integration, where prior stressors can either enhance, redirect, or disrupt the coordination between host transcriptional programs and microbial community dynamics.

Furthermore, our analysis identified fish with greater abundances of *Culicoidibacter* showed a moderate negative correlation with mortality, suggesting that individuals harboring greater relative abundances of this genus were less likely to die by the conclusion of the experiment. Conversely, we found the opposite effect with fish harboring the bacterial genera *Tundrisphaera* and *Cloacibacterium*, suggesting that higher levels of these taxa accompany, or possibly contribute to, heightened lethality. *Culicoidibacter* (formerly identified as ZOR0006) – an anaerobic, non-spore forming, Gram-positive bacterium – and *Cloacibacterium* – a facultatively anaerobic, Gram-negative bacterium – are common members of the zebrafish gut microbiome, whereas *Tundrisphaera* – a bacterium first isolated from tundra soils – is a less

common bacteria found in zebrafish gut microbiota communities [144,145,283,284]. These findings suggest that *Culicoidibacter* may act as a putative health indicator, while *Tundrisphaera* and *Cloacibacterium* may serve as putative disease risk indicators. Future work should seek to uncover the causal roles of these taxa in supporting zebrafish health mono- or co-colonization studies.

Although our multi-omic approach clarifies how prior exposures shape host-microbiome interactions, further work is needed to resolve the temporal and functional mechanisms that drive these patterns. Our transcriptional analysis was limited to the final time point, precluding assessment of how gene expression dynamics evolved throughout the stress and recovery periods. Similarly, mortality was only assessed at the conclusion of the experiment, preventing identification of when fish died relative to stressor exposure and limiting our understanding of temporal mortality patterns. Additionally, our 16S rRNA gene sequencing approach lacks functional resolution, as we cannot determine the metabolic capacity or functional potential of the microbiome to buffer hosts against parasite exposure or facilitate recovery from prior stressors. Lastly, our sampling design focused on tank-level rather than individual-level measurements, which may have obscured nuanced temporal trends and individual variation in host-microbiome responses that could provide deeper insights into stress-specific trajectories. Future work should integrate metagenomic sequencing across multiple time points to assess functional potential and metabolic output of the microbiome throughout stress and recovery phases. Additionally, transcriptomic profiling could track gene-expression trajectories during both stress exposure and recovery, clarifying the host-mediated historical contingency of the transcriptional patterns observed in this study. Furthermore, temporally integrated microbiome measures that capture how diversity and composition change over time could reveal dynamic

patterns not captured by single time point analyses. Finally, extending these analyses to other host-microbiome systems and organisms would test the generality of historical contingency effects and identify whether these patterns represent fundamental principles of host-microbiome ecology or are specific to certain systems or stressor combinations.

Collectively, our study demonstrates that historical contingency operates across multiple scales within host-microbiome systems, fundamentally influencing both resistance to future perturbations and resilience during recovery phases. Cumulative stress exposure produced dose-dependent increases in host mortality, with prior stressors either amplifying or dampening responses to subsequent challenges. Interestingly, prior antibiotic treatment produced a contradictory outcome, where prior antibiotic exposure damped infection outcomes, but heightened fish mortality implying that stress history can uncouple pathogen load and survival. Furthermore, the gut microbiome exhibits stress-specific response patterns, where antibiotics amplify parasite-associated changes, heat stress produces minimal compositional shifts, and combined stressors create unique microbial assemblages that persist beyond stress cessation. Host transcriptional responses show similar historical contingency, with distinct unique expression states that persist into recovery phases and reflect the type and number of prior stressors. Most importantly, our integrative analysis reveals that host-microbiome coordination itself is historically contingent, where prior stressors can enhance, redirect, or completely disrupt the coupling between host transcriptional programs and microbial community dynamics. These findings have profound implications for understanding how organisms respond to the multiple, sequential stressors characteristic of the Anthropocene, suggesting that stress history must be considered when predicting host-microbiome system resilience and recovery potential. Future work integrating temporal dynamics and functional potential will be essential for developing

predictive models of host-microbiome responses to environmental change and informing conservation and management strategies in an increasingly stressed world.

Methods

Fish Husbandry

Adult zebrafish *(Danio rerio*) of the 5D genetic strain (date of birth 11 Aug 2022) were used in this study. The 5D strain is an outbred wild-type line that has been maintained at the Sinnhuber Aquatic Research Laboratory (SARL) for over 15 years, where it was developed as a specific-pathogen-free (SPF) colony with no recorded history of *Pseudocapillaria tomentosa* infections [285]. Fish were reared in a single-pass, flow-through system supplied with dechlorinated Corvallis municipal water. Water temperature was recorded each morning and held between 25 °C and 28 °C; conductivity was kept within 90 – 130 µS cm^{-1}, total ammonia remained below 0.25 ppm, hardness did not exceed 0.25 ppm, and pH was stable at 7.6. These parameters, together with dissolved oxygen, were verified weekly to ensure compliance with established specific-pathogen-free (SPF) husbandry standards. The light cycle followed a 14 h light : 10 h dark regimen. Fish received Gemma Micro 300 (Skretting) twice daily at 1.5 % body mass except during designated exposure periods. All procedures conformed to the Institutional Animal Care and Use Committee (IACUC) at Oregon State University (permit number: 2022-0280).

Experimental Design and Timeline

We reared 720 adult zebrafish across 24 tanks and exposed fish to one of eight pairwise combinations of antibiotics, elevated temperature, and/or the intestinal helminth Pseudocapillaria tomentosa. Antibiotic exposure occurred from days 0-14, heat stress from days 15-29, and

parasite exposure from days 30-60. At each sampling time point between days 0-30, fecal and intestinal samples were collected for microbiome analysis (n = 72; 3 per tank). At the final time point (day 60), fecal and intestinal samples were collected, body size measurements recorded, final integrated mortality calculated, and infection prevalence and burden assessed on all surviving fish. A subset of intestinal samples (n = 72) across each disturbance regime from the final time point were selected for host transcriptomic analysis (n = 24 parasite unexposed samples, n = 48 parasite exposed samples).

Exposure Regimes

Antibiotics: streptomycin, ciprofloxacin, and ampicillin were each incorporated at 50 µg g^{-1} body weight day^{-1} into a 12 % (w v^{-1}) gelatin feed (Gell Belly Food Mix, Florida Aqua Farms) that also contained Gemma Micro 75. The medicated diet was delivered twice daily; uneaten feed was siphoned after 30 min to minimize system loading.

Temperature: thermal stress was induced with submersible titanium heaters (Hygger HG-8021) installed in each tank. Water was warmed from 28 °C to 35 °C at 3 °C day^{-1}, held at 35 °C for the designated 15-day interval, then returned to 28 °C using the same incremental schedule. Temperature loggers verified ±1 °C stability throughout the exposure.

Parasite: *Psuedocapillaria tomentosa* egg culture was maintained over past 10 years by Kent Laboratory [157]. Culture is maintained by cohabing niaive adult zerbafish with *P. tomentosa* infected fish (> 21 dpe) every 30 days.To generate a continuous effluent exposure, larvated eggs originating from a donor tank containing > 21 d post-exposure infected fish were delivered via a gravity manifold to the twelve parasite-challenge tanks; tank positions under the manifold were rotated every three days to equalize dose.

Dissections

Fish were euthanized in an ice-water slurry (< 4°C) until unresponsive. Under a stereomicroscope, micro-scissors opened the coelomic cavity; fine forceps freed the intestine, which was transferred to a glass slide containing ~500 µL system water. A 24 × 60 mm coverslip was applied for wet-mount parasite enumeration or tissue subsampling. Intestines for microbiome or transcriptome analyses were flash-frozen in liquid nitrogen and stored at -80°C.

Mortality

Integrated final percent mortality was calculated by dividing the number of collected samples by total number of samples per treatment group.

Infection assessment

Fish were histologically assessed for infection and total number of worms counted. Infection prevalence was calculated by dividing the number of fish with positive identification of infection by the total number of samples.

Fecal sample collection

At each sampling time point, fecal samples were randomly collected from 3 fish per tank. The only exception was at the final time point fecal samples were collected from all remaining surviving fish across all tanks. To collect fecal samples, individual fish were placed into a 1.4 L tank until they defecated. Fecal material was collected into a 1.5 mL microcentrifuge tube, and

immediately spun at 10k rpm for 2 minutes. Excess tank water was removed, and samples were snap frozen on dry ice and stored at -80°C until processing.

Microbial 16S rRNA library preparation and sequencing

DNA was extracted from fecal samples using the Qiagen DNeasy 96 Powersoil Pro Kit following the kit protocol. 16S rRNA gene library preparation was performed on each DNA sample following a modified version of Earth Microbiome Project 16S Illumina Amplicon Protocol. The Schloss lab dual indexing primers were used to amplify the V4 region of the small subunit (SSU) rRNA gene, using Platinum II Hot-Start PCR Master Mix (2x) from ThermoFisher Scientific. PCR products were visualized on a 1.5% agarose gel to confirm the expected ~350bp amplicon. Quantification of PCR amplicons was performed using the Quant-iT 1X ds DNA HS Assay Kit (ThermoFisher Scientific), with fluorescence measured on a BioTek Synergy H1 Hybrid Multi-Mode Plate Reader at the CQLS. Amplicons (100µg per samples) from each fish sample, along with kit and water controls, were pooled to generate a composite 16S library. This pooled library was purified using the QIAquick PCR Purification Kit and quantified using the Qubit 1x dsDNA HS Assay Kit on the Qubit 2.0 fluorometer. Sequencing was performed at the CQLS on the Illumina MiSeq platform using a paired-end 2 x 300 bp configuration.

Host transcriptomics library preparation and sequencing

Total RNA was isolated from flash-frozen zebrafish intestinal tissue using the Qiagen RNeasy Mini Kit. Tissue homogenization was performed with a mortar and pestle under liquid nitrogen to ensure thorough disruption. RNA extraction followed the RNeasy Mini Kit protocol, including

on-column DNase I digestion to remove genomic DNA contamination. RNA was eluted in 60 µL of RNase-free water. Quantification of RNA was performed using the Qubit RNA HS Assay Kit on a Qubit 2.0 fluorometer. RNA integrity was assessed at the CQLS using the Agilent 2100 Bioanalyzer. Samples meeting quality criteria were used for library preparation, which involved poly(A) enrichment followed by library preparation using the NEBNext RNA-Seq protocol. Prepared libraries were evaluated for quality using the Agilent 2100 Bioanalyzer, pooled into a single library, and quantified via qPCR. Sequencing was conducted on the Illumina NextSeq 2000 platform using a paired-end 2 × 100 bp P4 flow cell.

Bioinformatic Processing and Statistical Analyses

All bioinformatic processing, statistical testing, and data visualization were performed in R (v 4.3.3)[201]unless noted otherwise. Raw 16S rRNA amplicon reads were quality-filtered, denoised, and assigned to taxa with the nf-core/ampliseq workflow [286], whereas RNA-seq reads were trimmed, aligned, and quantified using nf-core/rnaseq [287]. Subsequent statistical analyses were two-sided with a significance threshold of $\alpha = 0.05$; all randomization and resampling steps were initialized with set.seed(42) to ensure full reproducibility.

Mortality Analysis

Integrated final percent mortality was calculated at both treatment and tank levels using the uncleaned phyloseq object (prior to post-DADA2 processing) to ensure complete mortality information. For each treatment group, we calculated percent mortality as (dead fish / total fish per treatment group) × 100, where total fish per treatment group was set to 90. For tank-level analysis, total fish per tank was set to 30. Chi-square tests for independence were used to

compare mortality between exposure regimes across all treatments. Post-hoc pairwise Chi-square tests were conducted to identify specific group differences, with statistical significance determined at $P < 0.05$. Fisher's exact test was used for 2×2 contingency tables when sample sizes were small. Additional analyses included Chi-square tests to assess the association between number of prior stressors (History: 0, 1, or 2 stressors) and mortality, with pairwise tests and trend analysis using correlation tests to evaluate dose-dependent relationships.

For historical contingency analyses, separate Chi-square tests were conducted for parasite-exposed groups (A- T- P+ vs A+ T- P+, A- T+ P+, A+ T+ P+) and parasite-unexposed groups (A- T- P- vs A+ T- P-, A- T+ P-, A+ T+ P-). Interaction analyses between history of stressors and pathogen exposure were conducted using both Chi-square tests and logistic regression models with interaction terms.

Infection Outcome Analysis

Fish were histologically assessed for infection and total number of worms counted. Infection prevalence was calculated by dividing the number of fish with positive identification of infection by the total number of samples per treatment group. Normalized worm burden was calculated as mean worms per infected fish to account for differences in infection prevalence across treatments. For infection prevalence analysis, Chi-square tests were used to assess differences between treatment groups, with post-hoc pairwise Chi-square tests for specific comparisons. For total worm burden analysis, a negative binomial generalized linear model (GLM) using MASS::glm.nb was fitted to individual-level worm count data, followed by ANOVA using car::Anova for model significance testing. Post-hoc pairwise comparisons were conducted using emmeans with Bonferroni correction. For normalized worm burden analysis

(worms per infected fish), multiple statistical approaches were employed due to the ratio nature of the data: (1) one-way ANOVA on tank-level ratios, (2) Kruskal-Wallis non-parametric test, (3) permutation tests using coin::oneway_test with 10,000 resamples, and (4) bootstrap confidence intervals with 10,000 resamples. Post-hoc analyses included Tukey HSD tests for ANOVA and pairwise permutation tests for non-parametric approaches. Model diagnostics included assessment of dispersion parameters (theta), deviance analysis, and comparison with Poisson models to ensure appropriate model selection. All analyses were conducted with seed 42 for reproducibility, and statistical significance was determined at $P < 0.05$ with appropriate multiple testing corrections applied.

Alpha Diversity Analysis

Alpha diversity metrics were calculated at the genus level using three complementary measures: Shannon diversity (measuring both richness and evenness), Simpson's diversity (measuring dominance, weighted toward abundant taxa), and richness (count of unique taxa). Data normalization was performed using a custom function that first tested for normal distribution using Anderson-Darling tests ($p \leq 0.05$ indicating non-normal distribution). For non-normal data, Tukey's power ladder transformation was applied to approximate normality, followed by min-max normalization to scale values to 0-1 range. For normally distributed data, direct min-max normalization was applied. This normalization process created standardized versions of all diversity metrics (denoted with "_norm" suffix) for fair comparison across different diversity measures.

We calculated displacement scores as a measure of change from baseline, using mean initial baseline alpha diversity measures collected at day 0 for each unique exposure regime and

tank. Displacement was calculated as the difference between current diversity scores and reference scores (Displacement = Current Score - Reference Score). Generalized linear models (GLMs) with Gaussian family were built to determine whether parasite exposure or historical stressors associated with variation in displacement scores, with ANOVA tests used to evaluate model significance. Post-hoc Tukey's HSD tests were conducted for pairwise comparisons between treatment groups.

Beta Diversity Analysis

Dissimilarity among samples was quantified using Bray-Curtis and Canberra dissimilarity metrics to generate distance matrices. Personalization scores were calculated as a measure of how unique each sample's gut microbial community composition is relative to all other samples. For each sample, we calculated the median distance to all other samples (Beta.Score) and mean distance to all other samples (Beta.Score.Mean), with higher scores indicating more "personalized" (dissimilar from others) and lower scores indicating more "typical" (similar to others) communities. These scores were normalized using the same normalization approach as alpha diversity metrics.

Displacement of personalization scores was calculated as the difference between current personalization scores and reference scores. GLMs with the Gaussian family were built to assess the displacement of gut microbial communities. Permutational multivariate analysis of variance (PERMANOVA) using adonis2 was used to assess whether exposure regimes explained variance in gut microbial community composition. Homogeneity tests of dispersion (betadisper) were conducted to evaluate within-group dispersion, with ANOVA and Tukey's HSD tests used to assess significance of dispersion differences between groups. Principal coordinate analysis

(PCoA) and constrained analysis of principal coordinates (CAP) were performed for visualization of community structure differences.

Differential Abundance Analysis

Differential abundance testing was performed using MaAsLin2 (Microbiome Multivariable Association with Linear Models) to identify genus-level taxa whose relative abundance significantly differed between exposure regimes. MaAsLin2 was implemented with log transformation (LOG) and total sum scaling (TSS) normalization. The analysis was conducted using a fixed effects model with Treatment as the primary variable, using control groups (A- T- P-) as reference for baseline comparisons and parasite-only groups (A- T- P+) as reference for historical contingency analyses.

Statistical significance was determined at q-value < 0.05, with Benjamini-Hochberg multiple testing correction applied. Effect sizes were reported as coefficients representing log-fold changes in relative abundance. Analysis was conducted separately for different experimental comparisons: (1) all treatment groups compared to control, (2) parasite-exposed groups only (A- T- P- vs A- T- P+), (3) parasite-exposed groups with prior stressors (A- T- P+, A+ T- P+, A- T+ P+, A+ T+ P+), and (4) parasite-unexposed groups with prior stressors (A- T- P-, A+ T- P-, A- T+ P-, A+ T+ P-) to assess both response to parasite exposure and recovery from prior stressors.

Results were visualized using heatmaps showing the top 50 most significant differentially abundant taxa, with taxa clustered by hierarchical clustering and treatments ordered according to experimental design. Comprehensive comparison tables were generated including summary statistics (total significant taxa, positive/negative effects per treatment), top 10 most significant

taxa by treatment, overlap analysis between treatments, and direction of effect comparisons to identify shared taxa with consistent effects across different stressor combinations.

Transcriptomic Analyses

Salmon was used for transcript quantification with length-scaled counts, yielding 20,648 intestinal transcripts across 72 samples (24 parasite unexposed samples, 48 parasite exposed samples). Raw count data was imported into R and converted to a SummarizedExperiment object, with sample names cleaned and metadata integrated, including treatment information, worm counts, and phenotypic measurements. Pre-filtering was applied to remove low-abundance transcripts, excluding genes with less than 2 counts per million (CPM) in fewer than 3 samples. Additionally, 16 specific genes were excluded from analysis due to technical artifacts or annotation issues. Variance stabilizing transformation (VST) was applied for visualization and quality control purposes.

Quality control and sample assessment were performed using sample-to-sample distance calculations with Euclidean distance on VST-transformed data, visualized as heatmaps with treatment annotations. Principal component analysis (PCA) was performed to assess sample clustering and identify potential outliers, with samples annotated by treatment combinations (antibiotics, temperature, parasite exposure) and individual stressor components. Differential gene expression analysis was conducted using DESeq2 with a design formula of ~ Treatment (8 treatment combinations), size factors estimated using the median ratio method, and dispersion estimation using local fit type for large datasets. Statistical significance was determined at $P < 0.05$ with Benjamini-Hochberg multiple testing correction, and effect sizes were calculated as log2 fold change with shrinkage using apeglm.

Four main analytical comparisons were conducted to address different aspects of the experimental design. The "All Treatment Groups" analysis provided a comprehensive assessment across all 8 treatment combinations compared to control (A- T- P-). The "Parasite Effect" analysis examined the baseline parasite response (A- T- P+ vs A- T- P-). The "Historical Contingency" analysis evaluated the effect of prior stressors on parasite response (A+ T- P+, A- T+ P+, A+ T+ P+ vs A- T- P+). Finally, the "Recovery Analysis" assessed the effect of prior stressors on recovery (A+ T- P-, A- T+ P-, A+ T+ P- vs A- T- P-).

Functional enrichment analysis was performed using clusterProfiler for gene ontology (GO) analysis with the org.Dr.eg.db database for Danio rerio, focusing on biological processes (BP) with Benjamini-Hochberg multiple testing correction (FDR < 0.1), minimum gene set size of 5 genes, and maximum gene set size of 500 genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conducted using organism 'dre' (Danio rerio) with ncbi-geneid key type and Benjamini-Hochberg multiple testing correction (FDR < 0.1). Enrichment analysis was performed separately for up-regulated and down-regulated gene sets from each comparison, with gene ID conversion performed using Entrez IDs for compatibility with enrichment databases.

The analysis was optimized for computational efficiency using BiocParallel for parallel processing (utilizing 75% of available cores), memory optimization and garbage collection, caching of intermediate results, and task granularity optimization for large datasets. Results were visualized using volcano plots showing log2 fold change versus -log10(adjusted p-value), heatmaps of top significant genes across treatments, bar plots summarizing up/down-regulated gene counts, PCA plots for sample clustering assessment, and comprehensive summary tables

with statistical metrics. All results were systematically saved and organized by analysis type, including full results, significant results only, and enrichment analysis outputs.

Differentially Abundant Taxa and Differentially Expressed Gene Partial Correlation Analysis

Spearman correlations were calculated between all differentially expressed genes and differentially abundant taxa using the cor() function. Data were normalized using z-score transformation within each gene/taxon across samples to account for different scales of measurement. Significance was assessed using permutation-based p-values with false discovery rate (FDR) correction using the Benjamini-Hochberg method. Partial Correlation Analysis: To control for potential confounding effects of parasite load, partial correlations were computed using the nptest::np.cor.test() function with worm counts as the control variable. This analysis was performed on the top 100 gene-taxa pairs ranked by absolute correlation strength, using 1000 bootstrap replicates for significance testing. Multiple Testing Correction: All p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method implemented in p.adjust() with FDR < 0.05 for initial correlations and FDR < 0.1 for partial correlations.

Differentially Abundant Taxa and Mortality Correlation Analysis

Differentially abundant taxa (DAT) were identified using MaAsLin2 (Microbiome Multivariable Association with Linear Models) with significance threshold of $q < 0.05$. Results were imported from separate MaAsLin2 analyses for each research question and processed using readr::read_tsv(). Taxonomic names were standardized by replacing periods and underscores with hyphens and spaces. Mortality data were calculated at the tank level using the complete

dataset (prior to read filtering) to ensure accurate mortality representation. For each treatment-tank combination, percent mortality was calculated as: (total fish - surviving fish) / total fish × 100, where total fish was set to 30 per tank. Pearson correlation analysis was performed between differentially abundant taxa and mortality using the stats::cor.test() function. For each analysis, the top 10 differentially abundant taxa were selected based on MaAsLin2 q-values. Correlation coefficients, p-values, and sample sizes were calculated for each taxon-mortality pair. Significance was assessed at $\alpha = 0.05$.

All statistical analyses were conducted in R using the tidyverse ecosystem. Correlation results were summarized using the gt package for publication-ready tables. Heatmaps were generated using pheatmap with color-coded correlation coefficients and significance annotations. Individual taxon-mortality plots were created using ggplot2 with mortality bars and individual abundance points overlaid. All analyses used a consistent random seed (42) for reproducibility. Results were cached using knitr caching to improve computational efficiency. Data validation included checks for missing taxa between MaAsLin2 results and abundance datasets, with appropriate warnings for mismatches. The analysis utilized R version 4.x with the following key packages: phyloseq (v1.40.0), microViz (v0.10.0), tidyverse (v2.0.0), gt (v0.9.0), pheatmap (v1.0.12), ggplot2 (v3.4.0), and stats (base R) for statistical computations.

Figure 13: Experimental design schematic showing exposures and husbandry events during the course of the study.

Across 24 tanks, 720 adult zebrafish were sequentially exposed to antibiotics, heat stress, or parasites, alone or in combination, resulting in 8 unique disturbance regimes. **1)** Fish in the antibiotic exposure groups were exposed to antibiotics from day 0 to 14. **2-3)** Fish in the heat stress groups were gradually exposed to a temperature increase from day 15 to 18; 3-4) sustained at the elevated temperature from day 18 to 25, **4-5)** and then gradually reduced the temperature back to ambient conditions from day 25 to 28. **5-6)** Fish in the parasite exposed groups were continuously exposed to the intestinal helminth Pseudocapillaria tomentosa from day 30 to 60. **1-5)** At each sampling time point between days 0 and 30, fecal and intestinal samples were collected for microbiome analysis, and body size measurements were recorded (n = 72; 3 per tank). **6)** At the final time point, fecal and intestinal samples were collected, body size measurements.

Figure 13: Experimental design schematic showing exposures and husbandry events during the course of the study (continued)

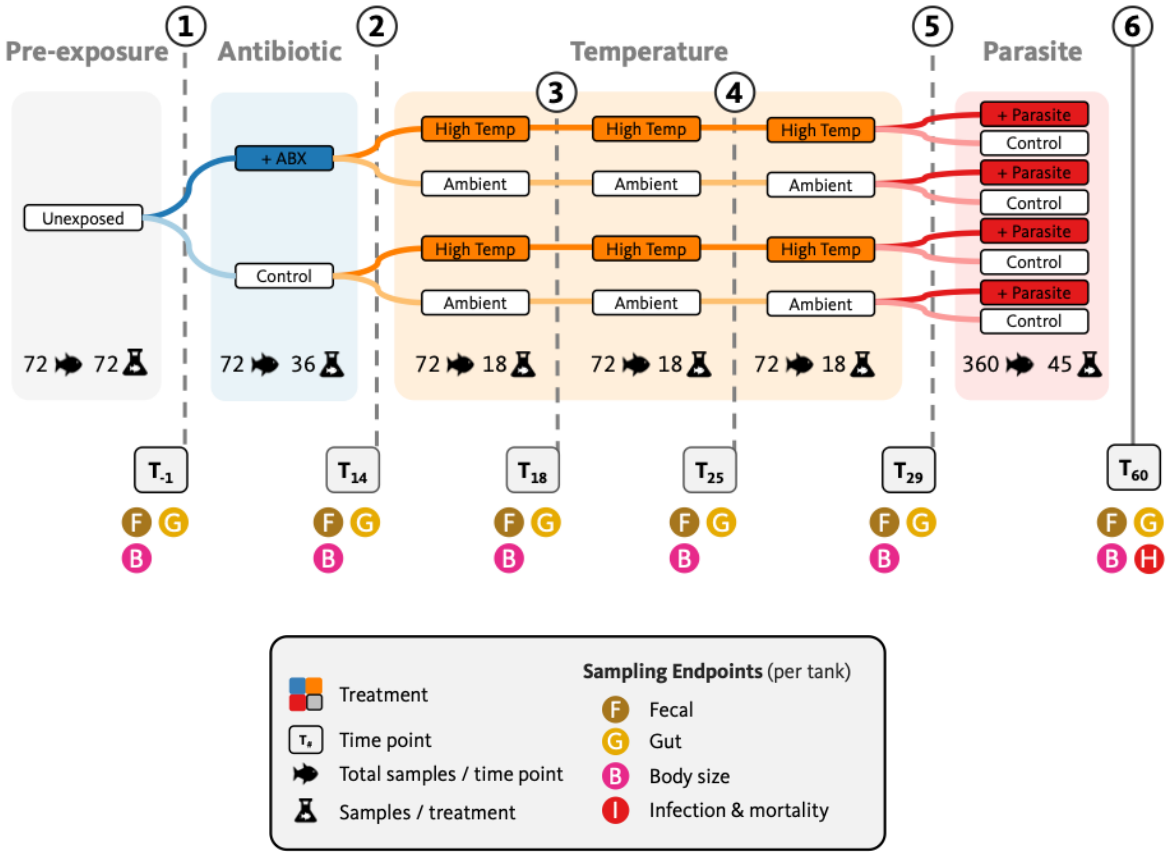


Figure 14: Effects of stressors on host physiology: mortality and infection outcomes.

(A) Integrated final percent mortality facetted by exposure regime across all exposure regimes. Integrated final percent mortality is calculated by dividing the number of collected samples by total number of samples per treatment group. Total number of mortalities are listed within parentheses. **(B)** Percent mortality by number of historical stressors between parasite unexposed and exposed fish facetted. **(C)** Infection prevalence and **(D)** mean worm counts and by exposure regimes exposed to parasites. Infection prevalence is calculated by dividing the number of fish with positive identification of infection by the total number of samples. Mean worm burden is calculated by dividing the total number of worms per exposure regime by number of positively infected fish. Total number of mortality and infected fish per exposure regime is listed within parentheses. A "*" indicates statistical significance below the "0.05" level.

Figure 14: Effects of stressors on host physiology: mortality and infection outcomes (continued)

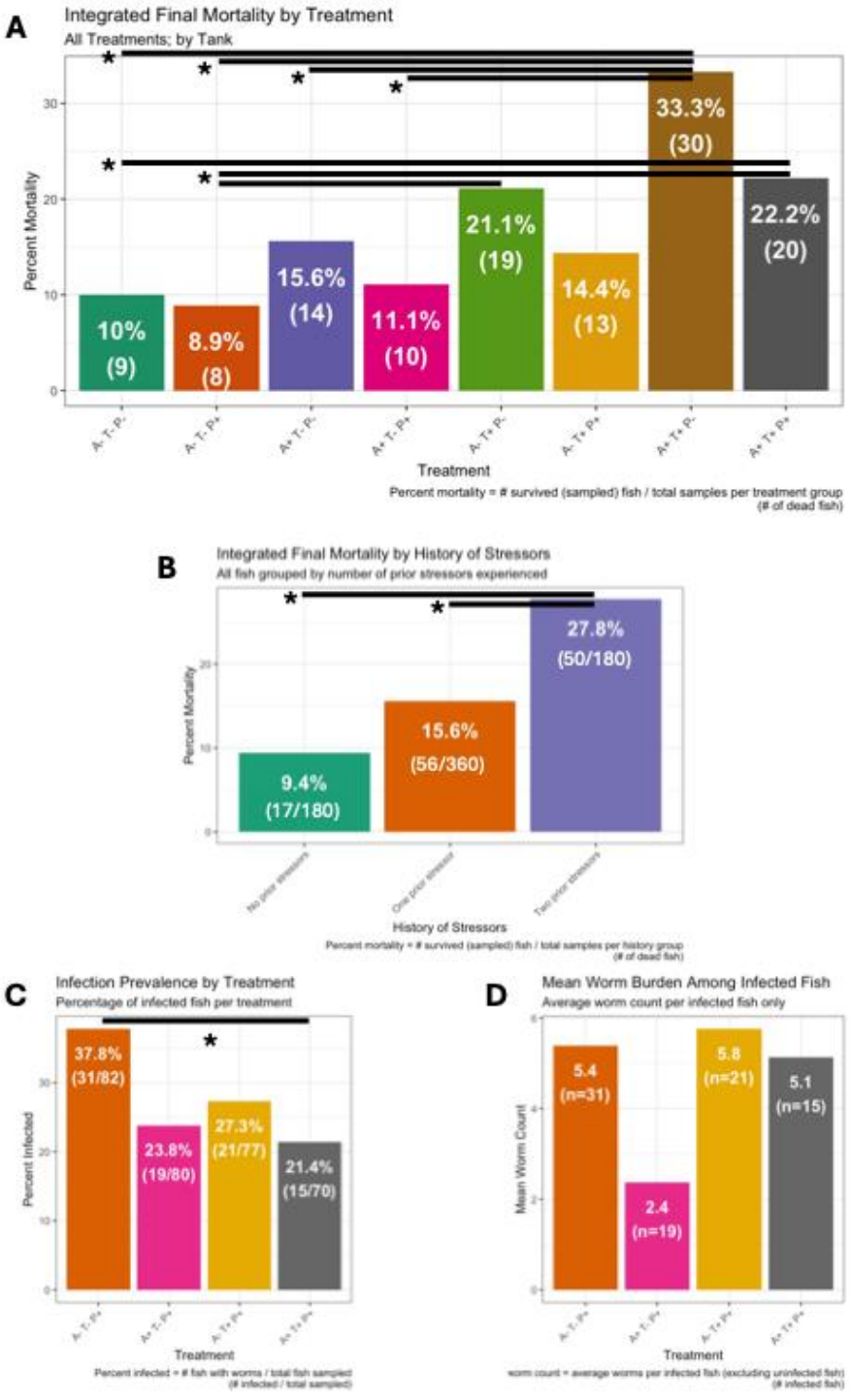


Figure 15: Effects of stressors on zebrafish gut microbiota.

Microbiome **(A)** alpha and **(B)** beta displacement scores at final time point (day 60). Capscale ordinations based on the **(C)** Bray-Curtis and **(D)** Canberra dissimilarity of gut microbiome composition constrained on the main effects of exposure regime (Treatment) at day 60. **(E)** A heatmap of model coefficient values of the top 50 statistically significant abundant gut microbial taxa identified by MaAsLin2 at day 60. The color of each cell represents the coefficient value and direction (red is positive, blue is negative). White colored cells indicate a significant effect was not observed. A "*" indicates statistical significance below the "0.05" level. Black arrows indicate direction of greatest change in the indicated covariates.

Figure 15: Effects of stressors on zebrafish gut microbiota (continued)

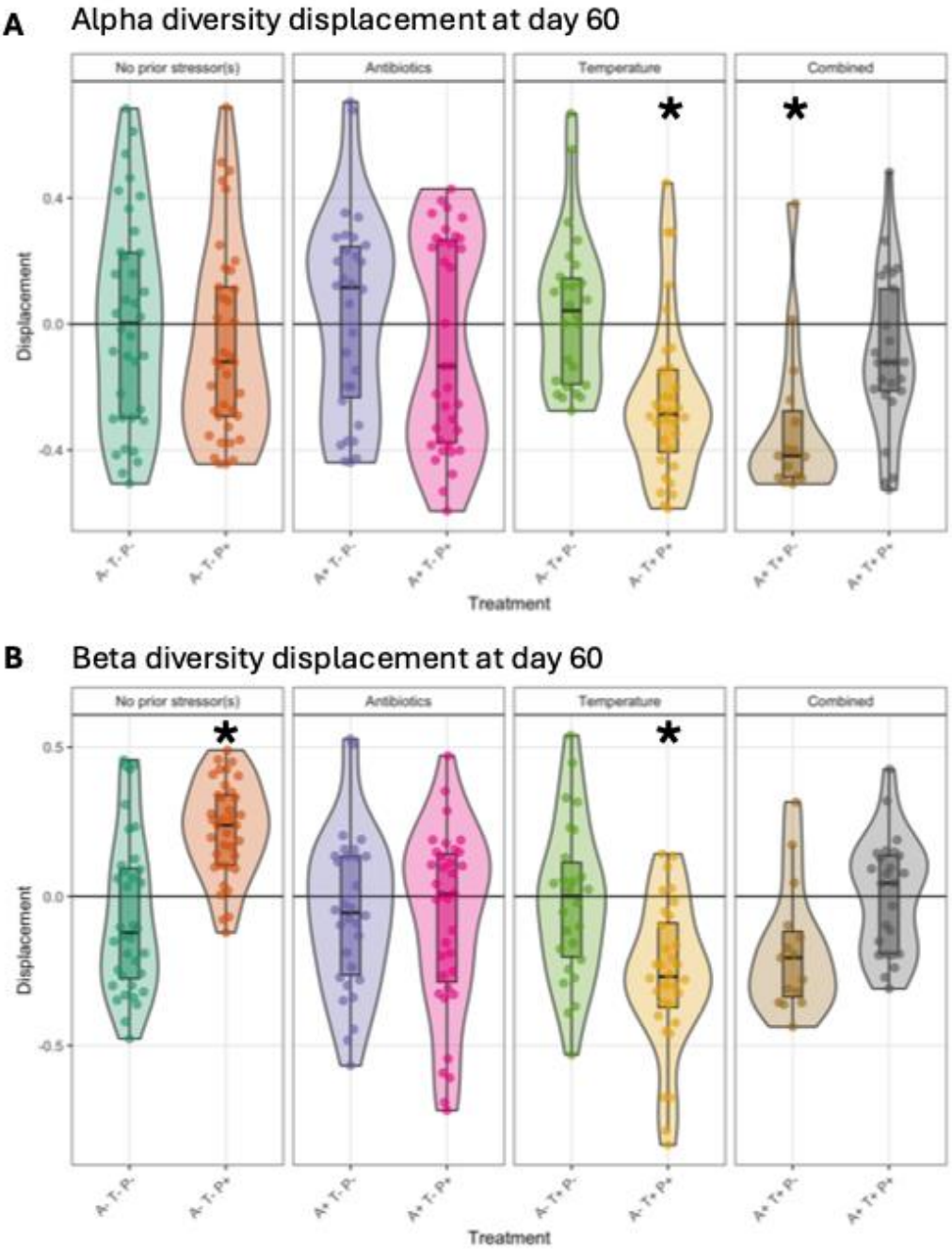


Figure 15: Effects of stressors on zebrafish gut microbiota (continued)

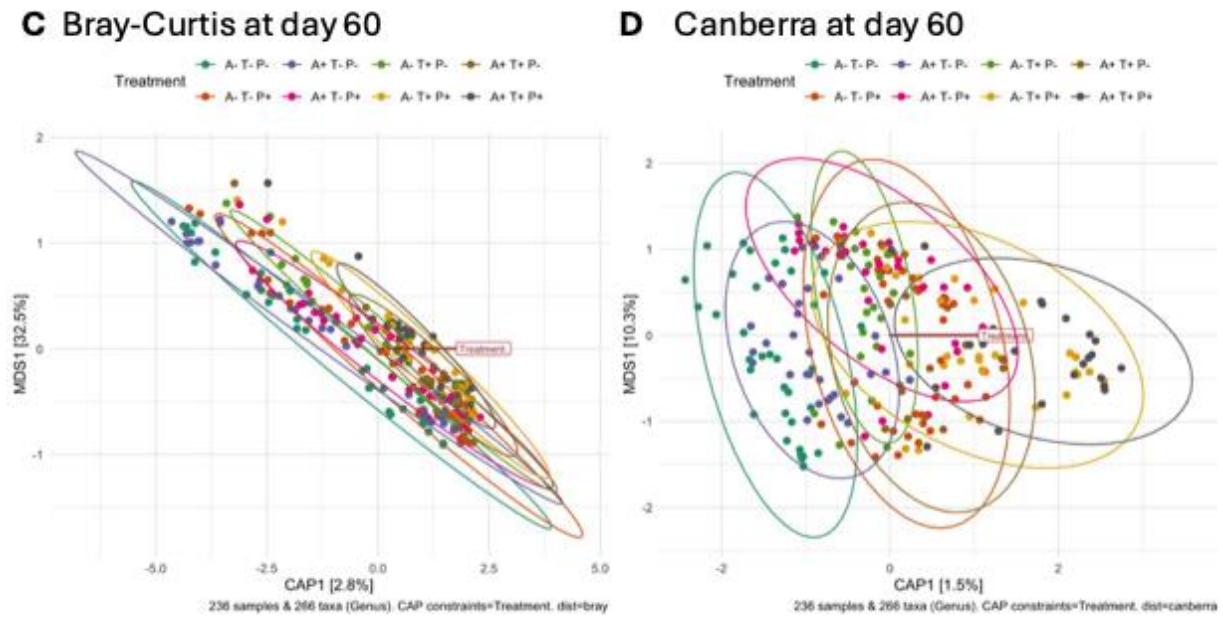
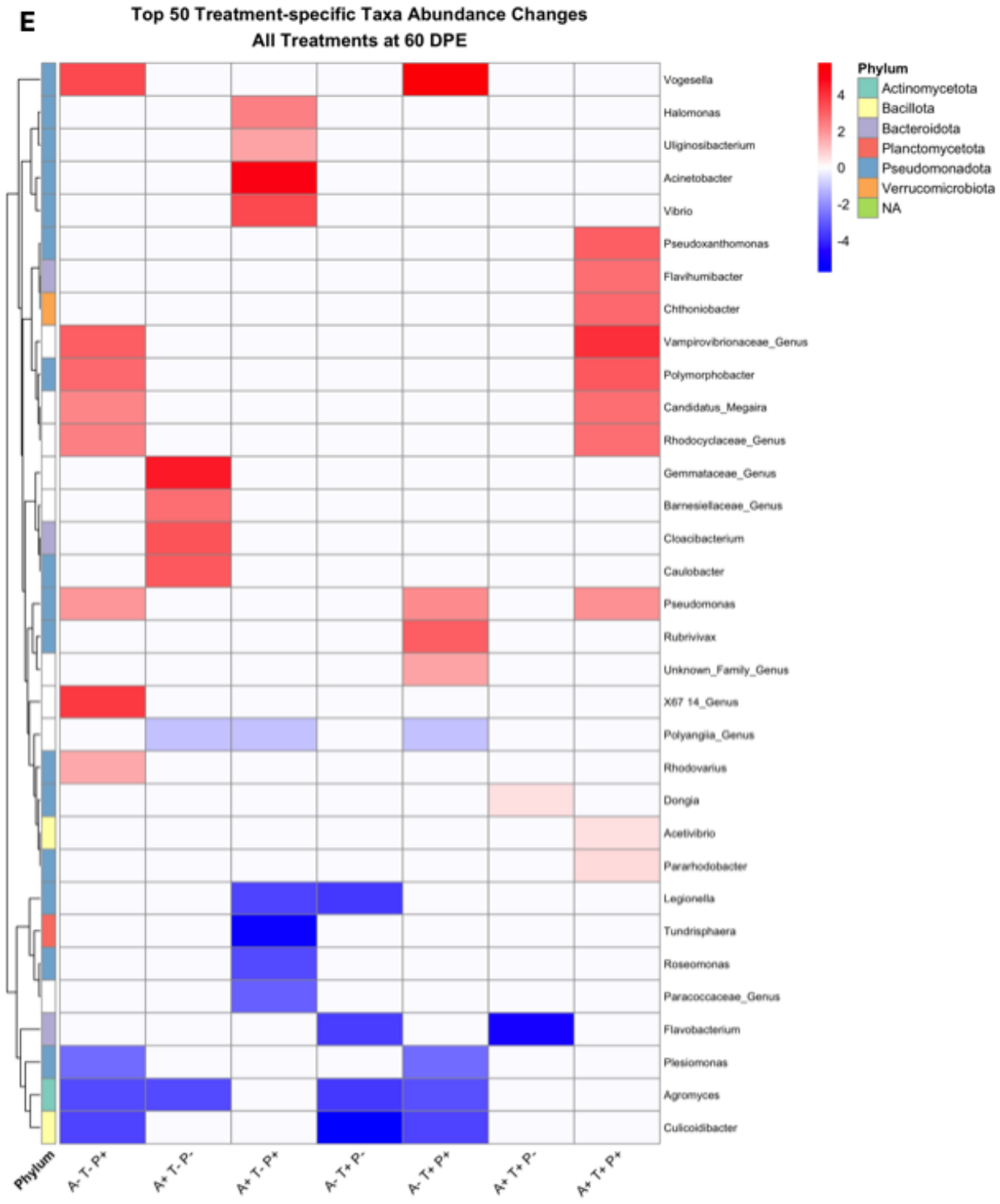


Figure 15: Effects of stressors on zebrafish gut microbiota (continued)



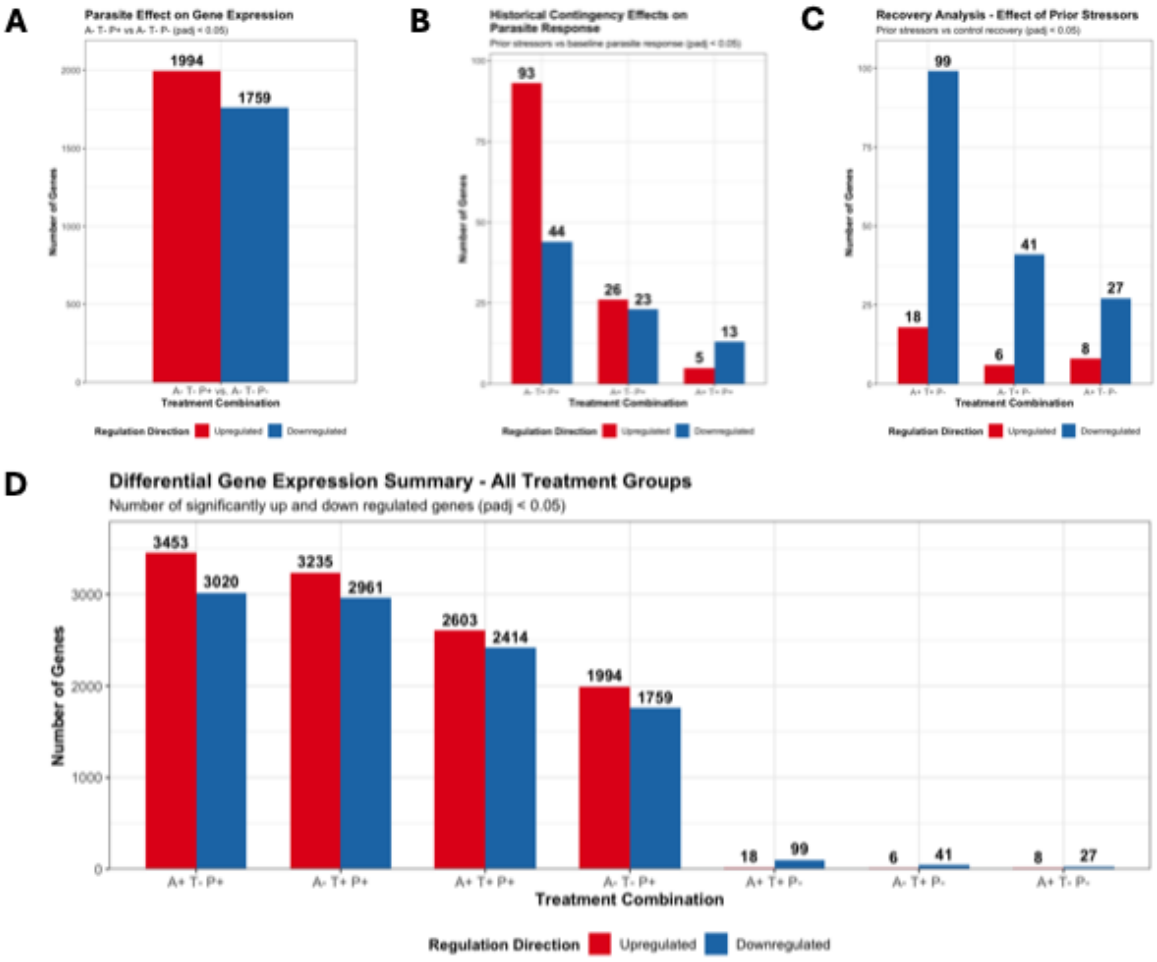


Figure 16: Effects of stressors on host intestinal gene expression.

Bar plots showing number of significantly up- (red) and down-(blue) expressed genes at day 60 between **(A)** control group vs parasite exposed only group; **(B)** parasite exposed only group vs parasite exposed + historical stressor exposure regime groups; **(C)** parasite unexposed group vs parasite unexposed + historical stressor exposure regime groups; **(D)** controls vs all other exposure regime groups.

Figure 17: Integrated analysis of gene-taxon and taxon-mortality correlations.

(A) Bipartite network graph showing top 10 significant partially correlated taxa and their associated gene pairs at day 60. **(B)** Significantly correlated taxa and mortality across all exposure regimes (Treatment) at day 60. Red indicates positive correlation and blue indicates negative correlation.

Figure 17: Integrated analysis of gene-taxon and taxon-mortality correlations (continued)

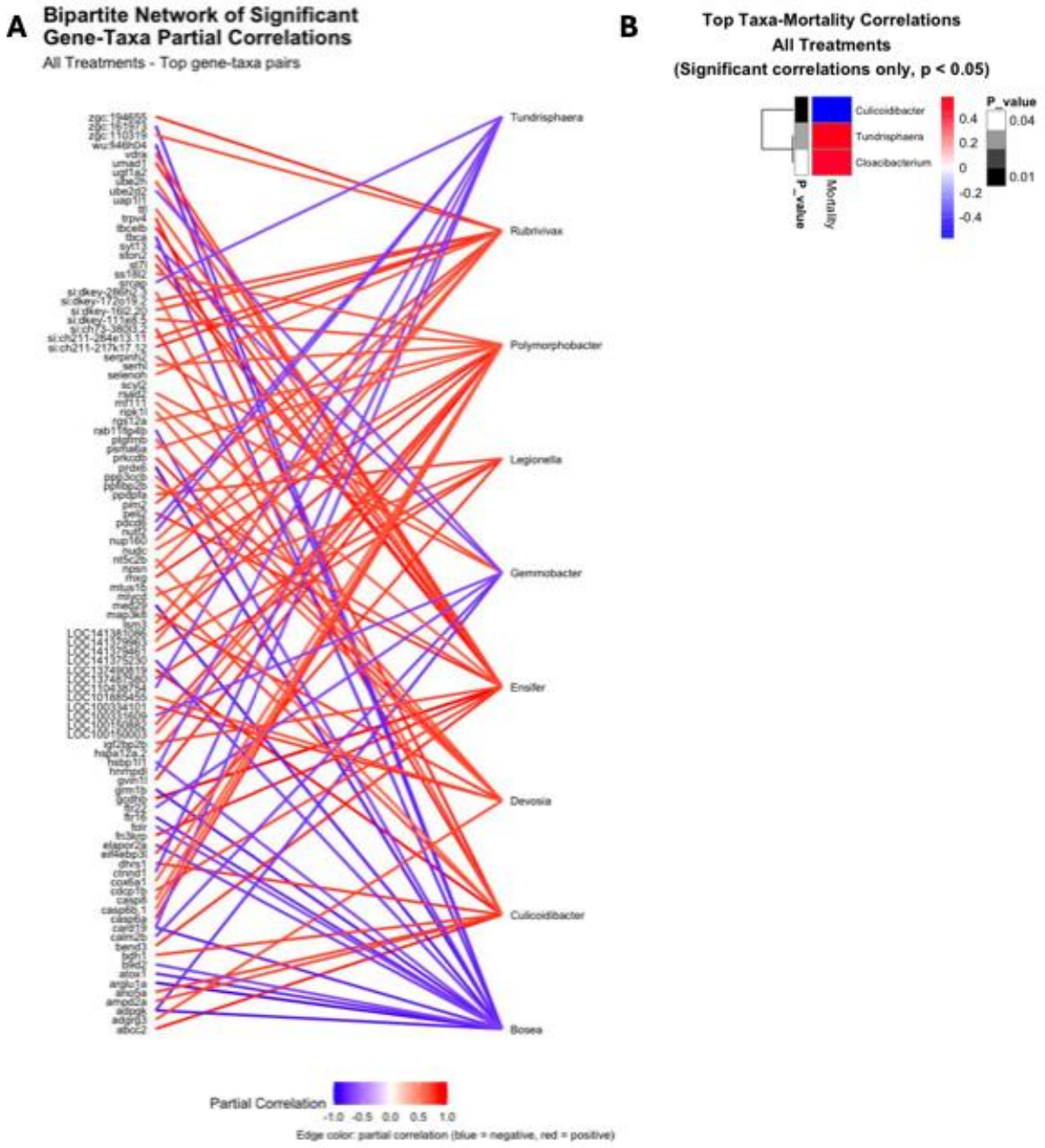


Table 1: Table of exposure regimes.

Filled in cells with "+" indicate exposure to antibiotics (A; blue), temperature (T; orange), and/or parasites (P; pink). White cell with "-" indicates no exposure, while color-filled cells containing "+" indicate exposure to a stressor. For instance, the row containing the control group (e.g., A- T- P-) is composed of only white cells indicating no exposure to any stressors, while the row of dual exposure group (e.g., A+ T+ P+) are all filled in indicating exposure to all stressors. Together, there are eight unique exposure regimes.

Exposure Regimes	Antibiotics (A)	Temperature (T)	Parasite (P)
A- T- P-	A-	T-	P-
A- T- P+	A-	T-	P+
A+ T- P+	A+	T-	P-
A+ T- P+	A+	T-	P+
A- T+ P-	A-	T+	P-
A- T+ P+	A-	T+	P+
A+ T+ P-	A+	T+	P-
A+ T+ P+	A+	T+	P+

CHAPTER 5

CONCLUSION

The research in this dissertation aimed to elucidate context-dependent principles of microbiome response to environmental stressors and clarify how modulating different dimensions of these stressors affects microbiome response to influence host health in light of the Anthropocene. More specifically, this work leveraged the zebrafish model system to investigate how diet affects microbiome sensitivity to pathogen exposure, how temperature and parasite exposure interact to shape microbiome dynamics and infection outcomes, and how historical contingency fundamentally reshapes host-microbiome system resilience and recovery to exposure to prior stressors. These investigations address critical gaps in understanding how organisms navigate the complex, sequential environmental challenges characteristic of our

rapidly changing Anthropogenic world, where microbial communities often shift well before macroscopic symptoms appear and function as early indicators of ecosystem health.

As detailed in Chapter 2, I showed that diet is not simply a background variable, but it actively shapes the successional pathways along which zebrafish gut communities develop. Fish maintained on different common laboratory diets followed distinct diversity and compositional trajectories, and those trajectories predicted how each community responded to *Mycobacterium chelonae* exposure. Some diets produced microbiome profiles that were more sensitive to infection, underscoring diet as a possible hidden driver behind inconsistent microbiome compositions reported across facilities. These findings motivate diet standardization from a best practice to a prerequisite for improved reproducible zebrafish microbiome research. Looking forward, work should test nutritionally defined diets, integrate metabolomics to pinpoint protective functions, and explore microbiome-targeted interventions that promote pathogen-resistant microbiome states. Beyond the laboratory, the results foreshadow a broader societal concern: climate-driven disruptions to global food supply could alter vertebrate microbiomes in ways that erode natural defenses against disease, making equitable food access a critical pillar of public health.

Chapter 3 tackles the challenge of disentangling direct thermal effects from host-microbe interactions in a changing climate. Longitudinal sampling revealed that water temperature and exposure to the intestinal helminth *Pseudocapillaria tomentosa* independently altered gut diversity, yet only the highest temperature tested suppressed worm development. We also found evidence consistent with temperature-induced arrested development in *P. tomentosa*, where exposure occurred but larval maturation stalled. While arrested development is common among parasitic nematodes, to our knowledge, this finding represents the first observation of arrested

development of a parasitic worm under elevated water temperature conditions in a host organism. Among infected fish, microbiome diversity displayed a non-linear, temperature-dependent relationship to parasite burden, and these patterns manifested in gut microbial community composition as multiple potential alternative stable states. The result refines the Anna Karenina principle: apparent dysbiosis can reflect unmeasured environmental context rather than intrinsic instability. The work challenges current expectations regarding warming waters increasing disease burden to a nuanced view where microbiome-mediated responses may result in unanticipated outcomes. Future efforts should incorporate microbiome data into climate-disease models so that management strategies account for local environmental context.

Chapter 4 extends beyond single stressors by demonstrating that past exposures define both resistance and resilience to new perturbations. Sequential exposure to antibiotics, heat, and parasites showed that mortality climbed with added stressors, although final infection burden was not strictly proportional to stress history. Prior antibiotics amplified parasite-induced taxonomic shifts, heat alone produced minimal changes, and the combination introduced idiosyncratic responses. Multi-omic integration uncovered stress-specific gene-taxon networks, with the bacterial genus *Culicoidibacter* emerging as a consistent health-associated member of the gut microbiome. These findings confirm that host-microbiome responses are historically contingent, implying that variation among studies may stem from unrecorded exposure histories. Moving forward, dynamic, history-aware models are needed to predict system state from stressor sequence and spacing. Testing the persistence of host-microbiome stress memory and evaluating interventions that rewrite maladaptive trajectories will clarify whether principles identified here generalize across other vertebrate host-microbiome systems. Furthermore, policy and restoration plans must consider not only how many stressors act, but also the order in which they arrive,

aligning with a One-Health framework that links environmental policy to microbial, environmental, animal, and human well-being.

Collectively, this research deepens our understanding of how organisms navigate environmental change, revealing that host-microbiome relationships are molded not only by present stressors but also by the history and specific dimensions—modality, magnitude, and timing—of those stressors. Because microbiome disruption never occurs in a vacuum, accurate predictions must account for past exposures and the unique contextual landscape each organism inhabits. The work, therefore, cautions against one-size-fits-all interventions and instead argues for flexible, history-aware frameworks and models that can be tuned to local conditions. Finally, by demonstrating that food security, pollution, and climate impacts are unevenly distributed, the dissertation highlights an equity-centered One-Health mandate: safeguarding microbial relationships necessitates coordinated action that encompasses environmental policy, animal management, and human public health. In sum, the dance between hosts and their microbial partners is choreographed by both past and present environments, and understanding that choreography is essential for mitigating the cascading effects of anthropogenic change, from individual well-being to ecosystem stability.

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Appendices

Appendix Supplemental Material 1: Supplementary figures, tables, data, and methods associated with Chapter 2 are available through the journal website (DOI: https://doi.org/10.1186/s42523-023-00254-8) [158].

Appendix Supplemental Material 2: Supplementary figures, tables, data, and methods associated with Chapter 3 are available through the journal website (DOI: https://doi.org/10.3389/frmbi.2025.1605168) [280].

Appendix Supplemental Material 3: Supplementary figures, tables, data, and methods associated with Chapter 4 are available through a public GitHub repository (https://github.com/sielerjm/Sieler2025__ZF_StressHistory).