Coyotes in cities and sponges in streams:

Microbiomes in the face of environmental change

by

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ABSTRACT

Animals live in close association with communities of microbes that play important roles modulating host nutrition, immunity, health, and behavior. Many of these microbes live on or in their animal host, most notably in the vertebrate gut, but microbiome studies now encompass disparate taxa from diverse environments. With the increasing awareness that no animal is devoid of commensal microbes comes a new appreciation of the role these microbes may play as animals adapt to ongoing and future environmental changes, which already have well-described ecological consequences. In this thesis, I complemented 16S rRNA gene sequencing with information about diet, health, and environmental conditions to preliminarily explore how different environments may affect the microbiome of two phylogenetically diverse organisms, coyotes (*Canis latrans*) and freshwater sponges (*Ephydatia muelleri*).

Coyotes are adapting to life in urban environments and now inhabit cities across North America. I profiled microbiome composition along the length of the gastrointestinal tract in 10 coyotes that were trapped for other reasons and confirmed that coyotes largely resembled other mammals, and additionally noted that fecal samples were not reliable indicators of the microbiome in upper intestinal environments. I then used a larger sample of 76 trapped or road-killed coyotes to evaluate which components of the microbiome are most strongly linked to diet, measured as both stomach contents and stable isotope signatures (δ^{13}C and δ^{15}N), and to health, measured as both body condition and infection by the zoonotic parasite *Echinoccocus multilocularis*. Healthy coyotes harbored Fusobacteria-rich gut microbiomes which correlated with protein-rich diets; despite the emphasis on diversity in microbiome studies, microbiome diversity did not correlate with body condition. I lastly compared the duodenal and fecal microbiomes, in relation to diet and body condition, between urban and peri-urban coyotes.

Urban coyotes consumed broader diets containing more anthropogenic food, resulting in greater divergence in the duodenal microbiome among individuals and a slightly altered fecal microbiome characteristic of carbohydrate-rich diets. These changes were associated with poorer average body condition and double the prevalence of *E. multilocularis*.

Freshwater sponges have received little attention in microbiome research, despite the ecosystem services they provide in aquatic environments and the considerable work that has been done on their marine counterparts. I sampled sponges from three streams on Vancouver Island, BC, and showed that sponges harbor microbial communities distinct from ambient water and adjacent biofilms. These communities were dominated by the families *Chitinophagaceae* and *Comamonadaceae*, which may play ecological roles degrading sponge-derived chitin and steroids. Several aspects of these communities appeared to be stream-specific, suggesting that sponge-microbe associations may be driven by ecological factors unique to each stream.

Collectively, my results provide a foundational understanding of how the host-associated microbiome relates to environmental conditions in two organisms living in vastly different habitats. They suggest the potential for the microbiome to figure importantly in the ongoing process of urban adaptation in coyotes and in possible future changes in limnological conditions for sponges, with implications for host physiology and behavior.

PREFACE

This thesis is an original work by Scott Sugden. Data used in all analyses for the coyote chapters was generated from a sample of 76 coyotes collected in and around Edmonton, AB from August 2017 to April 2018. Sponge samples were obtained from Vancouver Island, BC in July 2018. Sample preparation and data collection were performed by S. Sugden at the University of Alberta. Stable isotope and water chemistry samples were analyzed at the Biogeochemical Analytical Services Laboratory at the University of Alberta, and all DNA sequencing was performed by Microbiome Insights in Vancouver, BC.

Chapter 5 has been submitted for publication in *Applied and Environmental Microbiology* and is currently in review as "The freshwater sponge *Ephydatia muelleri* harbors a distinct and stream-specific microbiome" with Lisa Stein as the co-author. S. Sugden collected and analyzed the data and wrote the manuscript. L. Stein assisted with concept formation and manuscript edits. The chapter appears here as submitted, although references have been merged with the preceding chapters and presented as part of the complete bibliography.

The remaining chapters have not yet been submitted for publication in a peer-reviewed journal. S. Sugden is the sole author of Chapter 1. Chapters 2-4 are co-authored by S. Sugden, L. Stein, and Colleen Cassady St. Clair. Kyra Ford is an additional co-author for Chapter 4, which is currently being prepared for submission to *Nature Ecology & Evolution*. For all chapters, S. Sugden was responsible for data collection, analysis, and manuscript composition, and L. Stein and C. St. Clair assisted with concept formation and provided manuscript edits. K. Ford assisted with data collection in Chapter 4.

DEDICATION

I was young and I thought I was tough, and I knew it was beautiful and I was a little bit crazy but hadn't noticed it yet.

Norman Maclean, USFS 1919: The Ranger, the Cook, and Hole in the Sky

The profoundest distances are never geographical.

John Fowles, The Magus

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

ASV   **Amplicon sequence variant.** Alternatively referred to as "exact sequence variant." Modern computational technologies have made it possible to use known sequencing error rates to infer exact 16S rRNA gene amplicon sequences to the level of individual nucleotides, without the need for traditional clustering approaches that use a 97% identity threshold to identify OTUs (operational taxonomic units). See ref. 153 for details.

DIC   **Dissolved inorganic carbon**

DOC   **Dissolved organic carbon**

GLM   **Generalized linear model**. A form of regression where the statistical distribution of the dependent variable is specified in the model and does not need to be linear.

RDP   **Ribosomal Database Project**. A collection of 16S rRNA gene sequences of known taxonomy against which new amplicon sequencing data can be compared to determine species identity.

SRP   **Soluble reactive phosphorus**

TDS   **Total dissolved solids**

TN   **Total nitrogen**

CHAPTER 1

The microbiome of wild animals in the face of environmental change

1.1 ABSTRACT

The past two decades have seen an explosion of microbiome research catalyzed by the accessibility and affordability of high-throughput sequencing techniques and large-scale initiatives like the Human Microbiome Project. Microbiome studies now encompass disparate taxa from diverse environments, consistently demonstrating the abundance of microbes living on and in their animal hosts and the importance these microbes have for host digestion, immunity, health, and behavior. With this increasing awareness that no animal is devoid of commensal microbes comes an appreciation of the role these microbes may play in helping animals adapt to future environmental changes. The environmental factors that can influence microbiome diversity and composition are well-described, as are the ecological consequences of environmental change, but in order to generate testable hypotheses for how environmental change may affect host fitness via the microbiome it is necessary to unite these two often-independent lines of research. In this literature review, I use current knowledge of host-microbe relationships alongside the metacommunity concept and disturbance theory to explore how environmental changes, particularly those caused by humans, can affect an animal's microbiome with either positive or negative effects for host fitness. There is great potential for the microbiome to influence whether an animal thrives or suffers in the face of environmental change, and more studies explicitly testing these relationships will further solidify the importance of the microbiome in discussions of conservation biology.

1.2 INTRODUCTION

The overwhelming majority of biological history belongs to microbes. When the first multicellular plants and animals began to evolve from single-celled ancestors some 600 million years ago (1, 2), microbes had already occupied and shaped Earth's surface and chemistry for over three billion years (3). As new technologies rapidly expand our ability to study microbes at ever-decreasing costs, biologists have begun to appreciate the functional and phylogenetic diversity of the microbial world. A recent analysis of data from DNA-based phylogenetic studies estimated the existence of over 1000 bacterial phyla (4)—two orders of magnitude more than have been identified for plants (12 phyla) or animals (33 phyla) (5). Underlying this new appreciation of modern microbial diversity is the recognition that multicellular organisms evolved not only from a microbial world, but also in and with it, and have therefore been influenced by microbial activity since their evolutionary origins (6).

Historically, relationships between microbes and multicellular organisms were primarily considered in the context of pathogens and disease, a viewpoint driven in the 20th century by the germ theory of disease (7). Studies of cooperative interactions between organisms, loosely identified under the umbrella term "symbiosis" (8), were largely restricted to interactions between multicellular organisms (7), where symbiosis could be directly observed in cases such as the pollination of plants by insects (9) or the still-contested example of Egyptian plovers cleaning the teeth of crocodiles (7). With some notable exceptions, such as the interactions between plants and rhizobia (10) and select bacterial-insect relationships (11), symbiotic relationships between microbes and multicellular "hosts" were largely overlooked due to the technological limitations of observing these interactions microscopically and the inability to culture and characterize the vast majority of microbial species (12).

In the past few decades, culture-independent high-throughput DNA sequencing studies of tissues from plants and animals have consistently demonstrated that plants and animals share their bodies with an abundance of microbes from thousands of different species, to the extent that in humans, the number of microbial cells equals or exceeds the number of human cells (13–15) and microbial genes outnumber human genes 150-fold (15). Although the majority of current research addresses microbes in the guts of humans and other vertebrates, symbiotic microbes have been found associated with disparate taxa from diverse environments ranging from deep-sea sponges (16) and hydrothermal vent crabs (17) to terrestrial earthworms (18) and many

arthropods (19). The ubiquity of these symbiotic host-associated microbial communities warranted the creation of new vocabulary to describe them: the term "microbiome" emerged in the late 20th century to identify a community of microbes occupying a specific habitat, either in a host or the broader environment (20). Microbiomes have since been described for almost every biological system, from the human gut to the global ocean (21), and their prevalence has prompted the claim that all multicellular organisms live in association with some form of microbial community (22).

Results from studies of host-microbe symbioses using a variety of techniques, including DNA sequencing, gnotobiotic models, and microbiome transplant experiments, have shown that microbial symbionts can have profound effects on host physiology (6, 22–24), and that environmental variations such as changes in social contact, habitat biogeochemistry, or food source or abundance can quickly and substantially alter the structure and function of the microbiome (12, 25, 26) and therefore the fitness of the host (6, 22, 24). Many hosts provide valuable ecosystem services, and so as the rate of global environmental change continues to increase due to human activities (27–29), the effects of environmental change on host-associated microbiomes and host fitness are becoming a new focus for conservation biology (30, 31).

In this review, I focus on the relationships between bacterial symbionts and animal hosts, not because other taxa are undeserving of attention but because an exhaustive outline of host-microbe symbioses is beyond the scope of any single review. After briefly describing the structure and function of symbiotic bacterial communities within their metazoan hosts, I discuss the metacommunity concept and how both biotic and abiotic factors may shape an animal's microbiome. I then use a series of examples to address how changes in an animal's environment, with an emphasis on changes caused by human activity, can affect host-microbe symbioses by altering natural microbiome assembly dynamics. These disruptions can have either positive or negative effects on host fitness. I outline key objectives for future conservation-based microbiome research and then conclude by introducing the subsequent chapters of this thesis, which specifically evaluate how environmental change influences the microbiomes of coyotes and freshwater sponges.

1.3 STRUCTURE AND FUNCTION OF THE MICROBIOME

The physiological role of bacterial symbionts within an animal host is in part determined by where a symbiont resides within the host and what organs or structures it has access to, which is itself a function of a shared evolutionary history. In animal hosts with complex physiology, distinct microbial communities may populate different organs, tissues, or anatomical regions. Studies of the human microbiome and other vertebrates address communities in the gut, oral cavity, respiratory tract, skin, and vaginal canal (12, 13, 32). The gut microbiome has also been described in a variety of other invertebrates, including insects (19). Mucus layers both on internal and external surfaces of organisms may be populated by unique microbiota (33–35). Some organisms have developed specialized organs to house their microbiota, like the light organ in the Hawaiian bobtail squid (*Euprymna scolopes*) that is recolonized daily by bioluminescent *Vibrio fischeri* (8).

Within a host, microbes may be either endo- or ecto-symbionts. Endosymbionts live inside host cells and typically have reduced genome sizes reflecting a dependence on host metabolism (8, 36). This is particularly common in arthropods, where endosymbiosis often occurs as a specialized, species-specific interaction in which the two partners have established an obligate mutual dependency (36). Ectosymbionts live on the external or luminal surfaces of the host, such as on the skin or within the gastrointestinal or reproductive tracts, but remain physiologically separate from host cells (22). Many of these interactions are facultative, or non-obligate, symbioses, where the host benefits from a particular microbial service but does not require it for survival (36). The remarkable physiological diversity of these host-microbe relationships, and the existence of unique microbial communities in different niches within a single host, speak to the deep links between the evolutionary histories of microbes and multicellular organisms.

Over evolutionary time, microbial colonization of these host organs and tissues was facilitated by host-microbe metabolic exchanges, which led to a baffling array of metabolic partnerships and physiological interdependencies between a host and its microbiome. Genetic studies of metabolism have demonstrated that microbes carry out fundamental biological processes that are essential for multicellular life but impossible for eukaryotes to perform (7). All plants and animals require nitrogen in the form of ammonia or nitrate, but only microbes are capable of converting atmospheric nitrogen to these biologically accessible forms (37). Rich

communities of shrimp, clams, worms, and other aquatic animals can only survive in the otherwise inhospitable waters near deep-sea hydrothermal vents due to microbial sulfur oxidation and inorganic carbon fixation (38). Many herbivorous mammals utilize carbon derived from cellulose, but only prokaryotes and some protozoa possess the enzymes required to break the β(1-4) glycosidic bonds in the cellulose molecule (12). In light of the contemporary theory that all eukaryotes derive from only two prokaryotic lineages (39), it is not surprising that they would only possess a fraction of the total prokaryotic metabolic potential. The earliest eukaryotes and multicellular organisms would therefore have been dependent on molecules cycled by the prokaryotes in their environment, and the diverse physiology of multicellular organisms thus has been and continues to be shaped by the diverse metabolisms of the prokaryotes (40).

Today, the physiological importance of the microbiome for animal health can be broadly considered in three categories: nutrient exchange and cycling within an animal; chemical signaling and behavioral modification; and development and maintenance of the immune system. The first of these ideas is not new—microbial-mediated hemolysis in hematophagous insects (11) and microbial fermentation of cellulose in the ruminant gut (12) were some of the first accepted examples of host-microbe symbiosis studied in the 1940s and 1950s (7). By associating with microbes, many animals gain access to novel metabolic pathways that improve digestive capacity, facilitate waste removal, and supply them with symbiont-derived carbon and nitrogen compounds including essential amino acids (24) and short-chain fatty acids (41). More recent studies have proposed neurological links between gut microbiome and host appetite (42), diet preferences (43), and even social behavior (44), suggesting the existence of a "gut-brain" axis that may additionally modulate mental health (45). Secreted microbial compounds have even been implicated in the behavior of organisms that lack nervous systems (46). Resident microbes may also support the host's immune system by producing antimicrobial compounds (47, 48) and stimulating the early development of the immune system in fetuses or neonates (49). Ongoing research will likely continue to elucidate new functions of the microbiome and new mechanisms for host-microbe communication and sensing.

1.4 ENVIRONMENTAL DETERMINANTS OF MICROBIOME COMPOSITION: THE METACOMMUNITY CONCEPT

The structure and function of an animal's microbiome, and its ability to perform the above services, can vary among species, among populations, and even among individuals within a population (50), due to a variety of factors including host diet, life stage, genotype, habitat, and the physiology of the organ or organism of interest (31). These factors can operate at timescales from days to generations (25). To understand the basis of between-host variation in microbiome composition, microbiologists are increasingly turning to major theories in community ecology, which have been used for decades to describe and predict the abundance and distribution of macro-organisms (51). In particular, metacommunity dynamics provide a valuable framework for addressing how a given microbial community can be shaped by a complex suite of biotic and abiotic factors acting across multiple spatial and temporal scales in the host's environment (52, 53). Applied to microbiome research, the metacommunity concept specifically considers within-host behavior and physiology, between-host social interactions, and interactions between a host and its wider environment, as well as the interplay between these three factors, as the mechanisms by which a host-associated microbiome is assembled from a regional pool or "metacommunity" of available microbes (53, 54).

Within individual hosts, the best-studied determinant of microbiome composition is diet, which is itself a byproduct of host behavior and lifestyle. In both humans and animals, long-term diet has a significant and predictable influence on the composition of the gut microbiome both within and among species (23), and changes in diet can effect measurable changes on the gut microbiome in less than one day (25). The response of microbiota to diet is similar across mammalian lineages (55), leading to the formation of unique gut microbial communities distinguishing carnivores, herbivores, and omnivores (23). Carnivores generally contain fewer taxa specializing in amino acid degradation, whereas herbivores, especially ruminants, harbor highly diverse microbiomes that synthesize amino acids from fermented plant material (55). Diet-specific communities in humans are now being referred to as "enterotypes," with distinct enterotypes distinguishing processed Western diets from traditional hunter-gatherer diets (56). In organisms without guts, food source still influences the host microbial community, as has been observed in the differences between filter-feeding versus carnivorous sponges (57).

However, diet alone cannot explain all the inter-species variation in microbiota, as

several species do not harbor the microbiomes that might be predicted by their diet: for example, the gut microbiome of herbivorous giant pandas is more like most carnivores than any other herbivore (23), and the microbiome of carnivorous baleen whales has similarities to both carnivores and herbivores (58). Recent research has suggested that host genetics and physiology, independent of diet or other environmental factors, can also affect microbiome composition, although the mechanisms behind this correlation are not fully understood (59, 60). Gut physiology may naturally select for or against microbial species; for example, giant pandas share the simple gut physiology common to carnivores, potentially inhibiting their colonization by a herbivorous microbiota (61). Phylogenetically related primates with similar gut physiology likewise share similar microbiota despite evolutionarily divergent diets (62). Other conditions within the host, such as gut pH or nutrient availability, may naturally exclude microbial species for which those conditions are not optimal (53). Some hosts present barriers to colonization, like the symbiont-sorting organ in the bean bug intestine (63), and many mount anti-microbial defenses that can distinguish native symbionts from foreign bacteria, allowing them to combat pathogens and prevent additional species from joining the symbiont community (53, 54). Competitive and cooperative interactions between the microbes within a host may also shape microbiome composition, and the outcome of those interactions is often influenced by host physiology (53, 64).

Between hosts, social and familial relationships can facilitate symbiont transmission, ensuring conservation of symbionts within host populations but producing variance between them (65). Organisms can acquire microbial symbionts directly from their parents (vertical transmission) or from other individuals in their community (horizontal transmission) (66–68). Vertical transmission acts to conserve the microbiota across multiple generations of a single host species and, in some cases, host population, and is particularly common for obligate symbionts or endosymbionts (68). Horizontal transmission is a function of the amount of social contact between hosts and of host immigration between different habitat patches, and results in similar microbiota across socially interacting populations and variation between isolated populations of the same species (67, 69). For example, sympatric populations of chimpanzees and gorillas share more similar gut microbiota than allopatric populations (70), and co-habiting humans and dogs exhibit more shared skin microbiota within households than between households (71). It has even been proposed that the microbiome may manipulate host behavior to promote altruism in

order to facilitate horizontal transmission (66). Commonly cited mechanisms of horizontal transmission include direct physical contact and coprophagy, and these mechanisms have been shown to cross species boundaries: a recent study of predator-prey populations suggested that predators can acquire microbiota from their prey (69).

A second form of horizontal transmission occurs when hosts acquire microbes from their surrounding environment; this has alternatively been referred to as environmental transmission (72). Direct acquisition of symbionts from the environment has been empirically demonstrated in sessile marine invertebrates, like sponges, which have limited or no social contact and therefore must acquire many symbionts from the ambient water (46). Because of the relative ease with which genetic material can be exchanged between microbes via horizontal gene transfer, environmental transmission of symbionts can also include the transfer of new genes to the host microbiota independent of the acquisition of new symbiont species (46, 68). Habitat-specific patterns in microbiome composition further suggest that host habitat may be a source of symbionts, with diverse species from similar but geographically separate habitats exhibiting more similarity in microbiota compared to related species populating different, adjacent habitats (53, 54, 73). In the Galapagos Islands, microbiome composition in terrestrial and marine iguanas is more similar among allopatric conspecific hosts, which occupy similar habitats but have no current mechanism for host-to-host transmission, than among sympatric heterospecific hosts occupying adjacent, overlapping habitats, indicating some role of habitat-dependent symbiont acquisition that may be mediated by host behavior and lifestyle (74). Microbiome similarity decreases with geographical distance between allopatric conspecific iguanas, which suggests previous host immigration and host-to-host transmission events in these populations (74). Habitat- or location-dependent symbiont diversity has been demonstrated in several other species, including frogs (75), bees (76), and humans (71), and is perhaps most clearly exemplified by migratory birds, which exhibit unique seasonal microbiomes compared to non-migratory conspecifics (77).

Some environmental acquisition may be stochastic, leading to population-specific "ecological drift" in microbiome composition (78), but some may be deterministic, where particular bacterial species or genes are selected for from the environment due to some physiological benefit they confer (53). The guts of Japanese people harbor a symbiotic bacterium capable of digesting the agar in seaweed consumed as part of the traditional Japanese diet;

Westerners who do not consume seaweed do not have this symbiont (79). Changes in diet constitute another form of selective pressure that can alter the microbiota, promoting the growth of taxa capable of digesting the new diet (25). Successful acquisition of a symbiont within a host population additionally requires compatible within-host physiology and can be facilitated by horizontal host-to-host transmission, reflecting the complex interplay between the various mechanisms by which a microbiome is assembled from a metacommunity.

These assembly mechanisms apply not only to individual hosts living within defined environmental conditions but also operate across a variety of spatial and temporal scales. Host-microbe mutualisms have co-evolved over millions of years through varying patterns of climate, diet, disease prevalence, and host behavior, and are consequently well-adapted to expected habitat variability, including spatial and temporal variation in temperature and food availability (50, 52, 80). As food availability changes, many animals respond by altering their diet, with expected seasonal changes in gut microbiome (80–82). Hibernating species like the brown bear harbor distinct microbial communities during hibernation, dominated by species that modulate metabolism and adiposity (83). Germ-free mice inoculated with microbes from hibernating bears produce more fat than wild-type mice or germ-free controls (83), suggesting an essential and evolutionarily conserved benefit of this seasonally varying microbiome for the hibernating bear. In these and other examples, seasonal variations in the factors influencing microbiome assembly, and in the interplay between those factors, enable a degree of host phenomic plasticity that maximizes nutrient acquisition and energy consumption across time and space and hints at the deeper co-adaptive relationships between hosts and the microbial metacommunity.

1.5 ENVIRONMENTAL CHANGE MAY DISRUPT HOST-MICROBE SYMBIOSES

Due to the complex integration of microbial activity, host physiology and behavior, and external environmental factors in shaping host-microbe symbioses, Rosenberg & Rosenberg (2008) proposed that an animal with all of its associated microorganisms be considered a unit of natural selection (22). The host-microbe conglomerate is collectively referred to as a "holobiont," and the complete genetic potential of the holobiont as the "hologenome." According to this "hologenome theory of evolution," natural variation in the abundance or diversity of microbial genes, corresponding to changes in the presence or abundance of microbial taxa, parallels the natural variation produced by mutation in the host genome and can itself be an

object of natural selection (22, 24). Given the host's reliance on microbes for essential physiological processes, natural variation in host microbiomes results in differential fitness between individuals or populations, with some microbes providing a selective advantage in different environments. This theory has been used to explain the co-evolution of intricate host-microbe metabolic partnerships (36, 52, 84), such as the behavior- and location-specific fitness benefits of bacterial-mediated adiposity in hibernating bears (above) or the ability of the Mojave Desert woodrat gut microbiome to neutralize toxins in endemic Mojave Desert plants (85). Although the hologenome theory has received strong criticism as an evolutionary model (67, 86), largely due to its inability to account for imperfect heritability of symbiont communities, the concept that natural and environmentally-induced variation in host microbiomes confers habitat-specific fitness advantages (or disadvantages) has important implications for ecology and conservation biology.

Because of the rapid generation time of microbes compared to their hosts, genetic and compositional variation in the microbiome can occur much more rapidly than variation in the host genome (22, 24), and therefore has been proposed as a mechanism by which hosts can adapt to future environmental change (87). Borrowing from community ecology is again fruitful here: disturbance theory considers how macro-organisms and communities respond to environmental changes or perturbation (88), and can also be applied to host-associated microbial communities (51). Hosts and their symbionts are already well-adapted to natural seasonal and spatial environmental variation, but rapid, unexpected changes in habitat and food availability (pulse disturbances) or sustained long-term changes (press disturbances) may disrupt host-microbe symbioses with unpredictable effects on host digestion, development, immunity, and behavior (50). The outcome of any particular disturbance depends on a variety of competing factors including the rate at which the microbiome can evolve in composition or function, the rate and magnitude of the environmental change, how effectively host physiology can support different symbionts, and how the environmental change may affect the microbiome assembly process, such as between-host transmission dynamics or the microbes available in the regional species pool.

Contemporary human activity is driving dramatic changes in the biosphere at a rate faster than any extant organisms have previously faced (27, 30), making the microbiome, and its recently appreciated adaptive capacity and influence on host physiology and fitness, an emerging

focus of conservation biologists (30, 31, 87). Although the response of animal microbiomes to anthropogenic disturbance, and the consequences for host physiology, fitness, and behavior, remain largely unexplored, conservation biology has long recognized the negative influence humans have on ecosystem biodiversity at the population level and at a genetic scale. These influences can be evaluated in the context of metacommunity dynamics and the many environmental determinants of microbiome composition to generate testable hypotheses for how contemporary environmental disturbances may disrupt host-microbe symbioses.

Habitat loss and fragmentation due to anthropogenic land-use changes present one of the greatest threats to biodiversity: almost 40% of ice-free land is used for agriculture and an additional 37% represents non-contiguous wildlands fragmented by agriculture, and those percentages are only increasing alongside the need to provide food and textiles for a growing world population (28, 29). Empirical studies across multiple habitats and timescales suggest that habitat destruction and fragmentation have consistently negative effects on both organismal and genetic diversity by creating allopatrically isolated smaller populations prone to increased competition, inbreeding depression, and stochastic genetic drift (89–91). In terms of microbiome composition, habitat fragmentation would be expected to reduce between-host transmission opportunities and decrease local microbial diversity, thus decreasing the size of the local microbial species pool and therefore the taxonomic and functional diversity of the host microbiome. More diverse microbiota are contestably more stable over time (64, 92) and have been associated with healthy development and improved digestion (12), suggesting that habitat fragmentation may decrease host fitness by decreasing microbiome diversity, and may result in differential fitness between hosts in different habitat patches. Smaller host populations would additionally be more prone to stochastic ecological drift in microbiome structure. Early experimental evidence from monkeys (93) and amphibians (94) supports these hypotheses, demonstrating lower microbial α-diversity in fragmented host populations.

Anthropogenic land use can also affect biogeochemistry in adjacent habitats, with known negative effects on biodiversity and potentially variable effects on the microbiome. Eutrophication driven by agricultural runoff has led to toxic algae blooms and the consequent formation of anoxic zones in oceans and lakes, causing large die-offs of aquatic animals (28). Industrial wastes introduce heavy metals and other contaminants to the environment, and at sufficient local concentrations these contaminants are highly toxic to both plants and animals

(28). The ability of populations to recover from these die-off events is further challenged by genetic erosion (95). The effects of an altered biogeochemical environment on a host's microbiota, and the consequent effects on host fitness, are a hard-to-predict function of whether beneficial toxin-processing metabolisms are available in the local microbial gene pool and whether the host can successfully acquire them. For example, the marine sponge *Theonella swinhoei* has acquired symbionts capable of sequestering barium and arsenic, allowing it to survive otherwise toxic concentrations of those metals (96), whereas the taxonomic and functional diversity of the both the earthworm gut (18) and frog skin (75) microbiome decreases with metal exposure, with unknown effects on host fitness. How the microbiome responds to local nutrient loads is thus likely to be host species- or even population-specific.

Habitat loss and fragmentation and increased atmospheric, soil, and water pollution can all be caused by urbanization (97), which has additionally been associated with changes in food availability (98). Urbanization-induced changes in trophic interactions can be caused by local extirpations, introduction of urban-adapted prey species such as rats, and availability of anthropogenic food waste (98–100), and these substantial changes to animal diets would be expected to induce corresponding changes in gut microbiota. A recent comparison of rural and city-dwelling sparrows found that urbanization alters cloacal microbiome functional composition and decreases overall diversity (97), but did not test for differences in host fitness. Urban-dwelling and urban-impacted species from diverse taxa are more commonly infected with parasites than their rural counterparts (76, 101, 102), and gut microbiota can influence parasite susceptibility (103), but to date there are few studies directly addressing the connections between urbanization, host microbiome, and parasite load. While it is likely that diet-induced changes in gut microbiota precede parasite infection, longitudinal studies of individual hosts would be required to demonstrate the causal relationships between these variables.

Climate change represents a global-scale environmental pressure experienced to some degree by all living organisms, resulting in increased ambient temperatures, spread of non-native species, gain or loss of suitable habitat, and altered ecosystem nutrient fluxes (104–106). Early discussions of the hologenome theory of evolution emphasized how the phenotypic plasticity conferred by rapidly adapting microbiomes could facilitate adaptation to climate change (22, 24, 87), but the little experimental evidence that is available has largely failed to support this hypothesis. Host-microbe symbioses in corals and sponges are temperature-sensitive (107–109),

and sustained temperatures even 1-2°C above average result in accumulation of pathogens, expulsion of symbionts, and host death (108, 109). Similarly, lizards kept in large, semi-natural enclosures with 2-3°C warmer climates exhibit a 34% reduction in gut microbiome diversity and lower survival relative to average climates (110). For a holobiont to adapt to climate change, new symbionts need to be acquired, or current symbionts need to adapt to the changing conditions, before the magnitude of the disturbance proves lethal to the host. It would appear that for the few species studied to date, the rate of environmental change exceeds the adaptive capacity of the hologenome, but much work remains to be done to characterize how host-associated microbiomes respond to the various stresses associated with climate change.

The best model systems we currently have for directly evaluating how environmental change can both shape an animal's microbiome and affect host fitness, independent of any natural variation in season or habitat use, are captive and domestic animals. Several studies have used next-generation sequencing approaches to compare the microbiomes of captive or laboratory animals with those of their wild counterparts, and regardless of species or natural habitat, captive animals generally exhibit less diverse microbiota (31, 111, 112). Captive animals have fewer opportunities for between-host symbiont transmission, a confined and often homogenous environment from which to acquire symbionts, and a carefully monitored diet, all of which would reduce the size of the available microbial metacommunity. This is of particular importance to captive breeding programs attempting to rehabilitate endangered species (31): avoiding inbreeding depression has long been a challenge in small, managed populations, but experimental evidence suggests that reintroduction attempts may fail anyway if the organism lacks a sufficiently diverse microbiome (30). An understanding of the microbiome thus plays an important role in conservation biology, suggesting that varying diets and promoting social encounters between multiple hosts and host species may increase the success of reintroduction efforts. However, information gleaned from these studies of how environmental homogenization affects captive animals remains limited in scope, as results from one host species cannot be perfectly extrapolated to others (58, 62, 113).

1.6 APPROACHES TO STUDYING HOW MICROBIOMES RESPOND TO ENVIRONMENTAL CHANGE

Understanding how host-microbe symbioses can be disrupted by environmental disturbances, and how changes in the microbiome can influence host physiology and fitness, is crucial for evaluating the adaptive capacity of the host or holobiont in the face of the sustained environmental changes imposed by human activity. Considerable work has already been done to describe how animal populations respond to anthropogenic disturbance and how the host-associated microbiome shapes host physiology, but direct correlative and causal relationships linking disturbance to a host's microbiome and the microbiome to *in situ* fitness are only beginning to appear in the scientific literature. Untangling these relationships in natural habitats will require careful evaluation of the many feedback loops among the microbiome, host physiology, and host behavior, as well as thoughtful controls to account for natural and expected environmental variation. Our current awareness of how microbiomes are assembled from the microbial metacommunity to which a host is exposed can be used to design experiments testing how the well-documented challenges to ecosystem biodiversity, including habitat fragmentation and loss, eutrophication, and climate change, may affect the microbiome. Results from such studies could inform efforts not only to preserve global biodiversity for its intrinsic value, but also to maintain wild populations that serve keystone ecological functions and provide valuable ecosystem services to humans.

An effective approach to microbiome-oriented conservation research requires integrating established microbiome analyses with traditional ecological field experiments to accomplish three general objectives. First, differences in microbiome composition between host species in disturbed and undisturbed habitats need to be observed and characterized, and these differences must be distinguished from normal spatial and temporal variation due to habitat heterogeneity among undisturbed populations. Researchers need to identify wild animal populations that have no direct contact with areas utilized or impacted by humans and that do not consume domestic or anthropogenic foods (52). These wild populations can be profiled and then compared to populations affected by disturbance, taking care to quantify the types and magnitude of disturbance using existing tools for environmental assessment (114, 115). Methods for collecting and preserving samples suitable for microbiome analysis will depend on the organism of interest. For many organisms, the gut microbiome can be characterized using scat samples obtained non-

invasively from the environment; for others, more invasive sampling techniques like sedation or lethal harvesting may be required. Amplicon sequencing of the 16S rRNA gene and shotgun metagenomics are the most commonly used methods to then identify the taxonomic composition and metabolic potential of these host-associated microbial communities (116–118).

Second, any observed differences in community composition need to be connected to differences in community function. Determining how the microbiome influences host physiology remains challenging even in well-studied animals, including humans, and often require a mixture of both correlative experiments and manipulative experiments with controlled variables. Meta-transcriptomics and meta-proteomics, or quantitative PCR experiments for transcripts of functional interest, can reveal differences in host-microbe metabolic exchanges in different environments or under different experimental conditions. For preliminary investigation, microbiome metabolic profiles can be inferred from amplicon sequencing data using predictive software such as PICRUSt (118) or Tax4Fun (119). Additional experiments utilizing antibiotic treatments, germ-free organisms, or microbiome transplants from hosts into germ-free organisms can often elucidate important connections between the microbiome and host physiology, but these approaches admittedly have several limitations (120, 121). Most importantly, mice are physiologically different than many animal taxa, and results from mice cannot be considered perfectly representative of phylogenetically diverse taxa. Many of these experiments remain challenging for wild animals and would need to be further developed before they could be effectively used across the animal kingdom.

The third and most difficult objective is to demonstrate that these differences in community composition and function have an appreciable effect on host fitness, either positive or negative, independent of other environmentally-induced changes in host biology. Addressing this objective may require targeted longitudinal experiments observing how microbiome composition and host health and fecundity change within individual organisms over an extended period of measurable environmental change. Similar information can be obtained from cross-sectional correlative experiments, with sample sizes large enough to control for broad suites of life history factors such as diet and family structure. However, distinguishing causative from correlative relationships in these studies is difficult given the unavoidable degree of interconnectedness between life history, microbiome, and fitness. Experiments using antibiotic treatments, microbiome transplants, or germ-free model organisms to characterize the function of

the altered microbiota could again be enlightening in this context.

No laboratory experiment can perfectly replicate the intricate biotic and abiotic forces that affect wild animals in natural habitats; developing and refining techniques for *in situ* evaluation of host-microbe symbioses and host fitness will ultimately be the best avenue for a holistic assessment of how perturbation of these symbioses impacts host biology, either positively or negatively. Obtaining this knowledge, which will require extensive partnerships between ecologists and microbiologists, would add to our understanding of host-microbe co-evolution in its natural context and further integrate microbiomes into discussions of global change and of wild animal ecology and evolution. For those organisms in which anthropogenic disturbance negatively affects the host via changes in the microbiome, this knowledge will aid conservation biologists trying to protect biodiversity and ecosystem function.

1.7 THESIS OBJECTIVES, OUTLINE, AND IMPACT

The experiments outlined in the subsequent chapters of this thesis begin addressing the three objectives described above by examining the microbiome of two model animal systems, coyotes and freshwater sponges, in the context of environmental variation or change. As cities and metropolitan areas spread into the surrounding landscape, coyotes (*Canis latrans*), a generalist canid species, are adapting to life in urban environments and now inhabit cities across North America (122, 123). Urban-adapted coyotes eat more anthropogenic food than rural animals, exhibit behavioral differences that may make them more prone to human conflict (123–125), and are more likely to be infected with two zoonotic parasites, sarcoptic mange (125) and the tapeworm *Echinococcus multilocularis* (126), that can be transmitted to domestic animals and pets. *E. multilocularis* can also cause respiratory disease in humans (127). Freshwater sponges live in lakes and streams on Vancouver Island, British Columbia that are differentially affected by agricultural runoff (128, 129), logging operations (130), and climate change. Many oligotrophic sponge species rely on their microbiome for essential nutrients (16, 131–134), and so changes in nutrient availability may alter the microbiome with unknown consequences for host fitness. These two systems represent a comparison of phylogenetically diverse animals experiencing different environmental changes, with unknown consequences for the composition and function of the host microbiome and, furthermore, unknown consequences for the fitness and health of the host.

To begin answering the overarching and largely unexplored questions of how environmental variation or disturbance affects host-microbe symbioses, the specific aim of this thesis is to provide the first descriptive overview of the microbiome in both model systems, using high-throughput sequencing of 16S rRNA gene amplicons to determine the taxonomic composition of each microbiome from hosts living across environmental gradients. These taxonomic profiles are supplemented with environmental and physiological data, when possible, to generate testable hypotheses regarding the functional role of the microbiome in each host, the reasons why the studied environmental gradient may produce the observed changes in the microbiota, and how these changes affect the host. Underlying this work are the dual guiding hypotheses that 1) environmental variation experienced by the animal host produces distinct and replicable changes to its microbiome and 2) these changes to the microbiome have subsequent implications for host fitness.

The next three chapters focus on the coyote gut microbiome. Using data from 76 coyote carcasses collected opportunistically from Alberta fur trappers and the City of Edmonton, I build towards a robust correlative analysis of how the interplay among diet, microbiome composition, body condition, and parasite susceptibility is affected by urban habitat use. Because the coyote microbiome is previously unstudied, I begin in **Chapter 2** by providing a general overview of the composition and diversity of the coyote gut microbiome along the length of the intestinal tract in 10 coyotes. This profile addresses three general questions: how the coyote microbiome compares to other vertebrates, whether fecal samples are representative of the proximal intestine in a wild animal, and how individual life history and intestinal microenvironment interact to shape the microbiome. As far as I can ascertain, this is the first such profile in an animal outside of laboratory and clinical setting, and it provides an important foundation for subsequent examination of how environmental change may affect this natural microbiome composition.

Chapter 3 uses the entire sample of 76 coyotes to assess how variation in the microbiome relates to variations in diet and body condition. Microbiome profiles based on 16S rRNA gene amplicons are supplemented with body condition data obtained from coyote necropsies, dietary information obtained from stomach contents and stable isotope analysis, and parasite infection status determined using PCR. I specifically test the hypothesis, common to humans, that physiological health relates to microbiome diversity and the abundance of select probiotic taxa. Only fecal samples are used for this analysis, with the goal of generating results

applicable to future scat-based studies Diet, microbiome, and health are inextricably linked; identifying which dietary elements and microbiome signatures are associated with good or poor body condition builds on the general characterization of the "normal" coyote gut microbiome provided in Chapter 2 and provides context for the analysis, presented in Chapter 4, of how urban habitat use affects coyote diet, microbiome, and health.

Differences between urban and peri-urban coyotes are tested in **Chapter 4** using a further-expanded data set that also includes microbiome profiles from the small intestine. The chapter frames the expectation of a clear, linear relationship in which urban coyotes consume a broader diet containing more anthropogenic food, have a distinct microbiome reflecting this altered diet, and in turn exhibit poor health or decreased fitness. While the results of these analyses are not able to show direct causative relationships linking microbiome composition to fitness, they do suggest the potential for the microbiome to contribute importantly to disease and conflict in urban coyotes.

In **Chapter 5** I turn to the microbiome of freshwater sponges, which have received limited attention almost exclusively in Lake Baikal (135, 136). Despite hundreds of studies describing the microbiome of marine sponges, the existence of freshwater sponge-specific microbial communities, distinct from the communities found in ambient water, has been questioned by some authors (137, 138). I use 16S rRNA gene amplicons to characterize the microbiome of the sponge *Ephydatia muelleri* in the Sooke, Nanaimo, and Cowichan Rivers on Vancouver Island, with the goal of demonstrating that this sponge does indeed harbor a unique microbiome. Although I also evaluate physicochemical properties of the ambient water to test for evidence of eutrophication, the nature of this study is largely exploratory, and the limitations of sample size and geographical breadth preclude any robust evaluation of environmental disturbance. However, the ecosystem services provided by marine sponge microbiomes are well-documented (46), and by demonstrating the presence of freshwater sponge-specific microbiomes in these Vancouver Island streams I open the possibility that these microbial communities could play important ecological roles in mediating future eutrophication.

Collectively, the results presented in this thesis provide a preliminary characterization of two previously unexplored microbiomes, and the knowledge of how disturbance or variance may affect host-microbe symbioses adds to our growing awareness of the microbiological impacts of human activity. Both host-microbe systems addressed by this research also present relevant

objectives for conservation biologists. Understanding if and how disturbance of the coyote gut microbiome influences aggression or parasite susceptibility could inform management practices to mitigate human-coyote conflict and the spread of zoonotic disease. Filter-feeding by sponges and other sessile invertebrates controls water clarity and influences primary production, providing an ecosystem service that, if lost, would have significant downstream effects on other aquatic organisms (139). Preserving this service requires understanding the sponge's ability to adapt to environmental change, which may be mediated through its microbiome. For both systems, future studies to directly evaluate the consequences of a changing microbiome for host fitness will provide an additional base on which to ground management decisions. These ecological implications are discussed alongside potential future studies more thoroughly in **Chapter 6**.

As ecologists evaluate how human activity continues to reshape the planet on which we live, and as microbiologists continue to explore the intimate partnerships between microbes and animal hosts, there is no better opportunity to unite these two lines of research to inform conservation biology. That union is the fundamental objective of this thesis. Microbes have influenced animal evolution since animals first appeared on the planet and have persisted through the dramatic environmental changes that caused mass extinctions of many animal clades; it seems only fair for us to also consider how microbes will respond to contemporary and future environmental change as they continue to shape host evolution through their influence on animal physiology.

CHAPTER 2

High individual variation in a biogeographical profile of the coyote gastrointestinal microbiome

2.1 ABSTRACT

Most of our knowledge of the vertebrate gut microbiome comes from fecal samples; however, the gastrointestinal tract traverses several different physical, chemical, and environmental conditions from stomach to anus and the microbiome varies with intestinal microenvironment. Due to the difficulties involved in sample collection, the upper intestinal microbiome is poorly understood in wild animals despite its potential to inform broad interpretations about host-gut microbe relationships under natural conditions. Here, we used 16S rRNA gene sequencing to characterize the microbiome of wild coyotes (*Canis latrans*) along the length of the gastrointestinal tract, including samples from the duodenum, jejunum, ileum, caecum, ascending and descending colon, and feces. Microbial communities in the large intestine were distinct from those in the small intestine, with higher diversity and a greater abundance of anaerobic taxa. Fecal samples were not an adequate proxy for studying upper intestinal environments, as they contained only half the amplicon sequence variants (ASVs) present in the small intestine. Within each of the small and large intestine, individual identity explained six-fold more among-sample variation than intestinal microenvironment, suggesting that the selective pressures experienced by the coyote gastrointestinal microbiome are intestinal segment (small or large), followed by individual lifestyle context, and finally, weakly, the different microenvironments (or locations) within each segment. Our study appears to be the first such investigation conducted using free-living animals rather than livestock or laboratory organisms and provides a foundational understanding of the gastrointestinal microbiome in a wild canid.

2.2 INTRODUCTION

Much attention has been given to the role of the gut microbiome in human health and disease (140), and more recently microbiome research has expanded to include domestic and wild animals (52, 84, 141). Most microbiome research is conducted using scat or fecal samples, which are non-invasive and easy to obtain from any study organism. Fecal samples have been used to identify microbes associated with many different diets and health conditions across phylogenetically diverse animal taxa (36, 55, 58, 70). However, the physiology and function of the gastrointestinal tract varies greatly along its length (142), and the gut microbiome correspondingly varies with the intestinal microenvironment (143). In humans, few microbes survive in the highly acidic conditions of the stomach, where only 10^1-10^2 cells from less than 100 taxa persist per gram of stomach content, whereas the nutrient-rich colon houses 10^{12}-10^{13} cells per gram of content, representing hundreds of taxa and 70% of all microbes found in the body (144). Fecal samples only partially represent the microbial communities inhabiting the more proximal intestine, limiting the availability of region-specific information on communities that may play different roles in relation to different diets and health conditions (145, 146).

Attempts to directly study these upper intestinal communities and their relationship with their animal host are likewise limited in their breadth of application. In humans, studies of the upper intestinal microbiota often rely on biopsy samples, which limits the number of physical locations that can be studied in a single individual. Biopsy samples are also collected after bowel preparation, which temporarily alters the native intestinal ecosystem (147). In laboratory studies, all animals are housed in identical cages and fed the same diet in synchrony (148, 149), which enables the characterization of intestinal niche-specific microbiota but cannot discriminate the effects of variation in individual diet or other lifestyle contexts. Moreover, wild animals do not share the same microbiota as captive conspecifics (111, 112) and are difficult if not impossible to ethically sample (52). For these reasons, few studies have been able to profile the microbiome along the length of the intestine in any wild animal living in an uncontrolled environment, despite high potential to inform broad interpretations. Expanding our current knowledge of the fecal microbiome in wild animals to include information from these hard-to-reach upper intestinal environments could provide valuable insight not only on the utility of fecal samples for assessing microbiome composition and function elsewhere in the intestine but also on how the niche-specific nature of the intestinal microbiome is affected by among-individual differences in

lifestyle context.

In this study, we used high-throughput sequencing of 16S rRNA gene amplicons to investigate biogeographical variation in microbiome composition along the gastrointestinal tract of coyotes (*Canis latrans*), a wild canid that is prevalent throughout North America. We characterized the microbiome at multiple sites within both the small and large intestine, with a primary goal of evaluating the extent to which fecal samples are representative of these intestinal environments. We additionally sought to discriminate how the competing selective pressures of intestinal microenvironment and individual diet history interact to shape the microbiome among intestinal sites and among individuals. Our study appears to be the first such investigation conducted using free-living animals rather than livestock or laboratory organisms. Previous studies have reported similar characterizations in kenneled dogs fed standardized diets, concluding that, as in humans, the colonic microbiome is more diverse than the upper intestine, harboring more taxa at significantly different abundances (150, 151). Coyotes are generalist consumers of broad diets that range from mammalian prey through vegetation to anthropogenic food. While we expected to observe a similar increase in diversity along the coyote gastrointestinal tract, we hypothesized that individual diet and lifestyle context would play a larger role the explaining differences in microbiome composition than the more often-cited differences in intestinal microenvironment.

2.3 METHODS

2.3.1 Sample collection and necropsy

As part of a separate study analyzing the effect of urban habitat use on the coyote gut microbiome (this thesis, Chapter 4), we collected 76 coyote carcasses killed for other reasons in and around Edmonton, Alberta, Canada. For our examination of intestinal microbiome biogeography, we randomly selected 10 individuals, attempting to minimize variation in sex, body size, and habitat use. All 10 individuals were large (> 10kg) males killed by local fur trappers working in small forested areas outside of the city and had no visible signs of deformity or disease. Carcasses were frozen at -80°C for 5 days to neutralize zoonotic parasites before being transferred to -20°C until necropsy.

At necropsy, we isolated the gastrointestinal tract from each coyote. We extruded any fecal material present in the rectum before removing 10cm sections of the duodenum, jejunum,

ileum, ascending colon, and descending colon, and isolating the caecum. Each intestinal section was longitudinally transected along its anti-mesenteric side and the luminal contents were gently advanced into microcentrifuge tubes using sterile spatulas, taking care not to disturb the intestinal mucosa. Luminal contents were similarly removed from the caecum, resulting in a total of 7 samples per individual, save for two coyotes with no feces present in the rectum. We stored all samples at -80°C until analysis. For the purposes of this study, we refer to each sampling location as a sampling "site" that belonged to one of two intestinal "segments," either the small or large intestine.

2.3.2 DNA extraction and sequencing

We extracted whole community DNA from 100mg of each sample using the MP Bio FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA), following the manufacturer's instructions. An additional 5-minute incubation at 50°C was added prior to the final elution to maximize DNA yield. We tested for DNA purity using a Nanodrop spectrophotometer and quantified concentrations using Qubit 1.0 (Thermo-Fisher) before submitting samples to Microbiome Insights (Vancouver, British Columbia) for high-throughput sequencing of the V4 region of 16S rRNA gene. In brief, DNA samples were PCR-amplified in 50μl reactions with 2μl of template DNA using the universal bacterial primers 515F (5'-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3') and 806R (5'-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3') modified to include Illumina adaptors and sample-specific indices. Amplicons were normalized to equimolar concentrations and sequenced on an Illumina MiSeq using V3 chemistry and 250bp paired-end reads. We removed one sample that produced less than 1,000 reads, resulting in an average of 44,969 raw reads per sample. Raw, unfiltered sequence data has been deposited in the NCBI Short Read Archive under accession number PRJNA528765.

2.3.3 16S rRNA gene sequence analysis

16S rRNA gene sequence data was processed using the package *dada2* (152) as implemented in R 3.5.0. We truncated forward and reverse reads at 240bp and 160bp, respectively, and removed low-quality reads using the DADA2 default filtering parameters. We then used DADA2 to merge paired-end reads and determine exact amplicon sequence variants

(ASVs) using the pooled inference procedure. ASVs are determined based on predicted sequencing error rates and can resolve differences at the level of individual nucleotides, providing better taxonomic resolution than traditional clustering techniques that use a standardized 97% similarity cutoff (153). To assign taxonomy, we removed chimeric sequences using DADA2 and aligned ASVs against taxa in the RDP reference database (Release 11.5) (154) using the naïve Bayesian classifier method implemented in DADA2 (155). ASVs that were identified as chloroplasts or mitochondria were removed. We used the package *phangorn* (156) to generate a generalized time-reversible maximum likelihood phylogenetic tree for our data following the procedures described by (157). For our final analyses, we included only ASVs with a length of 250-256bp and averaged ASV abundances across 1,000 rarefactions to the minimum library size of 8,279 reads. Our final feature table included 1,111 unique ASVs and was imported into the R package *phyloseq* (158) for subsequent analysis.

2.3.4 Statistical analysis

We calculated ASV richness and Shannon's diversity index from rarefied data using *phyloseq* and used a two-way ANOVA with Tukey's honestly significant difference *post hoc* test to analyze large-scale differences between intestinal segments and smaller-scale differences among intestinal sites. For pairwise comparisons, we report p-values corrected for family-wise error rates. We validated our results by comparing linear mixed-effect models with and without individual identity as a random effect, where either richness or diversity was a normally-distributed dependent variable and intestinal site was the predictor. The amount of variance explained by the fixed and random terms was calculated using the package *MuMIn* (159). Inter-individual variation in diversity measures at each intestinal site was further assessed using the coefficient of variance.

Significant differences in taxon abundances between segments and among sites were calculated at the phylum, class, and family levels using the default settings of the package *ALDEx2* (160), which includes a centered log-ratio transformation of Dirichlet-distributed Monte Carlo samples of the unrarefied data and the Benjamini-Hochberg correction for false discovery rates. The between-segment comparison was evaluated using Welch's t-test and the among-site comparison with the native 'glm' method in *ALDEx2*.

We assessed the extent to which ASV abundances at individual sampling sites were reflected in the feces by calculating Spearman's correlation between mean stool ASV abundances and all other sampling sites. To enhance our biogeographical resolution of the microbiome, we extended this analysis to include all pairwise contrasts across all sampling sites. We additionally searched for ASVs that were present at intestinal sites but undetected in feces.

The relative explanatory power of individual lifestyle context and intestinal microenvironment in shaping overall microbiome composition was evaluated using non-metric multi-dimensional scaling (NMDS) based on the Bray-Curtis distance. We determined significant structuring effects using a permutational analysis of variance (PERMANOVA) with 1,000 permutations implemented using the 'adonis' function in *vegan* (161). Individual identity and intestinal site were included as predictors, and the R^2 value was used to assess the degree to which each predictor explained overall variation. This analysis was performed on all samples, as well as separately for the small and large intestine, and was repeated using the weighted UniFrac distance measure, which additionally considers phylogenetic relatedness in its calculation of ecological distance (162). To account for possible dispersion effects, we calculated multivariate dispersion using the 'betadisper' function in *vegan* and tested for homogeneity of group dispersions using the 'permutest' function with 1,000 permutations.

2.4 RESULTS

2.4.1 Diversity along the GI tract

We characterized the microbiome of luminal contents from six intestinal microenvironments ("sites"), in addition to fecal samples extruded from the rectum, in 10 large male coyotes. Species richness and the Shannon diversity index were calculated to profile variation in microbial biodiversity along the intestinal tract; individual rarefaction curves for both measures approached the saturation plateau (**Supplementary Fig. 2.1**), suggesting that our sequencing efforts adequately captured these communities. Sites in the large intestine contained an average of 80% more ASVs (142±51 vs. 79±40, mean±SD), corresponding with a 50% increase in diversity (2.81±0.66 vs. 1.80±0.53), relative to the small intestine (**Fig. 2.1a, b**; $t_{richness}$=5.42, df=65, $p<0.001$; $t_{diversity}$=6.71, df=65, $p<0.001$). The increase in richness and diversity between intestinal segments appeared to begin in the ileum: in pairwise comparisons among sample sites, all sites within one segment were similar to each other and significantly

different from sites in the other segment, excepting the ileum, which is located at the distal end of the small intestine and could not be statistically discriminated from any of the other five sites (**Supplementary Table 2.1**).

When examined individually, all 10 coyotes modeled the overall average trend of increasing diversity along the intestine (**Supplementary Fig. 2.2**). In linear mixed-effect models predicting richness and diversity based on intestinal site and individual identity, the effect of intestinal site explained 37.0% and 45.6% of the variance, respectively, compared to only 17.2% and 2.1% that could be explained by the random effect of individual. Inter-individual variation in richness and diversity was highest in duodenal and jejunal samples, as well as in feces (**Fig. 2.1c, d**), with lesser variation between those two regions. One exception to this trend was the caecum, where the coefficient of variance for the Shannon index peaked relative to other locations (**Fig. 2.1d**).

2.4.2 Taxonomic composition and ASV detection in feces

We assessed how the increasing diversity along the intestine was reflected in the abundance of different taxa. The dominant phyla at all seven sampling sites, in rank order, were Firmicutes, Fusobacteria, Proteobacteria, Bacteroidetes, and Actinobacteria, which together accounted for 99.8% of the intestinal microbiome (**Fig. 2.2**). All except Proteobacteria demonstrated significantly different abundances across the different sites after Benjamini-Hochberg correction for multiple comparisons. Firmicutes, represented almost exclusively by the class Clostridia, dominated in the duodenum and decreased by 50% along the intestine (p=0.003), while Fusobacteria, Bacteroidetes and Actinobacteria rose in abundance (p=0.040, p<0.001, and p<0.001, respectively). Proteobacteria were consistently present at 11.9% mean abundance across all intestinal sites.

At finer taxonomic resolution, eleven bacterial families were significantly differentially abundant among intestinal sites (**Fig. 2.3**). Apart from *Clostridiaceae*, which became six-fold less abundant from the duodenum to the descending colon, all these families were more abundant in the distal colon. Nine of these eleven families are obligate or facultative anaerobes; only *Erysipelotrichaceae* and *Helicobacteraceae* are aerobes or microaerophiles. Several additional families present at low abundances in the colon were not detected in the upper intestine, which was expected given the increased species richness in the colon, but six bacterial families detected

at various intestinal sites were unexpectedly not detected in any of the fecal samples (**Fig. 2.3**). Of these six, *Rhodobacteraceae* was present in the ileum and ascending colon at surprisingly high relative abundances (> 3%) for there to be no detection in the feces.

Based on the presence of these fecal-undetected taxa, we evaluated how well fecal samples represented other intestinal sites at the level of individual ASVs. Of the 759 ASVs with at least 0.001% mean relative abundance in our study, 361 were not detected in fecal samples. Most were either low-abundance taxa present in only one or two coyotes or were more abundant in the proximal intestine than the distal intestine, but several ASVs belonging to Alphaproteobacteria (including *Rhodobacteraceae*) and Verrucomicrobia that were detectable in colonic samples at modest abundances (>0.05%) in some individuals were undetected in fecal samples from those same individuals. For ASVs that were present in feces, we compared ASV abundances among sites using Spearman's correlation. Fecal ASV abundances were highly correlated with both colonic sites (descending colon R=0.64, p<0.001; ascending colon R=0.45, p<0.001) and became less informative towards the proximal intestine, with the largest decrease in Spearman's correlation coefficient occurring between the ileum and jejunum (**Fig. 2.4**). We additionally found that the most closely related sample site pairs, in terms of ASV abundances, were the caecum and ascending colon (R=0.64, p<0.001) and the caecum and descending colon (R=0.66, p<0.001).

2.4.3 Beta-diversity within and among individuals

We attempted to discriminate the relative effects of intestinal microenvironment and individual lifestyle context in shaping the coyote gut microbiome. The greatest variation in overall microbial community structure, evaluated using the Bray-Curtis dissimilarity index among individual samples, was explained by whether a sample came from the small or large intestine (**Fig 2.4a**; PERMANOVA F=21.01, R^2=0.342, p<0.001). Ileal samples clustered with duodenal and jejunal samples but shared compositional similarities with both the small and large intestine (**Table 2.1**). There were no significant differences in multivariate dispersion, or between-sample diversity, between segments or among sites (**Supplementary Table 2.2**).

When we removed the effect of intestinal segment by testing the three small intestinal sites separately from the four large intestinal sites, individual identity explained 56.1% and 64.2% of inter-sample variation in the small and large intestine, respectively. Only 9.3% and

4.5% of variation could be explained by sampling site (**Fig. 2.4b-d, Table 2.1**). This within-segment effect of individual identity also outweighed the effect of intestinal microenvironment when using the weighted UniFrac dissimilarity index, which accounts for phylogenetic relatedness and therefore smooths over samples that contain different ASVs from the same higher taxa (**Supplementary Fig. 2.3**). Coyotes were highly variable in the degree of similarity within their intestines: for some animals, the various sampling sites within each intestinal segment exhibited significantly higher multivariate dispersion relative to other coyotes for which the microbiomes of same-segment intestinal sites were hardly differentiable (**Supplementary Table 2.2**).

2.5 DISCUSSION

Obtaining information about the ecophysiology of the upper intestinal microbiome in wild animals is challenging but has the potential to yield valuable insights into how these communities respond to natural variation in diet and health. We characterized the diversity and taxonomic composition of fecal samples and six additional intestinal sites in ten wild male coyotes. Consistent with previous studies of wolves and domestic dogs (150, 151, 163), microbiome diversity was highest in the large intestine, which harbored more anaerobic taxa from a greater variety of phyla than the small intestine, which was dominated by Firmicutes. We used this intestinal profile to test the efficacy of fecal sampling for studying upper intestinal environments and found that almost half of the ASVs present in the small intestine were not detected in fecal samples, and those that were detected were not present at comparable abundances. Additionally, within each of the small and large intestine, the effect of individual diet and lifestyle context was six-fold larger than the effect of intestinal microenvironment in explaining microbiome composition. Our results contradict previous studies in animals with standardized diets and housing, which identified niche-specific intestinal communities and claimed that feces may still be an adequate proxy for evaluating the entire gastrointestinal microbiome (151, 164).

Overall, the coyote gut microbiome shared the same general taxonomic composition common to most mammals (113), with all intestinal sites containing Firmicutes, Fusobacteria, Bacteroidetes, and Proteobacteria as the dominant bacterial phyla. For unknown reasons the abundance of Fusobacteria throughout the intestine (6.2-24.3%) appears to be unique to canines

relative to other mammals (165). Our observations that the abundance of Firmicutes decreases along the intestine, making room for more Fusobacteria and Bacteroidetes, also agrees with results from domestic dogs (150, 151), although coyotes consistently harbored more Firmicutes relative to dogs at all intestinal sites. Members of the Firmicutes exhibit diverse metabolic specializations but are generally more responsible for protein degradation and consequently more abundant in carnivorous mammals compared to herbivores (113); coyotes consume a more carnivorous diet than domestic dogs and the universally higher abundance of Firmicutes likely reflects that distinction.

The specific structural and compositional differences we observed in microbial communities between the small and large intestine likewise follow known variation in intestinal physiology and function (142, 143). Most dietary proteins, lipids, starches, and simple sugars are absorbed in the small intestine, where pH is lower, transit time is shorter, cell turnover rate is higher, and conditions are more aerobic (142, 166). These conditions limit the number and types of microbes able to survive in the small intestine, leading to lower species richness and diversity. Because the host is responsible for most digestive action in this region, the breadth of metabolisms represented by the microbes that do persist is narrow (143). In the less harsh conditions of the large intestine, the host primarily absorbs water, vitamins, and electrolytes (142, 166), whereas the microbiota break down the diverse suite of undigested starch, unabsorbed sugars, and polysaccharides passed from the small intestine (143). These large intestinal communities experience fewer selective pressures and are correspondingly richer and more diverse, favoring anaerobes representing a wider variety of taxa and metabolisms (143). In our study, the transition from lower to higher diversity began in the ileum, which in dogs is where intestinal diameter begins to increase and villi become longer and wider, starting to resemble the wide and villi-free colon (167). Functionally, the ileum shares both the colon's role in water absorption and the jejunum's role in absorbing fully-digested carbohydrates and proteins (168), further supporting the ileal microbiome as a transitive community between the two intestinal segments.

Among-individual variation in alpha diversity was generally highest in the small intestine. Similarly high degrees of variability in upper intestinal microbial diversity have been observed in pigs (169) and mice (148). In mice, among-individual differences in intestinal physicochemical conditions are largest in the small intestine, which receives the most direct

influence from digesta and provides the least consistent or stable environment for bacterial growth (148). We did not measure intestinal physicochemical parameters here because we were unable to necropsy carcasses immediately after death, but we suspect similar heterogeneity would be found in our sample. Interestingly, the higher among-individual variance in small intestinal alpha diversity was not accompanied by a matching increase in among-individual beta-diversity, suggesting that these communities are still dominated by the same few taxa even if the total number of taxa they contain is more variable. It has been proposed that reduced microbial colonization of the proximal intestine stems from a co-evolutionary constraint imposed by the host to minimize host-microbe competition; hosts do this to ensure that the food items available to the most populous and diverse gut microbial communities are only those that are recalcitrant to host digestion in the small intestine (142). The fact that some individuals have a more diverse small intestinal microbiome than would be expected for this region could have important implications for host-microbe relationships and the degree of symbiosis relative to competition being experienced by the host.

Diversity in the caecum demonstrated an unexpectedly high coefficient of variation given that the caecum, which is a site of cellulose fermentation in herbivores, has limited function in carnivores whose diets contain little or no vegetation (142). Part of this variability was driven by one individual (coyote #37) with only two bacterial families, *Peptostreptococcaceae* and *Lachnospiraceae*, present in the caecum at 99% and 1% relative abundance, respectively. The caecum of this same individual was filled with over 50 helminths (tapeworms) greater than 1cm in length. However, even with this unique individual removed, the coefficient of variance in the caecum remained higher than other sites. Despite their carnivore physiology, coyotes are known to consume fruit, vegetation, and plant-based anthropogenic food (170), and the high variability in caecal diversity may reflect varying degrees of omnivory among the coyotes in our sample. Previous studies of the caecal microbiota have primarily focused on hindgut and foregut fermenters (171) but diet-induced changes in caecal microbiome composition have been reported for several of those taxa (172–174). Our results warrant further exploration of how intestinal parasites and generalist diets affect the carnivore caecum.

Although many of these observations regarding the structure and composition of the coyote gastrointestinal microbiome largely match profiles obtained from laboratory studies of livestock or captive vertebrates, two key aspects appear to be unique to the uncontrolled setting

tested here. One of our two primary objectives using our intestinal profile was to determine if fecal samples could be used to preliminarily assess the composition of other intestinal microenvironments in a wild animal. We conclude that while fecal samples may be a good proxy for sampling the colonic microbiome, they are only loosely representative of the small intestine and therefore cannot be relied upon to reveal lower-magnitude, yet functionally important, variation in communities residing above the caecum. Fecal ASV abundances did not correlate well with the duodenum or jejunum, and in ordination analyses fecal samples clustered with large intestinal samples separately from the small intestine. Our results disagree with previous studies in chickens (164) and monkeys (149) that claim feces may still be a good proxy for abundance-free assessments of microbiome membership, as almost half of the ASVs present in the coyote small intestine were not detected in feces. Although feces were more representative of small intestinal sites than samples from the caecum or ascending and descending colon, their representation was generally poor.

Our second major objective was to evaluate whether intestinal microenvironment or individual natural history played a larger role in shaping the gut microbiome in a sample where we did not control for diet, habitat use, or other aspects of natural history. Outside of the compositional differences distinguishing the small and large intestine, the effect of individual identity was six-fold larger than the effect of intestinal microenvironment. Among-individual variation was small relative to location-based differences in intestinal profiles for dogs (150, 151), pigs (169, 175), poultry (164, 176), and mice (148), but these animals experienced standardized diets and housing conditions. A single study of rhesus macaques (*Macaca mulatta*) found large differences among individuals in the microbial composition of the intestinal lumen, but it also found site-specific differences that our samples lacked (149). Our results may be more representative of wild animals for which the microbiome differs with diet (55), habitat use (177), asymptomatic disease (178), and environmental exposure (179). The implication of these differences between lab-based and wild studies is that functional understandings of the gut microbiome will require samples from regions in both small and large intestines. Our results suggest that the location of those samples within each region may not greatly affect the final conclusions.

An important limitation of our study is that we did not sample the mucosal microbiome at each sampling site. Mucosal microbes interact more directly with the intestinal villi and

epithelia, are less affected by transient passage of digesta, and may play a more direct role in transferring nutrients from digesta to host (34, 149), but they are also more difficult to reliably sample independently of the lumen even in euthanized animals (149), which is why we did not attempt to study them here. Work in humans and has shown that mucosal communities are more site-specific and contain more aerobic taxa than the adjacent lumen (180), and it remains possible that the mixture of selective pressures created by intestinal microenvironment and host life history are experienced differently by the mucosal microbiota. More work will be needed to sample these communities and determine how they experience the balance of selective pressures created in the gut.

We conclude that integrating the intestinal microbiome profile in free-living coyotes with prior knowledge of intestinal ecology and microbial metabolism from laboratory experiments offers a refined perspective on the factors that shape the gut microbiome in wild animals. Samples of the proximal intestine are difficult to obtain from free-living animals but ignoring this region likely will not capture all the functionally meaningful associations between mammals and their gut microbiota. These highly specialized gut microbial communities face selective pressures imposed by both their immediate microenvironment in the host intestine and their broader environment created by host diet, behavior, and environmental exposures. The resulting community thus reflects a balance of these two selective forces and so deducing the relative effects and importance of each pressure independently will be challenging for microbiome research on wild animals. In our study, intestinal segment (small or large intestine) conveyed the largest effect on microbiome communities, followed by individual identity, and finally, weakly, by the different microenvironments (or locations) within each intestinal segment. We consequently suggest that supplementing fecal samples with samples of the small intestine in traditional wildlife microbiome studies, when possible, could yield valuable insights into the nature of host-microbe associations in wild animals.

2.6 ACKNOWLEDGEMENTS

2.7 TABLES & FIGURES

Table 2.1: Permutational analysis of variance (PERMANOVA) results evaluating microbial community structure.

Permutational analysis of variance (PERMANOVA) was used to determine the relative effect of intestinal segment (small or large intestine), intestinal site, and individual identity in explaining differences in microbiome composition among samples. Results for all samples are shown on the left, and pairwise comparisons between intestinal sites are shown on the right.

	PERMANOVA					PERMANOVA		
Variable	F	R^2	p	Pairwise comparison		F	R^2	p
All Samples				Duodenum -	Jejunum	0.462	0.026	0.866
Segment	21.014	0.342	**0.001**		Ileum	1.828	0.097	0.089
Individual	4.447	0.180	**0.001**		Caecum	7.548	0.307	**0.001**
					Asc. Colon	7.047	0.293	**0.001**
Small Intestine					Des. Colon	7.902	0.317	**0.001**
Site	2.299	0.093	**0.020**		Feces	4.477	0.230	**0.003**
Individual	3.072	0.561	**0.001**					
				Jejunum -	Ileum	1.659	0.084	0.107
Large Intestine					Caecum	6.376	0.262	**0.001**
Site	1.200	0.045	0.220		Asc. Colon	5.919	0.247	**0.001**
Individual	5.705	0.642	**0.001**		Des. Colon	6.633	0.269	**0.001**
					Feces	3.659	0.186	**0.004**
				Ileum -	Caecum	2.796	0.134	**0.002**
					Asc. Colon	2.044	0.102	**0.041**
					Des. Colon	2.594	0.126	**0.006**
					Feces	1.205	0.070	0.247
				Caecum -	Asc. Colon	0.643	0.034	0.794
					Des. Colon	1.077	0.056	0.37
					Feces	0.713	0.043	0.75
				Asc. Colon -	Des. Colon	0.221	0.012	0.997
					Feces	0.241	0.015	0.997
				Des. Colon -	Feces	0.275	0.017	0.986
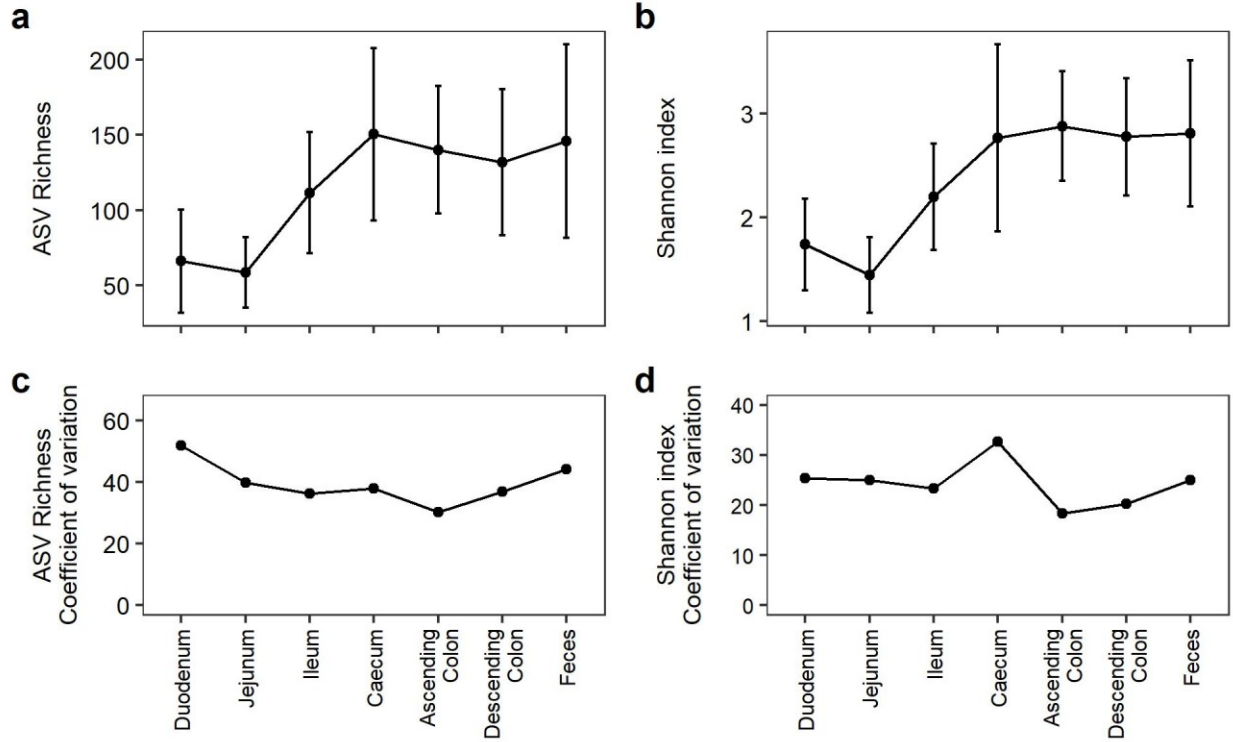
Fig. 2.1: Richness and diversity increase along the length of the intestinal tract.

ASV richness (**a**) and the Shannon diversity index (**b**) shown along the length of the coyote intestine. Corresponding coefficients of variation for each intestinal site are shown below (**c, d**). Richness and diversity generally increase along the length of the intestine and are more variable among individuals in the small intestine. The caecum presents an unusually high degree of variability in diversity
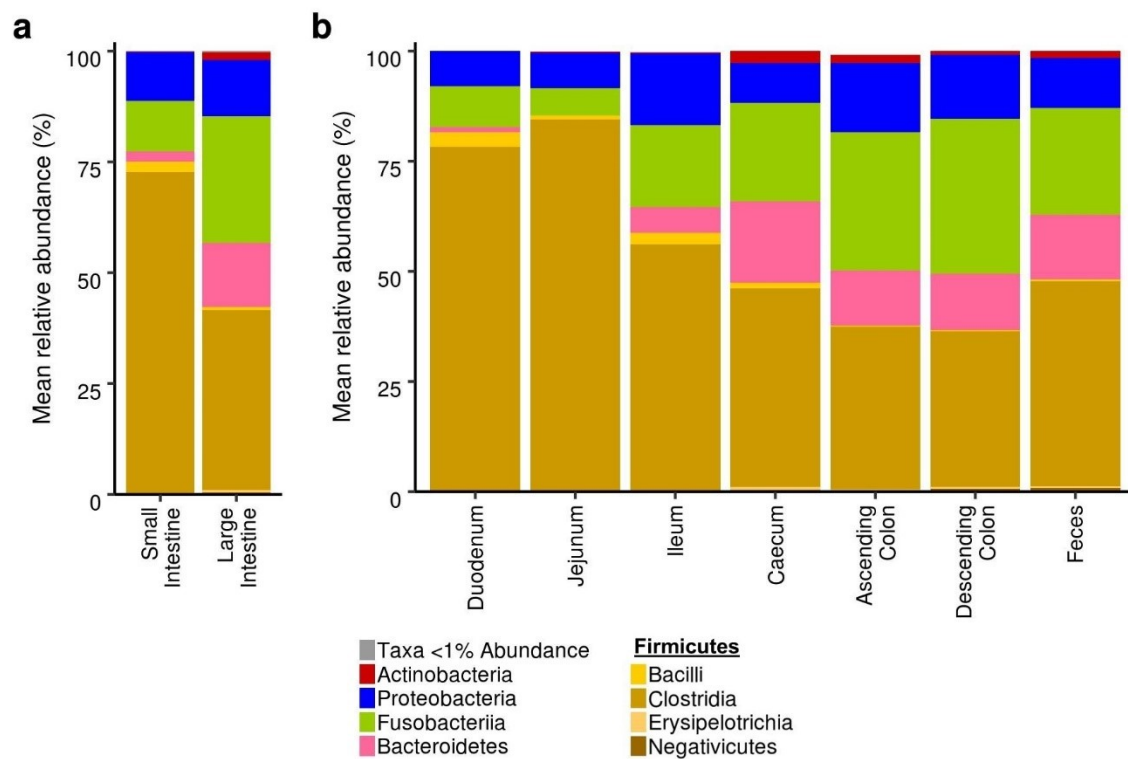
Fig. 2.2: Relative abundance of bacterial phyla at different intestinal sites.
Average relative abundances of bacterial phyla per general intestinal segment (**a**) and specific
sampling site (**b**). Phyla with a mean relative abundance less than 1% are classified as 'other.'
Firmicutes, the most abundant bacterial phyla, are further subdivided to the class level and
shown in shades of yellow.
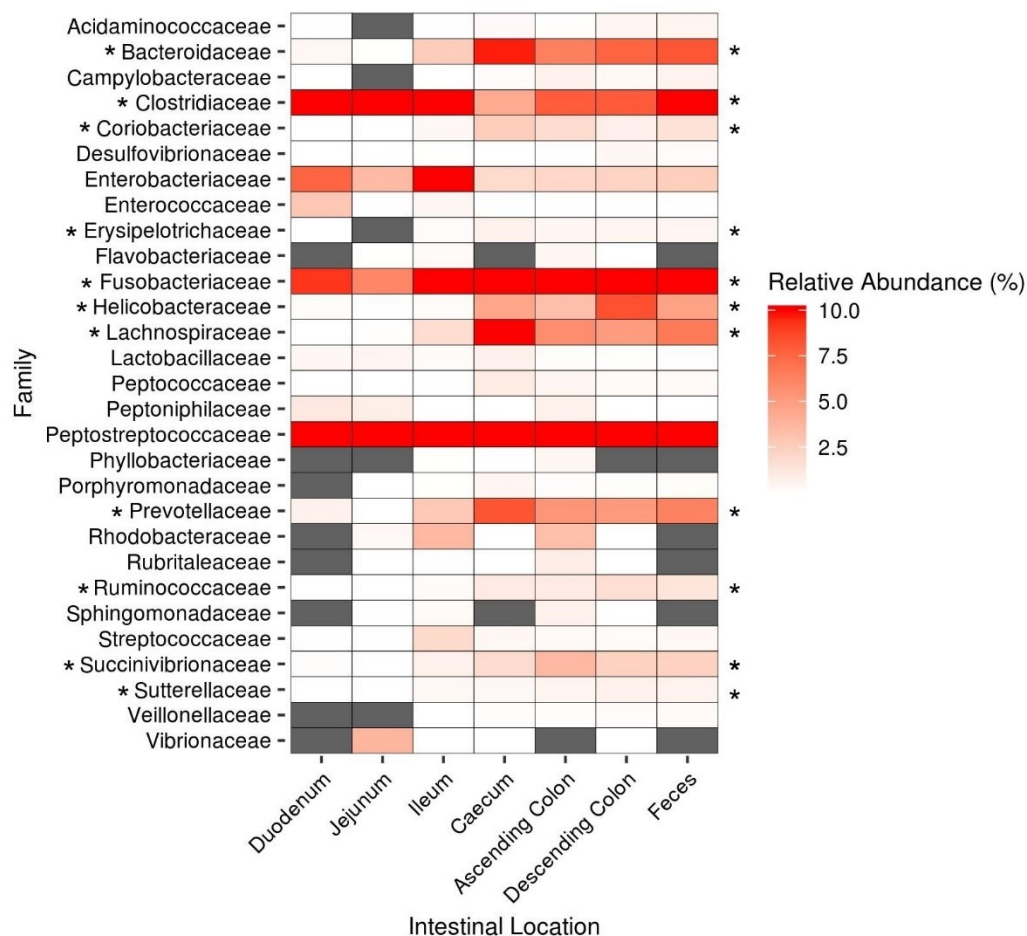
Fig. 2.3: Family-level relative abundances vary along the intestine.

Average relative abundances for different bacterial families along the intestinal tract, with abundance indicated in shades of red. Only families present at >0.01% relative abundance in the entire sample are shown, and grey boxes indicate sampling sites where a given bacterial family was not detected. Families whose abundances were significantly different among intestinal sites after correction for false discovery rates ($p < 0.05$) are indicated with an asterisk. Three families (*Clostridiaceae, Fusobacteriaceae,* and *Peptostreptococcaceae*) exceeded 10% abundance in some intestinal sites.
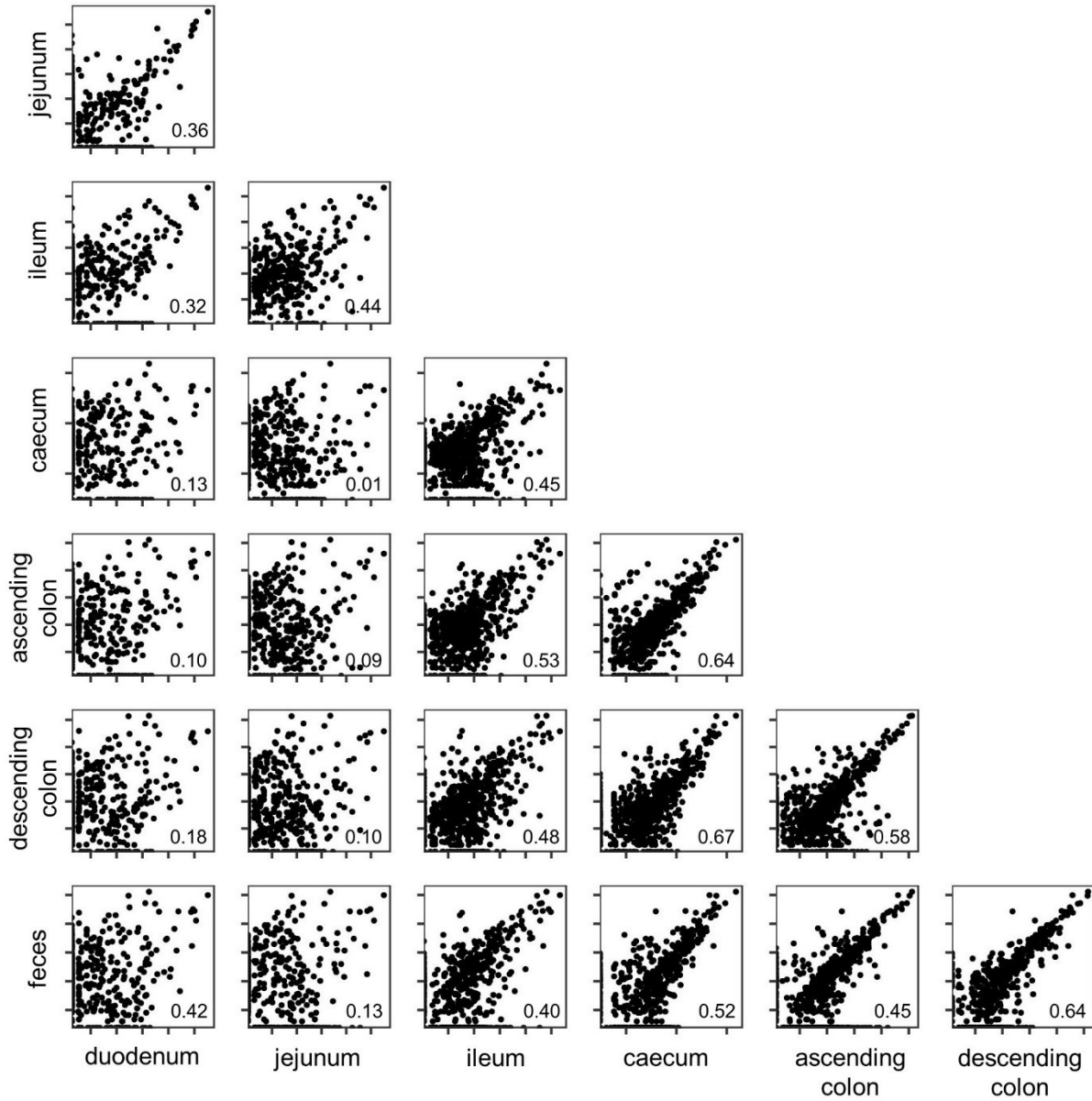
Fig. 2.4: Fecal sample ASV abundances most directly reflect the colonic microbiome.
Relative abundances of each ASV at each intestinal sampling site, averaged across ten individuals, are plotted in a pairwise correlation matrix. In each plot, each dot represents one ASV. Spearman's correlation coefficient is given in the bottom right of each graph, and both axes are log-transformed.
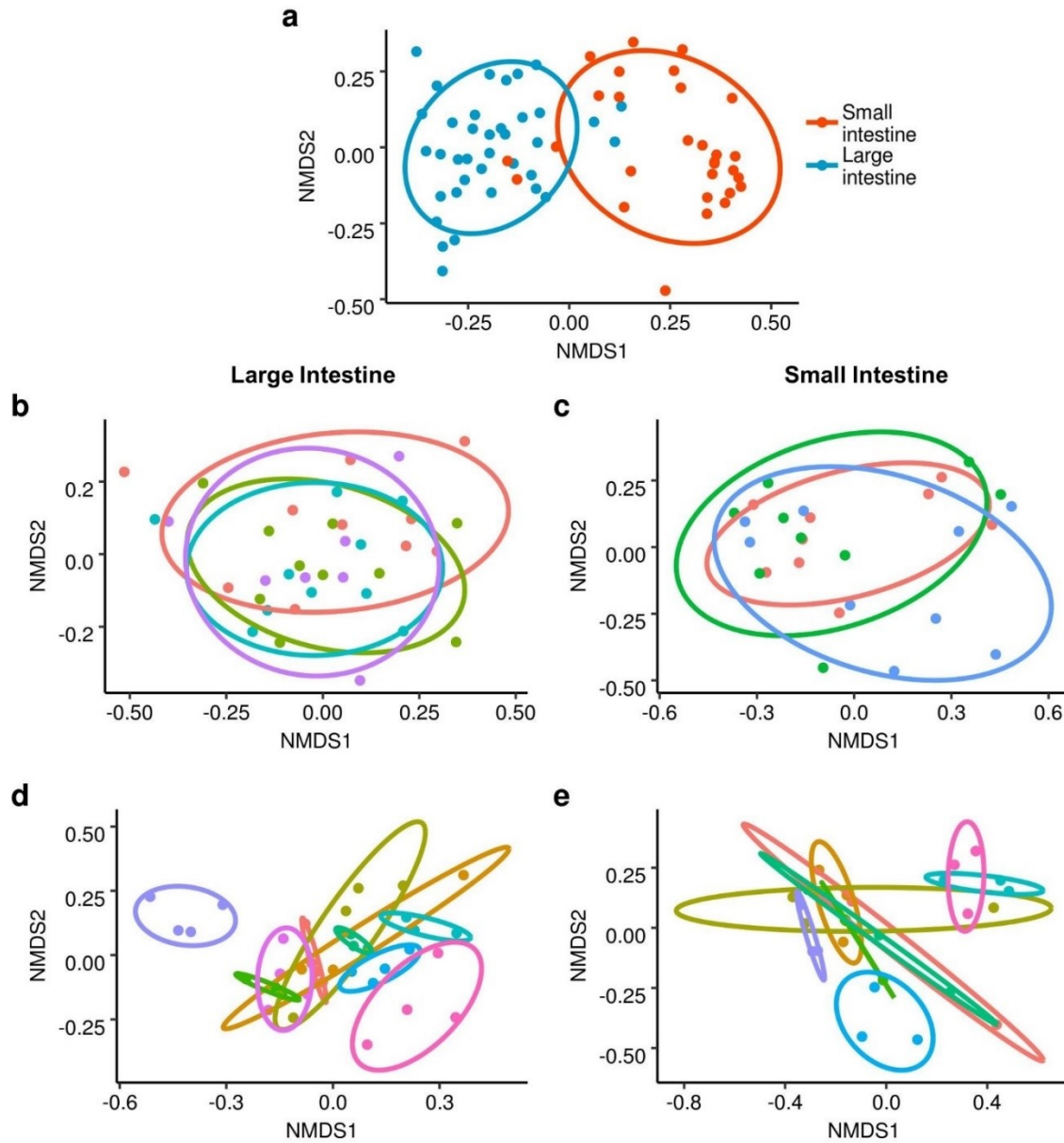
Fig. 2.5: Within the small and large intestines, individual identity explains clustering associations.

The microbiomes of the large and small intestine cluster independently of each other (**a**). In (**b**) and (**c**), samples within the large intestine and small intestine, respectively, are colored by intestinal sampling site, with no clear clustering associations. Panels (**d**) and (**e**) show the same plots colored by individual, with significant clustering associations distinguishing individuals. All plots are NMDS ordinations using the Bray-Curtis dissimilarity index, and ellipses denote 95% confidence intervals.

CHAPTER 3

Healthy coyotes harbor Fusobacteria-rich gut microbiomes driven by protein-rich diets

3.1 ABSTRACT

Studies of the gut microbiome in humans and laboratory animals continue to identify intricate and far-reaching relationships connecting the gut microbiome to host diet and health. However, these studies are performed in controlled settings that do not account for the variety of natural conditions experienced by free-living species and their results cannot be directly translated to different taxa. We used 16S rRNA gene amplicon sequencing to evaluate the microbiome of feces obtained from 76 coyote carcasses (*Canis latrans*) and tested its relationship to physiological health, measured as both body condition and infection by the zoonotic parasite *Echinococcus multilocularis*. We additionally accounted for diet by evaluating stomach contents and measuring stable isotope signatures (δ^{13}C and δ^{15}N) from claws. Healthy coyotes harbored Fusobacteria-rich gut microbiomes which correlated with protein-rich diets; Bacilli, especially *Streptococcaceae*, were the strongest indicator of poor condition and correlated with protein-poor diets. There was a significant co-occurrence of infection by *E. multilocularis* and evidence of anthropogenic food in the stomach, and both qualities were associated with lower body condition and increased gut microbiome richness. Despite the emphasis on diversity in microbiome studies, microbiome diversity did not correlate with body condition. Our results could potentially inform future scat-based assessments of coyote health and provide valuable information on the microbiological links between diet and condition in a widely distributed wild canid.

3.2 INTRODUCTION

Mammals have co-evolved with the thousands of bacterial species that colonize their gastrointestinal tracts, leading to the establishment of highly specific microbiomes that are intricately tied to host diet, energy acquisition, immune function, health, and behavior (6, 84). As methods for exploring the microbiome become both more sophisticated and accessible and our knowledge of microbiome structure and function continues to expand, we are increasingly able to point to specific relationships between the microbiome and host health. Variations in gut microbiome composition have been linked to conditions as diverse as obesity (181), cancer (182), autism (183), and anxiety (45), and individual bacterial taxa, including *Lactobacillus* and *Bifidobacterium*, are now commonly used as probiotics due to their known beneficial effects on host health (184). There is also a general consensus that higher gut microbial diversity is associated with health, though the specific mechanisms for this correlation are still being explored (185).

Our current understanding of the relationship between host health and the gut microbiome draws primarily from human studies and from controlled laboratory settings that use a few select animal species such as laboratory mice or monkeys (52). These approaches are valuable for identifying specific relationships among environmental factors, the gut microbiome, and health, but do not account for the variety of natural conditions experienced by wild animals to which the microbiome originally adapted. In addition, recent microbiome studies are finding that results from humans and other model organisms cannot be extrapolated to other animal species due to differences in host diet and gut physiology (58, 62, 113). Observations of livestock and animals living in captivity have provided valuable insights into the microbiome of non-model organisms (36, 55, 165), but even these animals live in homogenous, controlled environments and their microbiomes consequently differ from wild conspecifics (111, 112).

An understanding of how the microbiome influences health in wild animals living in spatially and temporally variable environments is a critical next step in the examination of host-gut microbiome co-evolution. In North America, coyotes (*Canis latrans*) provide an opportune system for exploring this relationship. They are a widely distributed generalist predator that has received additional attention in recent years due to their abundance in urban environments and the corresponding increase in reports of human-coyote conflict (170). They are also intermediate hosts for the zoonotic helminth parasite *Echinococcus multilocularis* (126), which is expanding

its range in North America and can cause human alveolar echinococcosis, a rare but severe zoonosis in the Northern Hemisphere (186). Knowledge of which microbial signatures are associated with body condition, parasite infection, or behavior in coyotes would not only lend insight into host-microbe co-evolution in wild canids but also have direct implications for monitoring and predicting host fitness in the context of potential aggression or the spread of *E. multilocularis* and other canid-borne zoonoses.

Here, we examined the relationship between the gut microbiome and host health in a sample of free-living coyotes obtained from both within and outside urban environments. We specifically aimed to 1) identify which components of the coyote gut microbiome are most indicative of host body condition and parasite infection status and 2) test the hypothesis that health is positively associated with gut microbial diversity. Because the gut microbiome cannot truly be considered independently of host diet, we accounted for diet using data obtained from both stable isotope analysis and visual examination of stomach contents. We predicted that the healthiest coyotes would have the most rich and diverse gut microbiomes, which would in turn be associated with protein-rich diets containing limited evidence of anthropogenic food. We additionally suspected that members of the probiotic genera *Lactobacillus* and *Bifidobacterium*, which have been recommended for enhancing gut health of domestic dogs (187), might be positively associated with coyote health.

3.3 METHODS

3.3.1 Sample collection and necropsy

We collected 76 coyote carcasses from Edmonton (Alberta, Canada) and the surrounding area between August 2017 and May 2018. Of these samples, 9 were road-killed, 3 were lethally managed, and the remaining 64 were obtained from local fur trappers. We skinned any coyotes that were delivered to us with their fur to ensure consistency in our physiological measurements. The sample contained 40 males and 36 females and included one coyote noticeably infected with sarcoptic mange. Carcasses were stored at -80°C for 5 days to neutralize any zoonotic pathogens and then transferred to -20°C until necropsy.

For each coyote, we measured the mass, body size (snout to base of tail), and girth around the ribcage, and we qualitatively assessed subcutaneous body fat on a scale of zero (no fat) to three. At necropsy, we removed the internal organs, measured the kidney fat index (KFI)

following published protocols (188), and recorded the mass of the spleen; the former metric measures body fat assimilation (188) and the latter can be used as an indicator of immune stress (189). To determine each coyote's recent diet, we opened stomachs and classified contents in two ways: whether the stomach was empty and, if not, whether it contained evidence of anthropogenic food, plastic, or paper. We also clipped the left hind outer toenail and stored it in a paper envelope for stable isotope analysis, and we removed the lower mandible for age determination. For microbiome analysis, we extruded fecal samples from the large intestine, which were then stored at -80°C until DNA extraction. Two coyotes did not contain extrudable fecal material, so we removed the contents of the colonic lumen by longitudinally transecting the antimesenteric side of a 10cm section taken from the distal colon. A concomitant study in our lab has shown that there are no significant differences in microbiome composition between feces and the colonic lumen (this thesis, Chapter 2).

3.3.2 Cementum aging

We determined the exact age of each coyote by counting cementum annuli. Lower canine teeth were removed from the mandible by soaking the mandible at 80°C for 6-8 hours. Teeth were fixed in a neutral solution of 10% formalin for at least 72 hours before being decalcified, sectioned, and stained following published methods (190), with two modifications: the 25% formic acid used for decalcification was buffered with 0.78 M sodium citrate, and teeth were sectioned to 12μm thickness before being mounted in Aquatex for visualization at 25X and 100X magnification. One researcher (S.S.) aged all the teeth based on the modal values of annuli counts taken from at least five locations along each tooth.

We chose to follow the aging criteria reported by Linhart et al. (191), who concluded that the first cementum annulus forms after 20 months. We distinguished coyotes in their first and second year based on tooth morphology: large pulp cavities and open root tips indicate coyotes in their first year, whereas smaller pulp cavities and closed root tips indicate coyotes in their second year. Coyotes that were at least 2 years old were aged exclusively based on cementum annuli. To increase precision, we assigned all coyotes a birth date of 1 May (192) and used the difference between birth date and death date to determine age to the nearest month.

3.3.3 Stable isotope analysis

We used stable isotope values (δ^{13}C and δ^{15}N) measured from claw samples to infer each coyote's habitual diet over the previous 8-10 months. δ^{13}C signatures can provide a reliable measure of anthropogenic food consumption because corn, which is ubiquitous in processed foods and livestock feed, has a distinctively high δ^{13}C value (193). δ^{15}N signatures are directly correlated with protein consumption (194). To obtain these measurements, whole claw samples were rinsed three times with a 2:1 chloroform:methanol solution to remove residual lipids and surface oils and then dried at 37°C for 5 days. After drying, we removed the distal 5mm of each claw, manually homogenized it into a fine powder using a pestle and mortar, and weighed 1.5 mg subsamples into tin capsules. Samples were combusted using a Vario Pyrocube and analyzed for δ^{13}C and δ^{15}N using an Isoprime Vision Mass Spectrometer at the Biogeochemical Analytical Service Laboratory (Dept of Biological Sciences, Univ. of Alberta). By convention, δ^{13}C results are reported relative to Vienna Pee Dee Belemnite and δ^{15}N results are reported relative to air.

3.3.4 DNA extraction and sequencing

We extracted whole community DNA from 100mg of each fecal sample using the MP Bio FastDNA Spin Kit for Soil following the manufacturer's instructions (MP Biomedicals, Santa Ana, CA). Fecal samples were thawed and manually homogenized prior to extraction, and before the final elution we included a five-minute incubation at 50°C to maximize DNA yield. Extracted DNA was stored at -20°C before being submitted to Microbiome Insights (Vancouver, BC) for sequencing. PCR amplification of the 16S rRNA gene was performed in 50μl reactions with 2μl of template DNA and barcoded universal bacterial primers 515F (5'-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA -3') and 806R (5'-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3'). Successful amplification was verified by agarose gel electrophoresis. Paired-end sequencing of equimolar concentrations of the PCR products was conducted on an Illumina MiSeq platform using V3 chemistry and 250bp reads. Sequencing depth ranged from 137 to 71,081 sequences per sample. Raw sequencing data has been deposited in the NCBI Short Read Archive under project number PRJNA528764.

3.3.5 Sequence processing

16S rRNA sequence data was processed to generate amplicon sequence variants (ASVs) using the package *dada2* (152), following previously described parameters for quality control and merging paired-end reads (this thesis, Chapter 2). Five samples with fewer than 4,000 reads were excluded from downstream analysis. We clustered high-quality, chimera-checked reads into unique ASVs and retained only ASVs with our target amplicon length of 250-256bp. To assign taxonomy, ASVs were aligned against taxa in the RDP reference database (release 11.5) (154) using the naïve Bayesian classifier method implemented in DADA2 (155). We removed six ASVs that were identified as chloroplasts or mitochondria. We used the package *phangorn* (156) to generate a generalized time-reversible maximum likelihood phylogenetic tree for our data following the procedures described by (157). ASV abundances were averaged across 100 rarefactions to the minimum remaining library size of 4,637 reads. Our final feature table, which contained 1,274 unique ASVs from a sample of 71 coyotes, was imported into the R package *phyloseq* (158) for subsequent analyses.

3.3.6 Parasite survey

We used PCR to test each coyote for possible infection with *E. multilocularis*. As part of a related study (this thesis, Chapter 4), we also had DNA samples extracted from the duodenal lumen of all 76 coyotes, and we used this DNA to inform our parasite diagnoses. DNA extracted from both duodenal and fecal samples was amplified in triplicate using *E. multilocularis*-specific primers Cest1 and Cest2 (195). PCR was performed in 25μl reactions with 0.2μM of each primer and 1μl of template DNA using cycling conditions described previously (195). We resolved PCR products in 2.0% agarose gels run for 35 minutes at 120V and post-stained with ethidium bromide. A coyote was considered positive for *E. multilocularis* if either the fecal or duodenal sample exhibited an approximately 395bp band in at least two replicates. Samples testing negative were diluted and tested again to control for possible PCR inhibition.

3.3.7 Statistical analyses

Data overview. All statistical analyses were performed in R 3.5.0. For each microbiome sample we calculated total ASV richness and the Shannon diversity index using *phyloseq*. We first used Student's t-tests to determine if any of our measures of body condition and microbiome

alpha-diversity varied significantly between sexes, and chi-square tests of independence to evaluate sex-specific differences in infection status and stomach contents. We then tested for significant correlations between alpha-diversity measures and each of our physiological measures obtained at necropsy using Spearman's rank correlation. We similarly used Spearman's rank correlation to identify individual taxa whose abundances were associated with continuous physiological measures and stable isotope signatures.

To evaluate general relationships between health and overall microbiome composition (beta-diversity), we visualized inter-sample microbiome similarity using non-metric multidimensional scaling (NMDS) based on the Bray-Curtis dissimilarity index. We used a permutational analysis of variance (PERMANOVA) with 1,000 permutations to ensure there were no sex-specific differences in overall microbiome composition. We then mapped our continuous physiological covariates onto the ordination using the 'envfit' function in the *vegan* package and measured the strength of the association using the goodness of fit statistic (R^2).

Generating a single index of health. Because many of our physiological measures were collinear, such as mass and girth, we reduced the dimensionality of our data by performing principal components analysis (PCA) on our biometric measurements. We extracted the axis loadings of the first principal component as a single composite metric of body condition. To ensure our results were robust to the variables we chose to include in this calculation, we repeated this procedure several times using different combinations of biometric measures. We additionally used the residuals in a linear regression of mass against body size, which serve as another common single index of body condition. All composite metrics were highly collinear and achieved similar results in downstream analyses (**Supplementary Figs. 3.1, 3.2**), so for simplicity only one PCA-generated metric is shown in this study. Two coyotes were necessarily excluded from analyses using these metrics because we lacked the data to calculate them (e.g., for one coyote delivered without a head we could not measure mass or size).

Taxa that correlate with health. To correlate body condition with microbiome composition, we calculated Spearman's rank correlation between taxon relative abundances and our body condition index, for each taxonomic level (phylum through genus). For these correlations, we only considered taxa that were present in at least 20% of samples with a mean relative abundance greater than 0.1%. We visualized the relationships between health and taxon abundance by ranking taxon-specific correlation coefficients from least to greatest and then

plotting correlation coefficients against correlation rank, producing a gradient of taxa from "unhealthy" to "healthy."

The abundances of individual taxa with significant correlation coefficients ($p < 0.05$) were then used as the dependent variable in negative binomial regressions against stable isotope measures, *E. multilocularis* infection status, presence of anthropogenic food in the stomach, and the body condition index, in order to determine which of these measures most strongly predicted the abundance of each taxon. Continuous predictors were centered and standardized to facilitate comparison. Models were ranked based on their Akaike information criterion scores corrected for small sample sizes (AICc), and we considered the best model(s) those with a ΔAICc less than two. To compare the relative effect of each predictor, we used a model-averaging technique where coefficients in each top-ranked candidate model were adjusted by model weight and averaged across all models (196).

Effects of *E. multilocularis* infection. We also tested for any direct relationships between *E. multilocularis* infection status and each of body condition and microbiome alpha-diversity using Student's t-tests. Differences in beta-diversity were visualized using NMDS based on the Bray-Curtis index and assessed using a PERMANOVA with 1,000 permutations. Taxa that were significantly differentially abundant between groups were identified at the class and family level using the default settings of the package *ALDEx2* (160), which includes a centered log-ratio transformation of the unrarefied feature table prior to differential abundance testing using Welch's t-test with the Benjamini-Hochberg correction for false discovery rates. These analyses were repeated using the presence of anthropogenic food in the stomach as the dependent variable.

Microbiome diversity and health. We lastly evaluated our hypothesis that gut microbiome diversity is strongly associated with overall health by ranking candidate generalized linear models and comparing predictor coefficients. Using our composite health index as a normally distributed dependent variable, we built models that included stable isotope measures, parasite infection status, presence of anthropogenic food in the stomach, and microbiome alpha-diversity as predictors. We controlled for any variation between sexes by including sex as an additional predictor. We checked variance inflation factors (VIF) to ensure predictors were not collinear (VIF < 2) and we centered and standardized continuous predictors. Coefficients were averaged across top-ranked models as before. We followed a similar approach to evaluate the

strongest physiological and behavioral predictors of gut microbiome diversity, substituting the Shannon index as a normally distributed dependent variable and body condition index as a predictor.

3.4 RESULTS

We evaluated the relationship between coyote gut microbiome and body condition while also accounting for differences in sex, diet, and age. In our sample of 76 coyotes, males were on average 25% larger than females by mass and 10% larger by size and girth, although some of this difference may be attributed to the average male being one year older as well (**Table 3.1**). There were no sex-specific differences in measures of immune system stress or fat assimilation, nor were there any differences in assimilated diet, which we evaluated using stable isotope signatures (**Table 3.1**). Twenty-four percent of coyotes contained evidence of anthropogenic food in their stomachs, including twice as many females as males (χ^2=4.40, df=2, p=0.061). The overall prevalence of *E. multilocularis* was 40%, and coyotes with anthropogenic food in their stomach were 1.6 times more likely to be infected (χ^2=4.40, df=2, p=0.036).

The fecal microbiome was dominated by the phyla Firmicutes (mean±SD, 52.0±26.0%), Fusobacteria (18.5±16.6%), Bacteroidetes (13.4±13.4%), and Proteobacteria (12.7±16.4%), and contained an average of 126±51 amplicon sequence variants (ASVs) per individual. Females had slightly more ASVs per individual, with no change in the Shannon diversity index (**Table 3.1**). Contrary to our expectations, there were no strong or significant correlations between microbiome richness or diversity and any of the physiological measures taken at necropsy (**Fig. 3.1a**). The only trend that approached significance was a weak negative association between girth and gut microbiome richness (Spearman's R= -0.203, p=0.089). Similarly, when we fit our continuous measures of body condition and diet as vectors onto an NMDS ordination of the fecal microbiome samples, no single measure explained more than 8% of the variation in community composition (**Fig. 3.1b**). The best-fitting vectors were the relative amount of subcutaneous fat (R^2=0.080, p=0.187) and spleen mass (adjusted for body mass; R^2=0.080, p=0.193). Sex also did not explain any of this variation (**Supplementary Fig. 3.3;** PERMANOVA F=1.172, R^2=0.017, p=0.239).

We tested if the abundances of different bacterial families correlated with physiological health. Aside from the abundance of *Bifidobacteriaceae* being lower in individuals with larger

spleens, the families *Bifidobacteriaceae* and *Lactobacillaceae* were either negatively or only weakly correlated with other measures of condition, respectively, and neither was associated with a protein-rich diet (**Fig. 3.2**). The same trends were true when we specifically examined the probiotic genera *Bifidobacterium* and *Lactobacillus* (data not shown). Instead, we noted that *Fusobacteriaceae* were consistently positively associated with all measures of health, decreased with spleen mass, and increased with protein consumption. One family of *Clostridiaceae* was strongly associated with the kidney fat index, and correlations with *Enteroccoccaceae* and *Streptococcaceae* were universally negative (**Fig. 3.2**).

Because many of our physiological measures were collinear, we repeated these analyses after generating a single metric for body condition. Our composite health index explained 64.7% of the total variation in biometric measures among individuals and effectively captured every measure except spleen mass (**Fig. 3.3a, Table 3.2**). Using this health index, we found the relative abundances of the classes Fusobacteria and Betaproteobacteria were significantly correlated with higher body condition, and the relative abundance of Bacilli with poorer body condition (**Fig 3.3b, Table 3.3**). Within these classes, *Fusobacteriaceae*, *Sutterellaceae*, and *Streptococcaceae*, respectively, accounted for the strongest correlations (**Table 3.3**). In generalized linear models, the abundances of Fusobacteria and Bacilli were most strongly predicted by $\delta^{15}N$ signature, which correlated positively with Fusobacteria and negatively with Bacilli (**Supplementary Fig. 3.4, Supplementary Table 3.1**), while no single predictor seemed to indicate the abundance of Betaproteobacteria. The genera *Bifidobacterium* and *Lactobacillus* were both negatively correlated with our body condition index (data not shown).

We also used our body condition index and microbiome information to test for specific relationships with each of our two binary variables, parasite infection status and the presence of anthropogenic food in the stomach. As expected, infected individuals and individuals that had recently consumed anthropogenic food scored 2.6 and 4.5 times lower in our body condition index, respectively (**Fig. 3.4a, b**; $t_{infected}=2.07$, df=68, p=0.043; $t_{stomach}=2.23$, df=69, p=0.029). However, contrary to our hypothesis, these individuals also harbored 25% more ASVs in their microbiome ($t_{infection}=-2.42$, df=69, p=0.018; $t_{stomach}=-2.18$, df=69, p=0.033), with a corresponding slight but not significant increase in diversity (**Fig. 3.4a, b**). Despite the significant co-occurrence of *E. multilocularis* infection and anthropogenic food consumption, the effects of these two binary variables on ASV richness were independent and not additive when

we evaluated pairwise comparisons across their four potential outcomes (**Supplementary Fig. 3.5**). Neither infection status nor stomach contents explained any significant variation in overall community composition (PERMANOVA $F_{infected}$=0.554, R^2=0.008, p=0.954; $F_{stomach}$=1.70, R^2=0.024, p=0.050) (**Fig. 3.4c, d**), nor were there any specific bacterial taxa that were significantly indicative of either quality after correction for false discovery rates. However, we note that coyotes consuming anthropogenic food contained an average of nine-fold more Eysipelotrichia (4.67 vs. 0.59%, p=0.022, q=0.228), 2.5-fold more Bacilli (10.05 vs. 3.69%, p=0.034, q=0.241), and 25% fewer Fusobacteria (14.34 vs. 19.95%, p=0.126, q=0.384).

Comparing model-averaged parameter estimates revealed which of microbiome composition, recent and assimilated diet, and parasite infection status best explained overall variation in body condition. Aside from sex and age, which were present in all the top-ranked models, the strongest predictors were the presence of anthropogenic food in the stomach, which had a negative relationship with health, as well as protein consumption ($\delta^{15}N$ signature), which had a positive relationship (**Fig. 3.5a, Supplementary Table 3.2**). Gut microbiome diversity did not appear in any of the top-ranked models. Interestingly, when we reversed the models to determine which parameters best explained gut microbiome diversity, the strongest predictor was also the presence of anthropogenic food in the stomach, with a positive effect (**Fig. 3.5b, Supplementary Table 3.2**). Even so, the coefficients for many of these predictors in both models were non-significant and several candidate models containing different predictors were within 2 AICc points of each other, suggesting that there may be other variables not measured in our study that further explain the observed variation in body condition.

3.5 DISCUSSION

Understanding and predicting past and future co-evolution between animals and their gut microbiomes requires an understanding of which aspects of the microbiome are associated with host fitness in different wild host species. We evaluated the gut microbiome of coyotes, a widely distributed generalist canid, in the context of their body condition and infection by the helminth parasite *Echinococcus multilocularis.* Despite considerable evidence from humans and laboratory animals that gut microbiome diversity and higher abundances of the genera *Bifidobacterium* and *Lactobacillus* promote health, we found no evidence for any of these relationships in coyotes. Instead, Fusobacteria were the strongest microbial indicator of higher

body condition, and their relative abundance was positively correlated with protein assimilation. *Streptococcaceae* were similarly indicative of lower body condition and negatively correlated with protein assimilation. There was a significant co-occurrence of infection by *E. multilocularis* and evidence of anthropogenic food in the stomach, and both qualities were associated with lower body condition and, unexpectedly, increased gut microbiome richness. Although we observed typical mammalian sex-based differences in physiology, with males being larger than females, there was no indication that any of the observed health-specific variation in microbiome diversity and composition were driven by sex.

Fusobacteria are considered a common member of the gut microbiome in healthy canines, and their relative abundance in our study (18.5%) is within the range of previous reports in wolves (9.2-30.5%) (163, 197), foxes (20-30%) (171), and domestic dogs (10-16.6%) (150, 151, 198). The reasons for their abundance in canines remain unclear, as many other mammals, including pigs (175), cats (165), and all studied ruminants (199), harbor few or no Fusobacteria in their intestinal ecosystem, and in humans Fusobacteria have been linked to inflammatory bowel disease and colorectal cancer (200). To our knowledge this is the first time Fusobacteria have been directly linked to quantitative measures of body condition in canines. Our observation that Fusobacteria are positively correlated with protein consumption agrees with a previous observation that dogs fed protein-rich diets harbored more Fusobacteria than dogs fed carbohydrate-rich kibble (198), and is likewise consistent with the moderately lower abundance of Fusobacteria in coyotes consuming protein-poor anthropogenic food.

It is tempting to view the positive correlations between protein consumption, a Fusobacteria-rich gut microbiome, and physiological health as an obligate cause-and-effect relationship, which would contribute to a positive feedback loop where healthy coyotes are better able to obtain protein-rich prey and in turn less likely to engage in conflict-prone behavior (124). Our observation that coyotes who recently consumed anthropogenic food were generally less healthy seems to support this hypothesis. However, this may not be universally true. Of the three coyotes in our study who contained no ASVs assigned to Fusobacteria, one was lethally managed after attacking and killing a large domestic dog. Conflict-prone coyotes in Edmonton are more likely to have poor body condition and consume less protein (125), and the absence of Fusobacteria would only further suggest this, but this individual appeared average or above average in all measures of health and protein assimilation and was distinguishable only by its

unique microbiome. A more recent study found that Fusobacteria were less abundant in aggressive relative to non-aggressive kenneled pit bulls (201), suggesting that the relationship between Fusobacteria and health may not be exclusively physiological. Untangling the mechanisms linking Fusobacteria to diet, health, and behavior, especially given the unique nature of this relationship in canines relative to other studied animals, will require more targeted investigation.

The implications of Betaproteobacteria and Bacilli, specifically *Streptococcaceae*, as additional correlates with physiological health are less precedented in canine microbiome studies. Betaproteobacteria were minimally abundant in our sample, only exceeding 2% abundance in four healthy individuals, and we consequently hypothesize that their observed correlation with health is more likely circumstantial than ecologically informative; at the phylum level, Proteobacteria were not strongly or significantly correlated with health. Bacilli, including *Streptococcaceae*, have been associated with obesity in humans (181) but their role in other animals is not well-established. However, we note that the members of Bacilli and Erysipelotrichia that were most negatively correlated with our body condition index were also those present at moderately higher abundances in coyotes consuming anthropogenic food. Some of these same taxa are known for carbohydrate fermentation and are regularly found in processed foods (25). Of the covariates we measured, decreases in protein assimilation most strongly predicted Bacilli abundance. These directional associations between the consumption of anthropogenic food, reduced protein assimilation, the abundance of carbohydrate-fermenting taxa, and physiological health in a carnivorous mammal present a negative feedback loop complementary to the positive cycle discussed above. This negative feedback has previously been considered independently of the microbiome as a "vicious circle" of low-quality diet and poor body condition (125).

The lack of any significant association between health and overall gut microbiome richness or diversity is surprising considering the frequency with which diversity metrics are cited in animal microbiome studies (202) and the reproducible observations of lower bacterial diversity in human patients suffering from a variety of health conditions (203–206). In our study, coyotes who recently consumed anthropogenic food fostered the most speciose fecal microbiomes. Anthropogenic foods contain a wider variety of more complex macronutrients than prey, including sugars, starches, and other complex fibers, and therefore require additional taxa

to digest them, which is why herbivores and omnivores consistently have more speciose microbiomes than carnivores (202). Because most anthropogenic food consumed by coyotes is protein-poor (124), any diet-induced increase in microbial species richness would not be expected to produce a corresponding increase in body condition.

We speculate that the relationship we observed between anthropogenic food consumption and *E. multilocularis* infection is primarily an artifact of habitat use, as urban-dwelling coyotes both consume more anthropogenic food and exhibit higher rates of infection (126; this thesis, Chapter 4). *E. multilocularis* is transmitted through small mammals, not anthropogenic food, but small mammals are more abundant prey in urban environments, where anthropogenic food is also more accessible (207). The lower body condition of coyotes seeking anthropogenic food may further affect their susceptibility to infection (124). Documented examples of microbial-mediated parasite susceptibility do exist (208, 209) and it remains possible that diet-induced changes to the coyote microbiome may interact with *E. multilocularis* to promote or inhibit parasite establishment. More rigorous evaluations of microbiome-helminth interactions, using visually quantified parasite burdens rather than a PCR-based presence/absence determination, will be needed to elucidate these potential relationships.

An important subsidiary observation in our study is that the connections between *E. multilocularis* infection, microbiome species richness, and anthropogenic food consumption did not persist when we used $\delta^{13}C$ signatures, our measure of a longer-term anthropogenic diet, in place of stomach contents. Neither species richness nor infection status could be predicted by $\delta^{13}C$. Diet-induced changes in the microbiome can appear within hours of a meal yet revert equally quickly after digestion as the microbiome returns to a stable state (25), which may be why we observed no long-term effects here. Coyotes naturally harbor a carnivorous microbiome (this thesis, Chapters 2, 4) and even coyotes consuming anthropogenic food regularly capture small prey (210). Anthropogenic meals likely constitute a novel and short-lived perturbation to the naturally carnivorous microbiome that may not be reflected in a longer-term measure like $\delta^{13}C$, whereas protein-rich diets support the resident carnivorous microbiota and are more likely to be consistent over extended time.

Our conclusions admittedly come with several limitations, including their basis in correlative evidence rather than direct experimental manipulations. We additionally rely on a presence/absence classification system for stomach contents that neglects both time since

ingestion and quantity of food types consumed, which may each have important downstream effects on the microbiome (25). However, we argue that the robust correlative approach used here, incorporating multiple complementary measures to gain information on host diet and body condition, nonetheless represents an accessible way to perform exploratory microbiome research in truly wild populations where controlled experiments may not be practical. Moreover, our results speak to the importance of first understanding the functional value of commonly used microbiome measures in target host species, given that neither microbiome diversity nor the abundances of taxa commonly referenced in human studies were necessarily positive attributes in coyotes.

Continuing to interrogate these host-specific host-gut microbe interactions in animals across the phylogenetic tree will not only further expand our understanding of host-microbe co-evolution, but also potentially generate valuable tools for wildlife biology. For example, our work implicates Fusobacteria as a positive indicator for diet, health, and possibly even behavior, and more refined testing of observations such as this could provide easy and non-invasive methods for assessing ecological populations and inferring their condition. Much work remains to be done for this form of ecological assessment to become viable and reliable, but we believe a better understanding of the microbiome in wild animals could have important and under-appreciated implications for wildlife and conservation biology.

3.7 TABLES & FIGURES

Table 3.1: Variation in body condition and microbiome alpha-diversity between males and females.

Measure	Mean		Student's t-test		
	male	female	t	df	p
Mass (kg)	11.88	9.47	-4.87	68	**< 0.001**
Body size (cm)	89.30	83.85	-0.39	68	**< 0.001**
Girth (cm)	48.62	44.09	-4.50	69	**< 0.001**
Age (yr)	3.65	2.06	-2.76	65	**0.007**
Adj, speen mass*	1.72	1.82	0.61	68	0.546
KFI	0.590	0.481	-1.45	69	0.152
$\delta^{13}C$	-22.74	-22.59	0.70	69	0.485
$\delta^{15}N$	8.99	8.69	-1.87	69	0.066
ASV Richness	116.1	137.0	1.74	69	0.086
Shannon index	2.66	2.77	0.59	69	0.56

* Adj. spleen mass is the absolute spleen mass (in grams) divided by total body mass (in kg).

Table 3.2: Physiological measures captured in our composite body condition index.
To determine which physiological measures were most strongly represented in our composite body condition index, we calculated Pearson's correlation coefficient between variables included in the index and the axis loadings for the first and second principal components. Only the first principal component was used as an index of condition.

Measure	Pearson's R	
	PC1	PC2
Mass	0.855	-0.315
Body size	0.891	0.303
Girth	0.876	-0.452
Adj. spleen mass	0.067	-0.015
KFI	0.254	-0.427

Table 3.3: Correlations between taxon abundances and body condition.

Results of Spearman's correlations for the three bacterial classes with significant associations between relative abundance and health. Representative families for each of these classes are also shown, as well as the mean relative abundance of each taxon.

Class / Family	Spearman's correlation R	p	Mean abundance (%)
Bacilli	-0.334	**0.007**	4.76
Streptococcaceae	-0.303	**0.016**	2.37
Enterococcaceae	-0.123	0.336	0.68
Lactobacillaceae	-0.111	0.387	1.47
Betaproteobacteria	0.264	**0.037**	0.47
Sutterellaceae	0.210	**0.099**	0.46
Fusobacteria	0.274	**0.030**	18.51
Fusobacteriaceae	0.274	**0.030**	18.51
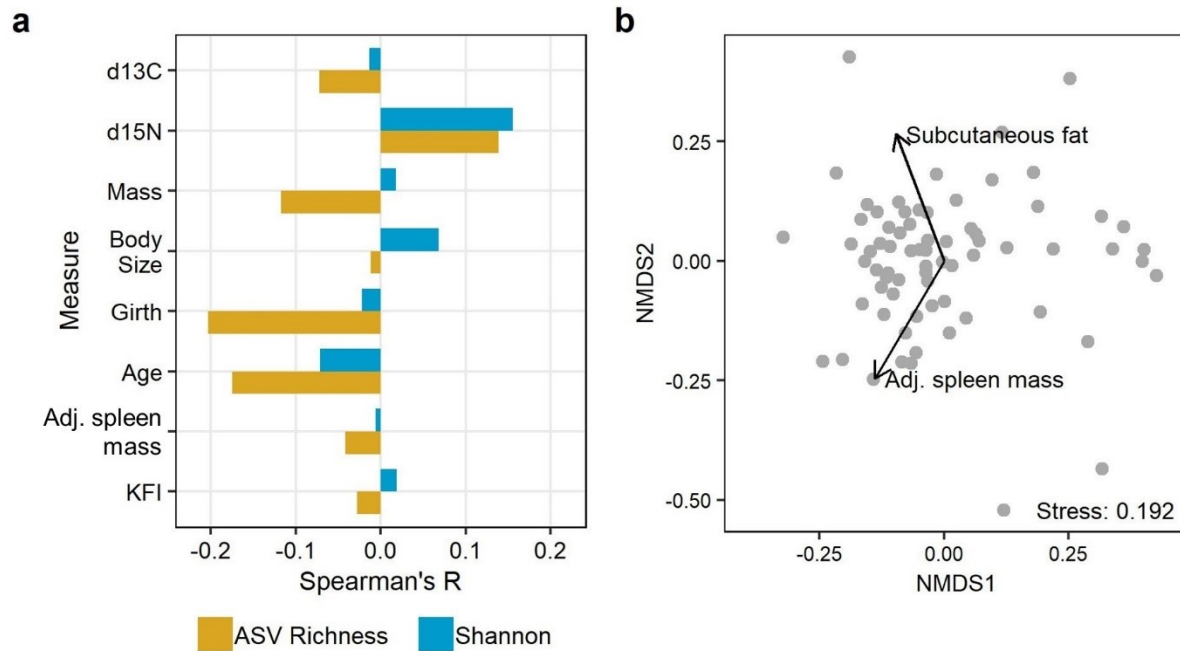
Fig. 3.1: No significant relationships between health and microbiome alpha- and beta-diversity.

(**a**) Spearman's rank correlation between the continuous measures of diet (δ^{13}C, δ^{15}N) and condition and each of microbiome richness and diversity (Shannon index). No correlations were significant ($p < 0.05$). (**b**) Non-metric multidimensional scaling of fecal microbiome samples using the Bray-Curtis dissimilarity index. Vectors show the directional effects of physiological variables explaining more than 5% of the variation. "Adj. spleen mass" refers to the quotient of spleen mass (in g) and body mass (in kg).
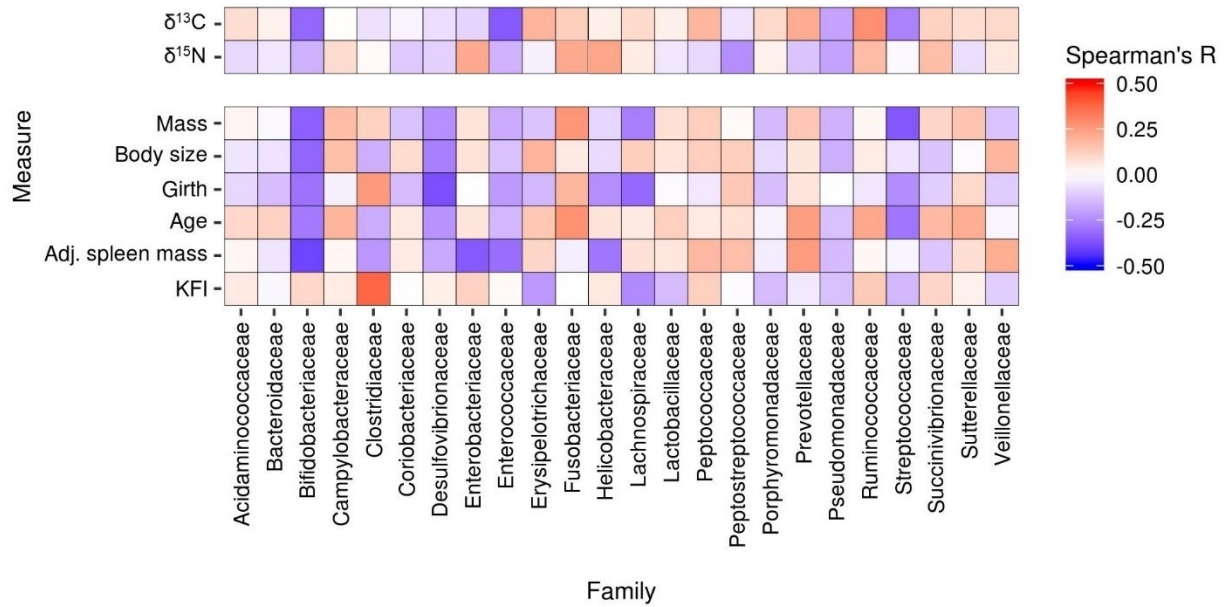
Fig. 3.2: Spearman correlations of family-level relative abundances against body condition.
Heat map depicting Spearman's correlation coefficient between relative abundances of bacterial families and each of stable isotope signatures and physiological measures. No correlation coefficient exceeded 0.5. "Adj. spleen mass" is used as before.
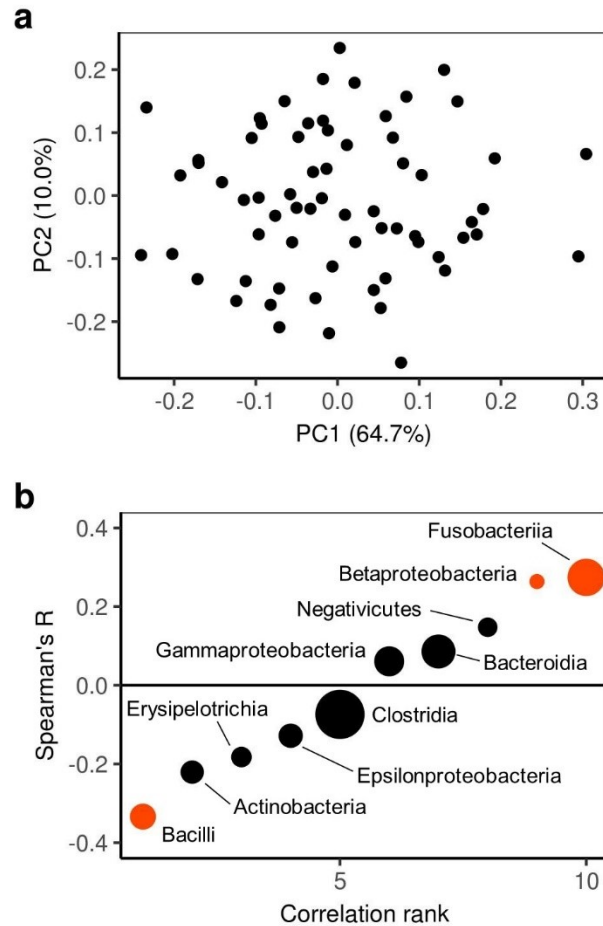
Fig. 3.3: Fusobacteriia, Betaproteobacteria, and Bacilli are the strongest indicators of condition.

(**a**) Principal component analysis of the different physiological variables measured in our study. We used values for the first principal component as a singular body condition index. (**b**) We calculated Spearman's correlation coefficients between our body condition index and taxon relative abundances. Taxa are ranked by their correlation coefficient so that "unhealthy" taxa appear in the bottom left and "healthy" taxa in the top right. The size of points represents mean taxon relative abundances, and taxa with significant correlation coefficients ($p < 0.05$) are shown in red.
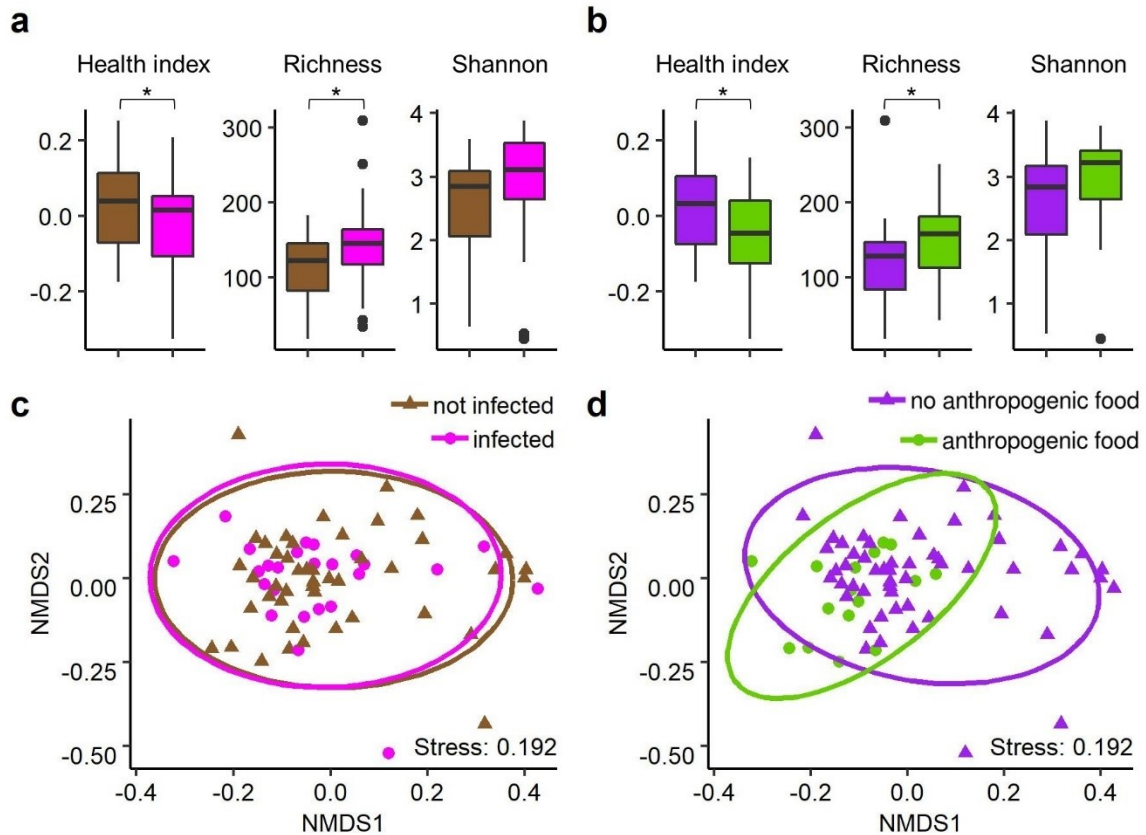
Fig. 3.4: Infected coyotes and those consuming anthropogenic food are less healthy and have more rich microbiomes.

Body condition index ("health index"), fecal microbiome richness, and Shannon diversity for (**a**) coyotes infected with *E. multilocularis* and (**b**) coyotes with evidence of anthropogenic food in their stomach. Asterisks indicate statistically significant differences (Student's t-test; * $p < 0.05$). (**c, d**) NMDS ordinations of the fecal microbiome using the Bray-Curtis distance, with samples colored by (**c**) infection status or (**d**) stomach contents. Ellipses represent 95% confidence intervals.
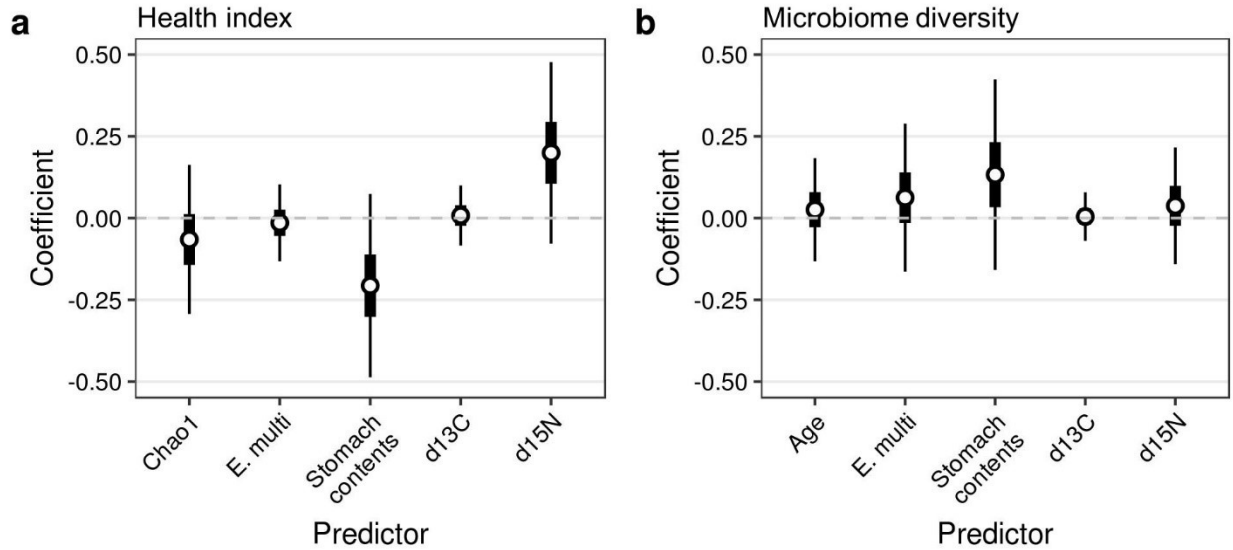
Fig. 3.5: Body condition and gut microbiome diversity are most strongly predicted by protein consumption and stomach contents.

(**a**) Forest plot of model-averaged predictor coefficients in a normally-distributed generalized linear model predicting body condition. Sex and age (not shown) were included as covariates to control for natural variation in condition. Other predictors included gut microbiome richness and diversity, *E. multilocularis* infection status, presence of anthropogenic food in the stomach, and stable isotope signatures. (**b**) Same as in (a), except using gut microbiome diversity (Shannon index) as the dependent variable and body condition index as a predictor. Sex and age were also considered as predictors rather than controls in this model. In both plots, boldface and narrow lines indicate 50% and 95% confidence intervals, respectively.

CHAPTER 4

Urban coyotes have more divergent microbiomes and poorer body condition

4.1 ABSTRACT

Generalist species able to exploit anthropogenic food sources are increasingly common in urban environments. Coyotes (*Canis latrans*) now live in cities across North America where they consume more anthropogenic food, are more likely to be unhealthy or diseased, and are increasingly common in reports of human-wildlife conflict. These different health and behavioral outcomes may be related to diet via the gut microbiome, which has far-reaching effects on animal nutrition and physiology but has not been studied in urban-exploiting mammals. We used stomach contents, stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$), 16S rRNA gene sequencing, and physiological measurements taken at necropsy to correlate diet with duodenal and fecal microbiome composition, body condition, and parasite infection status for a sample of 76 coyotes. Urban coyotes consumed broader diets containing more anthropogenic food, resulting in greater divergence in the duodenal microbiome among individuals and a slightly altered fecal microbiome characteristic of carbohydrate-rich diets, containing fewer Fusobacteria and more Bacilli and Erysipelotrichia. These changes were associated with poorer average body condition and double the prevalence of the zoonotic parasite *Echinococcus multilocularis*. We speculate that spatial and temporal variation in the extent of urban habitat use, reflected in the greater diversity of anthropogenic food, prevents adaptive co-evolution between the diet and microbiome of urban-adapted individuals, with downstream consequences for body condition, disease susceptibility, and possibly conflict-prone behavior.

4.2 INTRODUCTION

Urbanization is causing dramatic changes to terrestrial ecosystems, with more land predicted to be developed between 2000 and 2030 than in all of previous history (100). For many species, the well-documented selective pressures created by the expanding urban landscape (28, 29, 98) lead to local extirpations and consequent decreases in local diversity (211), but several generalist species can thrive in urban environments (122, 212). The success of these urban generalists is largely enabled by behavioral adaptations, foremost of which is broadening their diets to exploit abundant but often variable sources of anthropogenic food (212, 213). Urban habitat use has loosely been associated with physical costs (101), but the direct consequences of eating urban food are little understood despite their likely downstream effects on human-wildlife interactions including dependency, conflict-prone behavior, and the spread of zoonotic diseases (213).

In North America, coyotes (*Canis latrans*) are becoming a common resident of several major cities, which has coincided with increased reports of human-coyote conflict (170). Their generalist diet and behavioral plasticity contribute to their success in the urban landscape: coyotes can hunt solitarily or in small groups (214), and though they traditionally consume ungulates, rodents, and insects, they can also survive on fruit and anthropogenic food (170). The extent of anthropogenic resource use varies among cities and among individuals for reasons that may include sex, age, health, and resource availability (125, 210, 215–217). Coyotes are also carriers of zoonotic parasites, including sarcoptic mange (*Sarcoptes scabiei*) (125) and the intestinal helminth *Echinococcus multilocularis* (126). Both parasites can be transmitted to domestic pets, and the latter can cause alveolar echinococcosis, a rare but severe zoonosis, when transmitted to humans. Recent studies have suggested that these parasites are more common in urban animals (124, 126).

We hypothesized that the gut microbiome may play a pivotal role linking the consumption of anthropogenic food by urban coyotes to changes in their health, behavior, and parasite susceptibility because it is necessarily altered by changes in diet (23, 36) and has far-reaching effects on nutrient absorption, immune system function, behavior, and overall fitness (6, 52, 141). The recent proliferation of host-associated microbiome research continues to demonstrate the biological importance of the gut microbiome across diverse animal hosts (21; this thesis, Chapter 1). Gut microbes may enable (24, 87) or inhibit (67, 110) adaptation to new

environments, and directional shifts in microbiome composition have been observed in urban passerines (97, 218) but have not yet been studied in any urban-adapted mammal. Due to natural physiological differences among animal taxa, results of microbiome research in one host species or model organism cannot reliably be extrapolated to another (62, 113), making it important to directly study the microbiome of urban-adapted hosts in the context of their physiology and behavior.

In this study we tested the hypothesis that anthropogenic food consumption by urban coyotes causes a distinct shift in microbiome composition with consequent declines in physiological condition. Using coyotes killed for other reasons in and around Edmonton, Alberta, Canada, we inferred recent diet via stomach contents and assimilated diet via stable isotope analysis. We characterized the microbial community of both fecal and duodenal lumen samples based on previous observations that the small and large intestinal microbiota can respond differently to the same stressor (32; this thesis, Chapter 2), and we evaluated condition as both morphometric measurements taken at necropsy and infection by *E. multilocularis*. Our results support past evidence that urban coyotes eat a broader diet of lower quality, but additionally show that it results in greater divergence in the duodenal microbiome among individuals, poorer average body condition, and double the prevalence of a zoonotic parasite. We speculate that the greater diversity of anthropogenic food, over both space and time, prevents adaptive co-evolution between the diet and microbiome of urban-adapted individuals, which leads to reduced body condition and increased susceptibility to disease and has the potential to alter rates of conflict-prone behavior.

4.3 RESULTS

Urban coyotes have a wider diet breadth containing more anthropogenic food

We examined 11 coyotes collected from Edmonton ("urban") and 65 coyotes from the surrounding area ("peri-urban") (**Supplementary Fig. 4.1**) and used two complementary techniques to infer each coyote's diet. First, we examined the stomach contents of each coyote for the presence of anthropogenic food, including recognizable food items, plastic, or paper, and found that urban animals had a significantly higher prevalence of anthropogenic food in their stomachs (**Fig. 4.1a**; 71.4% vs. 22.6%, χ^2=7.25, df=1, p = 0.007). When we quantified stomach contents by volume for a subset of our sample, urban coyotes contained an average of 2.6-fold

more anthropogenic food in their stomach (40.0±42.7% vs. 15.4±28.7%; Student's t=-1.59, df=19, p=0.128). Anecdotally, the types of anthropogenic food in urban stomachs were highly variable, with some stomachs containing fast food or pastries while others contained fruits and vegetables such as pineapple or broccoli (**Supplementary Table 4.1**). Anthropogenic food in peri-urban stomachs was primarily plastic wrappers or aluminum foil, contained fewer identifiable digestible items, and was also significantly more likely to co-occur with other natural prey species (χ^2=6.78, df=1, p < 0.01).

To estimate assimilated diet over longer time periods, we used stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) measured from claw samples. Stable isotopes can accurately and reliably capture trends in anthropogenic food consumption because corn, which is ubiquitous in processed foods and livestock feed, has a distinctively high $\delta^{13}C$ signature (193). $\delta^{15}N$ signatures can additionally be used as an index of trophic level and protein consumption (194). In our study, urban coyotes had a higher mean $\delta^{13}C$ signature than peri-urban coyotes (**Fig 4.1b**; -21.6±1.26 vs. -22.9±0.65; Welch's t=-3.68, df=13.31, p = 0.003) that also increased with age (**Supplementary Fig. 4.2**); coyotes less than one year old when they died would have grown part of their claws as nursing pups, limiting their direct exposure to any food type. There was no significant difference in mean $\delta^{15}N$ signatures between groups, though urban coyotes exhibited lower minimum and maximum $\delta^{15}N$ values (7.01-9.45 vs. 7.61-10.8). Stable isotope mixing models using the $\delta^{13}C$ and $\delta^{15}N$ values of various coyote diet items estimated that urban coyotes consumed four times more anthropogenic food than peri-urban coyotes (**Fig. 4.1c**) and had a two-fold larger isotopic niche (2.75 vs. 1.37), another indicator of diet breadth.

Urban coyote duodenal and fecal microbiomes respond differently to anthropogenic diets

We investigated how this shift toward a broader and more anthropogenic diet in urban coyotes affected their gut microbiomes using high-throughput sequencing of 16S rRNA gene amplicons from both duodenal and fecal samples. Our sequencing efforts yielded 3,049,240 high-quality reads comprising 1,538 unique amplicon sequence variants (ASVs). Across all samples, microbial communities were dominated by four phyla (Firmicutes, Fusobacteria, Proteobacteria, and Bacteroidetes) that collectively accounted for 99.5% of the duodenal community and 97.0% of the fecal community (**Fig. 4.2a**). No significant differences in alpha diversity were observed between urban and peri-urban samples of either type (**Fig. 4.2b, d**).

To evaluate differences in community composition, we examined bacterial community structure using non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity. We also tested for differentially abundant taxa using both significance tests and the Hedge's *g* measure of effect size. Rather than shifting directionally to match the change in diet, the duodenal microbial community in urban animals instead exhibited higher multivariate dispersion, or among-individual diversity, than peri-urban animals (PERMUTEST F=4.88 p<0.03) (**Fig. 4.2c**). This lower degree of consistency in community structure was supported by a number of additional analyses: 1) similar results were obtained with several other commonly used community dissimilarity indices (**Supplementary Fig. 4.3**); 2) cohesion, a microbiome metric designed to quantify interconnectedness among taxa (219), was lower in urban samples, suggesting there are fewer, or weaker, community-level interactions in the urban duodenal microbiome (**Supplementary Fig. 4.4**); and 3) members of the 19 ASVs representing the core duodenal microbiome comprised a smaller proportion of the urban duodenal community (**Supplementary Fig. 4.4**). Urban coyotes contained an average of 2.5-fold more Bacilli (31.3±33.8% vs. 12.4±23.5%, p=0.283, q=0.704) and half as many Clostridia (34.5±35.3% vs. 71.8±27.9%, p=0.004, q=0.085) as peri-urban coyotes, though no taxa were significantly differentially abundant after correction for false discovery rates.

The fecal microbial community in urban coyotes did not demonstrate the same dispersion effect (PERMUTEST F=0.050, p=0.831) but did exhibit an expected, albeit weak, directional shift (**Fig. 4.2e;** PERMANOVA R^2=0.029, p=0.020). Members of the class Erysipelotrichia were significantly enriched in urban fecal samples (2.49±4.88% vs. 1.40±5.42%, p=0.003, q=0.050), which also contained an average of three-fold more Bacilli (14.4±22.8% vs. 3.6±13.6%, p=0.272, q=0.533) and two-fold fewer Fusobacteria (10.14±13.7% vs. 20.16±16.7%, p=0.029, q=0.204). There were no significant differences in the other community metrics we evaluated (**Supplementary Fig. 4.4**).

Because high taxonomic variation in the gut microbiome can obscure functional redundancy, we also explored metagenomic profiles predicted from our sequencing data. These revealed no differences in the functional alpha- or beta-diversity of the microbiome (**Supplementary Fig. 4.5**). Diet-based differences in microbiome function often hinge on the abundance of amino acid biosynthetic and degradative enzymes, which respectively signify herbivory and carnivory; we checked the relative abundance of these pathways but found no

significant differences between urban and peri-urban coyotes (**Supplementary Fig. 4.5**). Compared to published data from other animals (55), the coyote fecal microbiome most closely resembled carnivores in alpha diversity measures (**Supplementary Table 4.2**). In taxonomic distance-based analyses with these other animals, the fecal microbiome of urban coyotes was shifted slightly closer to omnivores, though both urban and peri-urban coyotes still most closely resembled other carnivores (**Fig. 4.3, Supplementary Table 4.2**).

Based on the observed dispersion effect in the duodenal microbiome, we expected either higher $\delta^{13}C$ signatures or the presence of anthropogenic food in the stomach would predict the Bray-Curtis distance between a microbiome sample and its same-group centroid but did not find any significant association (**Supplementary Fig. 4.6**). However, we did find preliminary evidence to suggest that the urban coyotes consuming more atypical diets, based on their stomach contents, were those with the most divergent duodenal microbiomes (**Supplementary Fig. 4.7**). There were no significant correlations directly between taxon abundances and stable isotope signatures, though a related study in our lab has used generalized linear models (GLMs) to show that higher $\delta^{15}N$ signatures are linked to more Fusobacteria and fewer Bacilli in the fecal microbiome (this thesis, Chapter 3).

Urban coyotes assimilate less fat, are immune-challenged, and are more likely to carry parasites

Finally, we explored how physiological health in urban coyotes may change in relation to their more anthropogenic diet and altered microbiomes. Urban coyotes had half as much kidney fat, which we measured as the kidney fat index (KFI), a common indicator of body fat reserves (220) (**Fig. 4.4**; 0.30±0.18 vs. 0.57±0.32; Welch's t=4.38, df=31.1, p < 0.001). After controlling for body mass, urban coyotes also had 35% larger spleens (**Fig. 4.4.**; 2.31±0.66 vs. 1.70±0.59 g/kg; Welch's t=-3.01, df=14.5, p = 0.009), suggesting they may be experiencing more challenges to their immune system (189). Interestingly, an equally strong predictor of spleen size in GLMs was whether the stomach was empty (**Supplementary Fig. 4.8**). We lastly used PCR to test coyotes for the presence of the intestinal helminth *E. multilocularis* and found that urban coyotes were almost twice as likely to carry this zoonotic parasite (63.6% vs. 35.4%; χ^2=3.00, df=1, p=0.083). These changes in kidney fat, spleen size, and infection status were not confounded by any differences in agglomerated body mass, size, or age between the two

populations (**Supplementary Table 4.3**), nor were there any significant differences in the residuals of a linear regression of body mass against body length, a commonly used index of condition in animal ecology studies (**Fig. 4.4**; -0.16± 1.17 vs. 0.023±1.83, Student's t=0.32, df=71, p=0.748).

We considered KFI, spleen mass, and infection status as dependent variables predicted by both diet and microbiome and found that coyotes with the most divergent duodenal microbiomes were also those with the lowest KFI (Spearman's R=-0.29, p=0.02). In fecal samples, the abundance of Erysipelotrichia was also negatively correlated with KFI (Spearman's R=-0.24, p=0.047). No diet or microbiome features were strongly indicative of either spleen mass or infection status (data not shown; also see this thesis, Chapter 3).

4.4 DISCUSSION

Generalist species able to survive on anthropogenic food are becoming increasingly common in urban environments, with important implications for human-wildlife interaction and conflict, and the gut microbiome may play an important role mediating the relationships among diet, health, and behavior in urban-adapted animals. We compared populations of urban and peri-urban coyotes to test the hypothesis that urban coyotes, which are known to consume a broader and lower-quality diet containing more anthropogenic food (124, 210), have altered gut microbiomes that also correspond with poorer body condition. Urban coyotes in our study were more likely to consume anthropogenic food and to consume more of it, but there was no evidence of a definitive shift in microbiome composition; instead, the duodenal microbiome became more heterogenous among individuals and the fecal microbiome contained more Erysipelotrichia and Bacilli and fewer Fusobacteria. These measurable changes in the coyote microbiome coincide with observations that urban coyotes assimilate fewer nutrients in the form of kidney fat reserves, experience more challenges to their immune system, and are twice as likely to carry the zoonotic parasite *E. multilocularis*.

The types of anthropogenic food consumed by coyotes in our study match previously established dietary patterns in several urban animals (221), with urban coyotes consuming a greater diversity of carbohydrate-rich, protein-poor food items as well as nutritionally valueless items such as leather gloves. Our estimate for the prevalence of anthropogenic food consumption is three times higher than a previous scat-based analysis in Edmonton (124), possibly reflecting

the higher sensitivity of stomach contents for finding completely digestible foods not detectable in scats (193). Reliance on anthropogenic food is additionally underestimated by our stable isotope mixing model because some foods necessarily classified as "fruit" in our model are fruits, such as crabapples, that could only be obtained from urban environments. Coyotes consuming these urban-sourced fruits would still be utilizing an anthropogenic food source with the same potential consequences for food conditioning and dependency. However, we did not observe the strong negative correlation between $\delta^{13}C$ and $\delta^{15}N$ that has been observed in other urban-exploiting mammals (193, 222) and only two of our coyotes had $\delta^{13}C$ signatures in excess of -20.5, a previously established isotopic threshold for anthropogenic food dependency (210), suggesting that urban coyotes are still occasionally obtaining natural prey and are not specializing in one food type.

In the duodenum, which is more immediately affected by short-term diet (143) and naturally more variable than feces (148, 169, 176), these dietary patterns in urban coyotes caused individual communities to become more divergent from each other. Aside from the effect of diet breadth alone, one probable reason for this unexpected variance is a higher frequency of a behavior analogous to "prey-switching," where individual coyotes regularly alternate among a wider breadth of available food including both protein-rich natural prey and carbohydrate-rich anthropogenic resources. This form of prey-switching has been cited as a mechanism of diet subsidization in urban coyotes (210) and would promote microbiome dispersion in a cross-sectional sample such as ours because different microbes specialize in digesting different nutrients (113) and each coyote may be capitalizing on a different diet at any given time. It would also produce longitudinal variation within individuals as the primary food source varies.

The non-correspondence between specific dietary information and the degree of microbiome divergence for individual coyotes does not nullify the presence or importance of these microbiome effects but rather offers further support for how inconsistent diets may produce microbiome divergence. Microbiomes co-adapt with their host to specialize on specific diets (52), and while diet-specific changes can be observed in the microbiome within hours of a new meal (25), frequently alternating among food items of vastly different nutritional composition would create inconsistent selective pressures in the gut microbiome and prevent the microbiome from co-adapting to the current diet. Stable isotopes indicated greater overall inclusion of anthropogenic food in the urban coyote diet but cannot discriminate between anthropogenic

foods, such as breads and processed meats (223), that differentially affect the microbiome (224). Constant variance between nutrient types would disrupt natural co-evolutionary dynamics, creating a game of 'cat-and-mouse' between microbiome and diet in which we would not expect microbiome composition or function to perfectly match diet composition at any given time. Instead, the most divergent microbiomes seemed to correspond with the most atypical recent diets, further suggesting that the microbiome may be regularly shifting to follow the broader suite of diet items being consumed.

The fecal microbiome, which most closely reflects the more longitudinally stable community in the colon (143; this thesis, Chapter 2), did not become more variable, but did shift to favor more Erysipelotrichia and Bacilli and fewer Fusobacteria. Although Fusobacteria have been associated with inflammatory bowel disease and other dysbiosis in humans (36, 200), they seem to be an essential component of the healthy canine microbiome (150, 151, 198; this thesis, Chapter 3) and have been linked to protein-rich diets in canines (165; this thesis, Chapter 3). A recent experimental comparison of the fecal microbiome in dogs also reported that carbohydrate-rich kibble diets led to more Erysipelotrichia and fewer Fusobacteria relative to protein-rich meat diets (198). This same signature in urban coyotes presumably reflects a net increase in carbohydrate consumption even as urban coyotes switch more frequently among food items over space and time. Several groups of Bacilli are known for carbohydrate fermentation and are regularly found in processed foods, offering further support for this hypothesis (25). Despite these taxonomic changes, the fecal microbiome exhibited no change in predicted metagenomic profile and when compared to other mammals was still most like carnivores, suggesting only limited improvement in the functional capacity to digest carbohydrates.

It is likely that these different but appreciable changes in duodenal and fecal microbiome composition, as they relate to a presumably more broad and variable diet containing more carbohydrate-rich and processed foods, constitute a fourth element in what has previously been described as a 'vicious circle' of diet, body condition, and disease susceptibility in urban animals (125). If the microbiome is never perfectly matched to the immediate diet, as we hypothesize in the small intestine, or is incapable of efficiently metabolizing a large portion of an already low-quality diet, as we suspect in the large intestine, many components of the diet become unassimilable 'empty calories.' A similar form of functional constraint has been observed in animals with evolutionary recent divergence in their diets, such as the giant panda, which harbors

a carnivore-like gut microbiome despite its herbivorous diet (61) and consequently only metabolizes 8% of the cellulose in bamboo (225). Urban coyotes, particularly those with the most divergent duodenal microbiomes, correspondingly assimilated less kidney fat. Impaired nutrient assimilation in turn affects immune function (226), and urban coyotes were more immune-challenged. Coyotes with the largest spleens, independent of their habitat, were also those with empty stomachs, hinting at a possible relationship between disease and impaired hunting ability. Diseased coyotes are also reported more frequently at compost piles, an easily accessible but nutrient-poor source of food (125). This cycle may additionally be affected by environmental factors that we did not measure: studies in other animals have shown that exposure to new pathogens, parasites, or chemicals, all of which are more common in urban environments (98), can disrupt the gut microbiome (18, 75) with secondary downstream effects such as poor health and altered behavior (179).

Other aspects of health and behavior may additionally be affected by microbiome composition in unpredictable ways. In our study, urban coyotes were twice as likely to be infected with *E. multilocularis*, which can cause a life-threatening zoonosis if transmitted to humans. *E. multilocularis* is transmitted via small rodents, a naturally more common prey source in urban environments, but it is possible that disrupted co-evolutionary dynamics between host and microbiome could provide a fertile environment for parasite establishment (209). Behavior may likewise be affected by the microbiome: one urban coyote in our study was delivered to us because it attacked and killed a large domestic dog, an uncharacteristically aggressive behavior for an urban coyote. Although low-protein diets and poor health have been cited as predictors of conflict (124), this coyote was above average in all physiological characteristics and had an average $\delta^{15}N$ signature. Instead, this was the only coyote to not contain any Fusobacteria in both duodenal or fecal samples, and the duodenal microbiome was unusually dominated by Clostridia (99.9%). Documented relationships between the microbiome and behavior abound in humans and other animals (227), and more recently microbiome composition, including fewer Fusobacteria, has been directly associated with aggression in dogs (201). Aggressive behavior and the spread of *E. multilocularis* both have great implications for human-coyote interactions in urban areas, and we suspect urban-induced disruptions to the microbiome may play important but poorly understood roles in mediating those outcomes.

Our attempts to define linear and self-reinforcing connections between diet, microbiome, and health in urban coyotes admittedly mask the known gradient in the degree to which coyotes experience this cycle. The urban landscape itself is highly heterogenous, comprising both natural lands and developed areas (228), and the extent to which urban coyotes exploit this landscape is equally diverse (210). We speculate that individual behavioral choices in urban coyotes, both laterally across the population and longitudinally within individuals, generate a complex mosaic of spatially and temporally variable diets, lifestyle contexts, and environmental exposures to which the microbiome, partially constrained by host physiology (62), cannot perfectly co-adapt. In line with the 'Anna Karenina' principle for animal microbiomes (229), which states that disturbed or dysbiotic individuals vary more in microbiome composition than undisturbed or healthy individuals, we propose that an important consideration in any study of urban-exploiting mammals, or of stress responses generally, is the extent of variation observed among individuals, as the same outcomes, such as lower nutrient assimilation or aggression, may be caused by several unique and unpredictable factors. We argue that individually isolating causal factors ignores the heterogeneity of this evolutionarily new landscape and the diversity of ways in which animals respond to it, and the challenge remains to find experimental approaches that can capture the extent of variation in both the urban habitat and the animals that occupy it.

4.5 METHODS

Sample collection and necropsy

We collected 76 coyote carcasses between August 2017 and May 2018. Of these, 9 coyotes were roadkill collected within Edmonton city limits by the City of Edmonton Animal Care and Control Center. Another 64 coyotes were provided by local fur trappers working near Leduc and Beaumont, south of Edmonton. The last three coyotes were killed due to negative interactions with humans or domestic animals: 1) shot by a farmer near Calmar, AB; 2) shot by Edmonton Police Service after exhibiting potentially rabid behavior, though it later tested negative for rabies; and 3) trapped by contractors hired by the City of Edmonton after it killed a domestic dog. For the purposes of this study, the roadkill and latter two euthanized coyotes were considered "urban" (n=11) and remaining coyotes were considered "peri-urban" (n=65). These coyotes were also used as part of a related study examining the relationships between microbiome composition and physiological health (this thesis, Chapter 3).

All carcasses were frozen at -80°C for at least 5 days to neutralize any zoonotic pathogens, and then stored at -20°C. Carcasses were thawed for 12-14 hours at room temperature before being necropsied following established procedures (this thesis, Chapter 3). For each coyote we obtained physiological measures (mass, girth, size, age, kidney fat index, and spleen mass) as well as stable isotope signatures from claw samples (δ^{13}C and δ^{15}N) and a binary classification of whether anthropogenic food, including urban-derived fruits, was present in the stomach. Techniques for stable isotope sample preparation and aging using cementum annuli are described in this thesis (Chapter 3). For microbiome analysis, we extruded fecal samples from the large intestine and removed 10cm sections from the duodenum, which were stored at -80°C until DNA extraction. Two coyotes did not contain extrudable fecal material in the colon, so we removed the distal 10cm of the colon and processed them as we did the duodenal samples (see below).

DNA extraction and sequencing

Fecal samples were thawed and then manually homogenized using a pestle and mortar. To obtain samples of the duodenal lumen, we longitudinally transected each 10cm duodenal section on the anti-mesenteric side of the intestine and then advanced the luminal contents into microcentrifuge tubes using a sterile spatula. Whole community DNA was extracted from 100mg of either intestinal contents or fecal sample using the MP Bio FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) following the manufacturer's instructions, with modifications described in this thesis, Chapter 3. The V4 region of the 16S rRNA gene was PCR-amplified and sequenced on an Illumina MiSeq at Microbiome Insights (Vancouver, BC), as described in this thesis (Chapter 3).

Stomach contents

For the 11 urban stomachs and a subset of 15 peri-urban stomachs, we created a more detailed profile of stomach contents based on relative volumes. We removed all contents from each stomach, scraping the stomach lining with a dissecting spatula, and then rinsed the contents using an 850μm sieve with room temperature tap water to remove mucous and aid in identification. Stomachs containing less than 1g of food were considered empty. For non-empty stomachs, we classified each discernable food item as prey, anthropogenic food, or other fruit

and vegetation. We estimated the proportional representation of each food group by distributing contents to uniform thickness across an 11x17-inch grid and calculating the percentage of occupied grid squares containing each food group.

Stable isotope mixing models

To predict the proportion of different food items in coyote diets using stable isotope mixing models, we obtained stable isotope values for coyote diet items from (125), which includes various prey species, fruit, and anthropogenic food. We supplemented this data with published isotopic values for beef and chicken from fast food restaurants (223). Diet items were assigned to one of three categories (prey, fruit, or anthropogenic food), which were then used as source items in stable isotope mixing models with the stable isotope values obtained from coyote claws representing the consumers. Mixing models were run in the R package *MixSIAR* (230) using three Markov Chain Monte Carlo (MCMC) chains each of length 100,000 and accounting for both 'residual' and 'process' error following the recommendations of (231). We accounted for tissue-specific discrimination factors following (210). Isotopic niche breadth was also calculated using *MixSIAR*.

E. multilocularis detection

We used PCR to test each coyote for possible infection with *E. multilocularis*. DNA extracted from both duodenal and fecal samples was amplified in triplicate using *E. multilocularis*-specific primers Cest1 and Cest2 (195). PCR was performed in 25µl reactions with 0.2µM of each primer and 1µl of template DNA using the cycling conditions described in (195). We resolved PCR products in 2.0% agarose gels run for 35 minutes at 120V and post-stained with ethidium bromide. A coyote was considered positive for *E. multilocularis* if either the fecal or duodenal sample exhibited an approximately 395bp band in at least two replicates. Samples testing negative were diluted and tested again to control for possible PCR inhibition.

16S rRNA gene sequence processing

Sequences were processed following previously described procedures (this thesis, Chapter 3). We initially retrieved 4,325,108 total reads, ranging from 103 to 77,835 per sample (mean 28,455±16,338). After trimming, quality-filtering, and chimera-checking the raw

sequences, we used the R package *dada2* to determine exact amplicon sequence variants (ASVs). We assigned taxonomy to ASVs using the Ribosomal Database Project (RDP) reference database (release 11.5) and then removed 1) twelve samples with fewer than 4,000 reads; 2) six ASVs identifying as chloroplasts or mitochondria; and 3) ASVs that deviated from our target amplicon length of 250-256bp. For our final analyses, we averaged ASV abundances across 100 rarefactions to the minimum library size of 4,637 reads. Our final feature table was imported into the R package *phyloseq* (158) for subsequent analyses.

Statistical analyses

      Differences in diet and health. All statistical analyses were performed in R 3.5.0 and for all statistical tests we defined a significance threshold of $p < 0.05$. Differences in binary categorical variables between urban and peri-urban coyotes (such as anthropogenic food in stomach and infection status) were assessed using the chi-square test of independence without Yates' continuity correction. Continuous physiological measurements and stable isotope values were tested for homoscedasticity using Levene's test, and then differences between urban and peri-urban coyotes were assessed with the Student's or Welch's t-test for homoscedastic or heteroscedastic data, respectively.

      The three variables below our significance threshold of $p = 0.05$ (KFI, spleen mass, and $\delta^{13}C$) were separately re-evaluated using generalized linear models (GLMs) with a gamma distribution and inverse link with urban habitat use, stomach contents, stable isotope values, and other physiological measurements as predictors. We removed non-significant predictor variables and any collinear predictors with variance inflation factors (VIF) greater than two and chose the best candidate model based on the lowest Akaike information criterion score corrected for small sample sizes (AICc). For the model with $\delta^{13}C$ as a dependent variable, we used the absolute value of $\delta^{13}C$ to accommodate the gamma distribution.

      Differences in microbiome composition. Microbiome analysis was performed separately for duodenal and fecal samples. We calculated taxonomic alpha diversity, including multiple indices of richness, evenness, and overall diversity, using the 'global' function in the package *microbiome* (232), and tested for significant differences using the Kruskal-Wallis test with urban status as the dependent variable. Between-sample beta-diversity was estimated using the Bray-Curtis, Jaccard, and weighted and unweighted UniFrac indices calculated by the

packages *vegan* (161) and *phyloseq*. We visualized dissimilarity between samples using non-metric multidimensional scaling (NMDS) and assessed differences in community composition between urban and peri-urban coyotes using a permutational multivariate analysis of variance (PERMANOVA) with 1,000 permutations as implemented by the 'adonis' function in *vegan*. To account for possible dispersion or destabilization effects, we calculated multivariate dispersion using the 'betadisper' function and tested for homogeneity of group dispersions using the 'permutest' function with 1,000 permutations.

In addition to these alpha- and beta-diversity metrics, we considered two other aspects of microbiome structure: 1) We defined a core microbiome for each sample type as the ASVs present with at least 0.2% relative abundance in over 50% of samples. We then calculated the total relative abundance of core microbiome members in each sample using the 'core_abundance' function in the package *microbiome*. 2) Cohesion is a newly developed microbiome metric designed to calculate the interconnectedness among taxa within a bacterial community (219). To calculate cohesion for our samples, we first filtered our unrarefied feature table to only include taxa present in at least 7% of samples with a mean relative abundance of at least 0.0005%. We then calculated cohesion following (219), using the 'taxon shuffle' algorithm and 200 iterations per taxa to generate a null correlations matrix.

Significant differences in taxon abundances between urban and peri-urban coyotes were evaluated at the class level using the unrarefied feature table by first agglomerating taxa using the 'tax_glom' function in *phyloseq* and then testing for differential abundance using the default settings of the package *ALDEx2* (160). We evaluated differential abundance using Welch's t-test and corrected p-values for multiple comparisons using the Benjamini-Hochberg correction. Corrected p-values less than 0.05 were considered significant. To avoid discounting large differences in mean relative abundance that did not pass this stringent significance threshold, we additionally calculated Hedge's *g* as a measure of effect size that accounts for standard deviation and uneven sample sizes. We considered taxa with an effect size >1 or <-1 to be differentially abundant even if the adjusted p-value was not significant.

Metabolic inferencing. PICRUSt 2.0 (phylogenetic investigation of communities by reconstruction of unobserved states) (118, 233–237) was used to infer the metagenome functional content of our samples, with our rarefied feature table supplied as the raw data. We explored differences in functional diversity and composition using the same alpha- and beta-

diversity analyses used to explore taxonomic variation, described above. To compare differences in the abundance of amino acid metabolic pathways, we manually identified MetaCyc pathways involved in either amino acid biosynthesis or degradation and tested for differential abundance using the same agglomeration procedure and *ALDEx2* parameters described above.

Comparisons to other animals. We compared our coyote fecal samples to published data for other carnivores, herbivores, and omnivores (55). Because these published sequences were from a different region of the 16S rRNA gene and pre-aligned against the SILVA database (release 102) (238), we re-assigned taxonomy to our DADA2-processed ASVs using a closed-reference clustering approach against the SILVA (release 102) reference database at a 97% identity threshold in QIIME 1.0. ASVs that did not align with the database were discarded, resulting in a loss of 20% of our ASVs. We agglomerated the remaining operational taxonomic units (OTUs) to the family level and then merged our new feature table with the published data and rarefied the final feature table to the minimum number of reads. Alpha diversity was calculated separately for each data set prior to agglomeration, to ensure comparisons remained at the level of individual OTUs, and beta-diversity was calculated for this merged sample as before. Distances between different groups (urban coyotes, peri-urban coyotes, carnivores, herbivores, and omnivores) were calculated as the distance between group centroids using the Bray-Curtis metric and significant clustering associations were assessed using a pairwise PERMANOVA adjusted with a Bonferroni correction.

Diet, microbiome, and health. To determine relationships between microbiome composition, diet, and health in duodenal samples, we calculated the Bray-Curtis distance from each sample to the overall centroid using the 'betadisper' function in vegan. This distance was correlated against stable isotope signatures and physiological measures using Spearman's rank correlation and against binary stomach contents using Welch's t-test. We also quantified diet divergence by using the Bray-Curtis distance to ordinate our subset of stomach samples based on the relative volumes of their contents and then similarly calculating the distance from each sample to the centroid. This measure was correlated against microbiome divergence using Spearman's correlation.

For both duodenal and fecal samples, we calculated Spearman's correlation between stable isotope values and relative abundances of individual taxa; for these correlations, we only considered taxa present in at least one-third of all samples and excluded samples as outliers if the

taxon relative abundance was greater than three standard deviations above or below the mean. We additionally considered results from GLMs that we previously evaluated as part of a related study in our lab assessing the overall relationships between diet, fecal microbiome composition, and physiological health independent of habitat use (this thesis, Chapter 3).

Data availability

Raw, unfiltered sequence data associated with this study has been deposited with the NCBI under the project number PRJNA528764.

4.6 ACKNOWLEDGEMENTS

4.7 TABLES & FIGURES
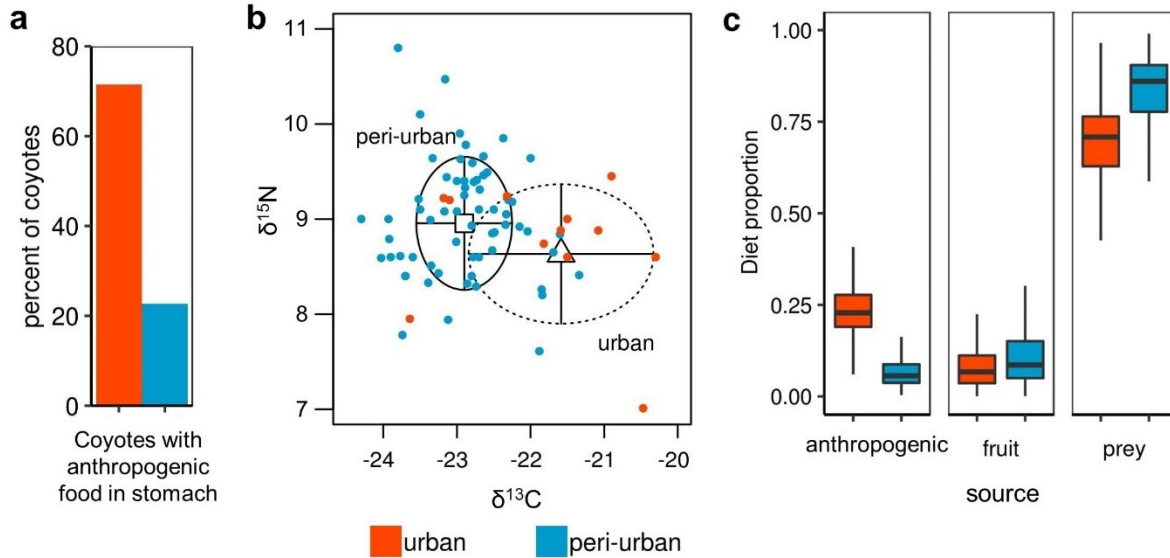
Fig. 4.1: Urban coyotes consume more anthropogenic food.

(**a**) Remnants of anthropogenic food were found more frequently in the stomachs of urban coyotes. The left axis indicates the number of stomachs containing anthropogenic food, expressed as a percentage of the total number of non-empty stomachs for each sample group. (**b**) Stable isotope values measured from claw samples. Urban coyotes have a significantly higher $\delta^{13}C$ signature relative to peri-urban coyotes, a slightly lower range of $\delta^{15}N$ signatures, and a wider isotopic niche. Hollow shapes and error bars represent means and standard deviations, respectively. Points for individual urban (red) and peri-urban (blue) coyotes are depicted in the background. (**c**) Anthropogenic food comprises a larger proportion of the urban coyote diet, based on a three-source Bayesian stable isotope mixing model. Bars show the 50, 75, and 95% confidence intervals.
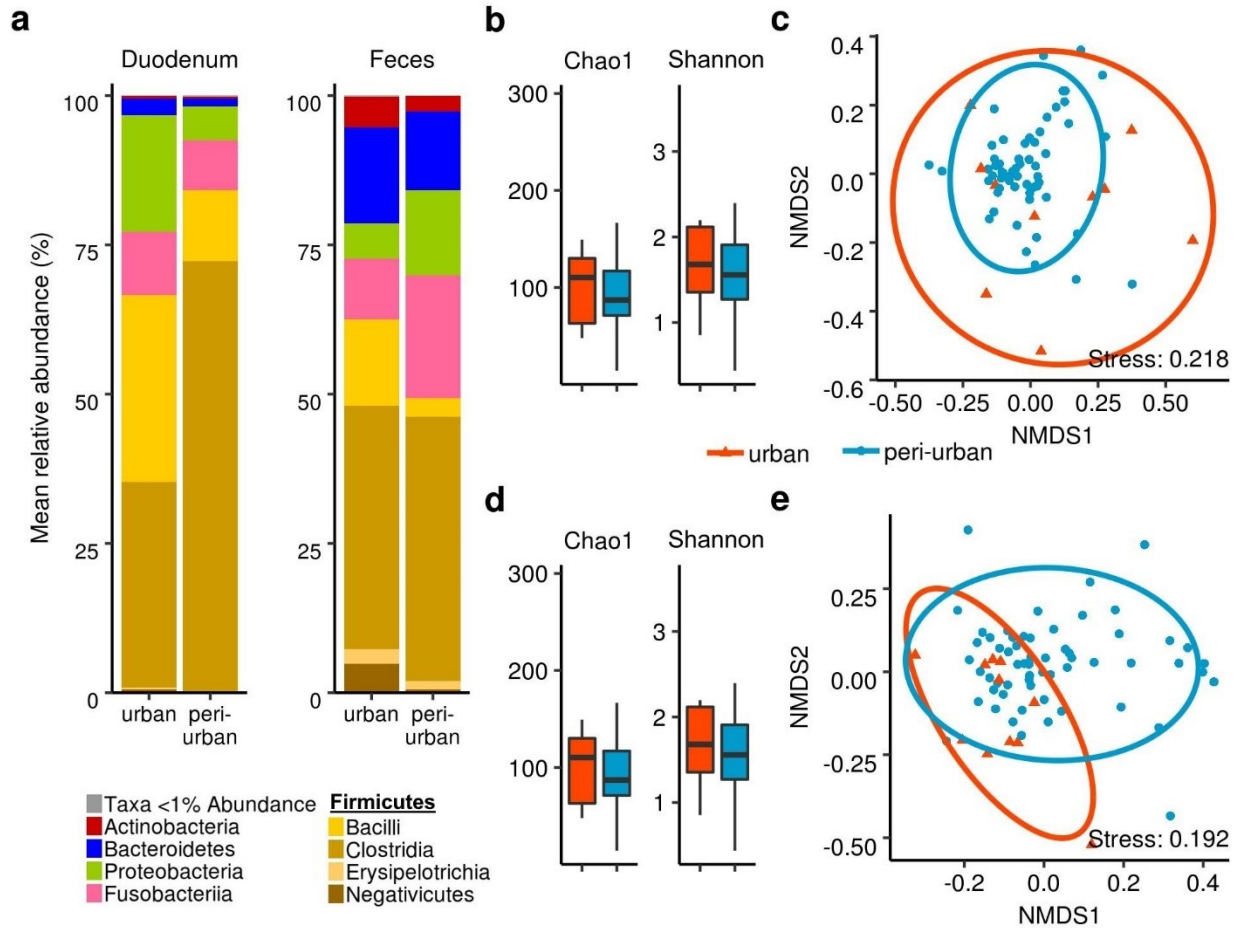
Fig. 4.2: Duodenal and fecal microbiomes respond to urbanization.
(**a**) Average relative abundance of each taxon per sample type (duodenum or feces) in urban and peri-urban coyotes. Taxa with a mean relative abundance of <1% are categorized as 'other.' Firmicutes (yellow shades) and Bacteroidetes (blue shades) are separated to the class level; all other taxa appear at the phylum level. (**b, c**) There were no significant changes in duodenal alpha diversity between urban (red) and peri-urban (blue) coyotes (**b**), but non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distances, coded by urban habitat use, reveal that the duodenal microbiome in urban coyotes is more dispersed or heterogeneous (**c**). (**d, e**) The fecal microbiome in urban coyotes similarly exhibits no change in alpha diversity (**d**) but exhibits a weak but significant compositional shift (**e**). In (**b**) and (**d**), plots show the Chao1 richness index and Shannon diversity; bars depict medians and interquartile ranges. Ellipses in (**c**) and (**e**) represent 90% confidence intervals.
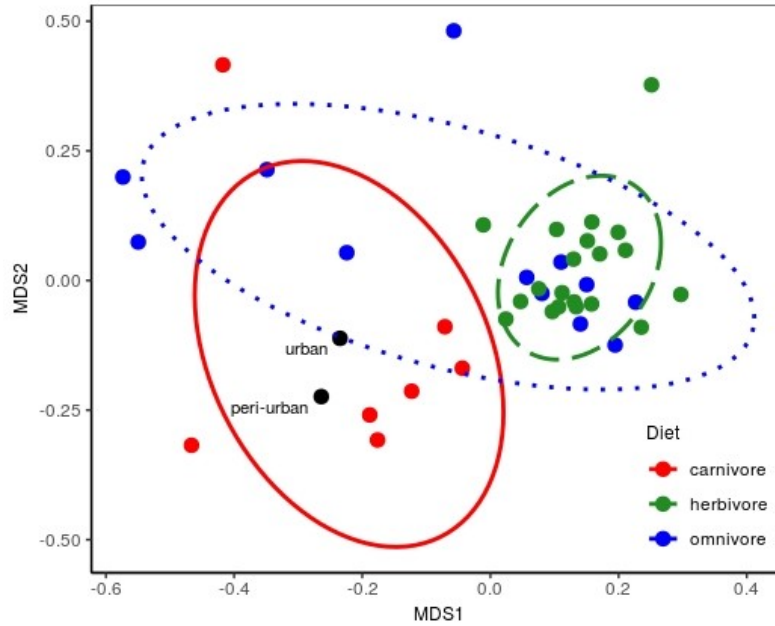
Fig. 4.3: Both urban and peri-urban coyotes most closely resemble other carnivores.
NMDS ordination using Bray-Curtis distance between the fecal microbiome of 39 different
mammal species colored by diet, using data from (55). For simplicity, urban and peri-urban
coyotes are represented as a single point showing the centroid of their respective samples.
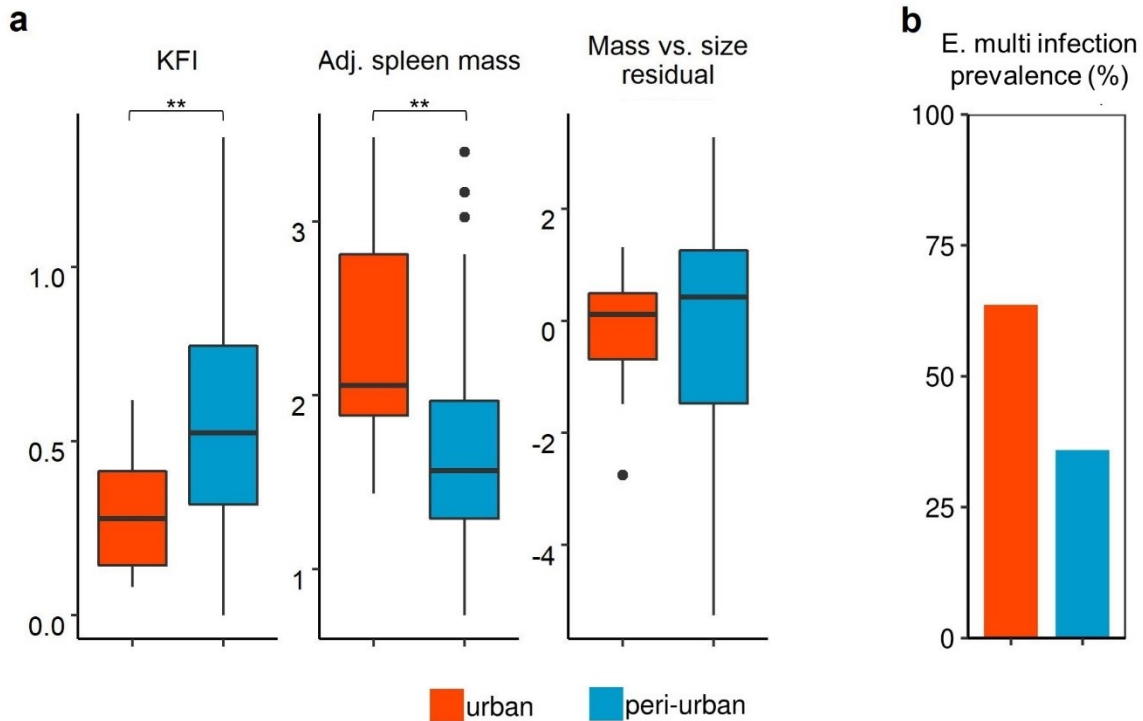
Fig. 4.4: Urban coyotes assimilate less fat, have larger spleens, and are more likely to be infected with a zoonotic parasite.

(**a**) Boxplots depicting differences in the kidney fat index, spleen mass, and residuals from a regression of body mass against body size (a common measure of condition) between urban and peri-urban coyotes. Spleen mass was divided by body mass to control for variation in body size. Asterisks represent pairwise contrasts using Welch's t-tests (**, $p < 0.01$). (**b**) Urban coyotes were also were twice as likely to carry the intestinal helminth *Echinococcus multilocularis*.

CHAPTER 5

The freshwater sponge *Ephydatia muelleri* harbors a unique and stream-specific microbiome

5.1 ABSTRACT

Sponges are known for hosting large and diverse communities of microorganisms that contribute to host metabolism and can produce industrially valuable bioactive molecules. Despite ongoing interest in the ecology and biotechnological potential of the sponge microbiome, most research has targeted marine sponges; freshwater sponges, which may also harbor microbiomes of ecological and biotechnological significance, have been the focus of less than a dozen studies. Here, we use high-throughput sequencing of 16S rRNA gene amplicons to characterize the microbiome of the freshwater sponge *Ephydatia muelleri* in relation to its environment. Using samples collected from the Sooke, Nanaimo, and Cowichan Rivers on Vancouver Island, British Columbia, we show that the sponge microbiome is compositionally distinct from the ambient water and adjacent biofilms. Individual sponges harbor on average over 1,000 unique amplicon sequence variants (ASVs), dominated by members of the phyla Proteobacteria, Bacteroidetes, and Actinobacteria. Two-thirds of the sponge microbiome is comprised of the families *Chitinophagaceae* and *Comamonadaceae*, which may play ecological roles degrading sponge-derived chitin and steroids. We additionally note that several taxa were differentially abundant in sponges taken from different streams. These patterns in abundance were not matched by variation in the water or biofilm samples, suggesting that other environmental conditions may shape the composition of these communities. Overall, this study demonstrated that freshwater sponges may represent an important yet underappreciated component in a comprehensive understanding of the ecology, evolution, and biotechnological value of the sponge microbiome.

5.2 IMPORTANCE

Freshwater sponges have been largely unrecognized in the field of sponge microbiome research, and even the existence of freshwater sponge-specific microbiomes has been questioned by previous authors. This study showed that the freshwater sponge *Ephydatia muelleri* harbors a community of microbes that is distinct from the ambient environment and variable between individuals from different rivers. These results raise exciting new questions about the evolution, ecology, and biotechnological value of freshwater sponge-associated microbial communities and suggest that freshwater sponges are an important component in a more comprehensive evaluation of the global sponge microbiome. Our findings are the first exploration of the microbiome of any freshwater sponge in North America and to our knowledge only the second study to analyze the sponge microbiome using high-throughput Illumina sequencing, which is now the benchmark for exploratory microbiome analysis.

5.3 INTRODUCTION

Sponges (Phylum: Porifera) have garnered considerable scientific attention for their ecological, evolutionary, and microbiological significance. They are the oldest extant multicellular animals, dating back over 600 million years (239), and as sessile benthic filter-feeders they collectively process thousands of liters of water per day, facilitating the transfer of essential nutrients between the benthic and pelagic zones in aquatic ecosystems (46). In the past few decades, marine sponges have additionally become known for harboring dense and phylogenetically diverse communities of microorganisms, with sponge-associated microbes comprising almost 40% of sponge body weight and reaching population densities 2-4 orders of magnitude higher than ambient seawater (240). These microbial communities, often housed within the sponge mesohyl, contribute to host metabolism and produce antimicrobials and other biologically active compounds that are beneficial to the sponge host (46). Many of these compounds have been isolated for their biotechnological value, including several that are now in clinical trials or on the market for their anticancer and antifungal properties (47).

Despite ongoing interest in the marine sponge microbiome, limited attention has been given to freshwater sponges (Haplosclerida: Spongillina), which first evolved from marine sponges approximately 300 million years ago (241, 242) and provide similar ecosystem services in freshwater habitats. Freshwater sponges have been found in both lotic and lentic systems from

a wide breadth of latitudes, elevations, and bathymetric conditions, and in those habitats colonize various substrates including bedrock and boulders, woody debris, and man-made surfaces such as cement or metal (243). They can tolerate extreme physicochemical conditions by forming gemmules, dormant clusters of embryonic cells that also comprise a form of asexual reproduction (243).

Some authors presumed that sponge-microbe associations would have been lost when sponges first invaded freshwater habitats (4, 8), but several studies have shown that some freshwater sponges are in fact associated with unique, albeit smaller, microbial communities that may also produce potentially valuable bioactives. Researchers in the 1980s characterized intracellular algal symbionts in the temperate sponges *Ephydatia fluviatilis* (244) and *Spongilla lacustris* (245), and more recent work has described microbes associated with endemic sponges in Lake Baikal (135, 136, 246, 247) . Antimicrobial compounds have been isolated from bacterial communities associated with *E. fluviatilis* (15, 16) and the Amazonian sponge *Metania reticulata* (250). However, another study found that the bacterial community associated with *S. lacustris* in Lake Staffelsee, Germany largely resembled the ambient water and was conspicuously absent from the sponge mesohyl (137). These results raise questions of how ubiquitous sponge-microbe associations are in freshwater environments, which microbes are permanent sponge residents rather than transients from the ambient water (251), and how freshwater sponges and these microbes interact.

Fully addressing the ecological questions posed by the freshwater sponge microbiome and prospecting its biotechnological value requires the use of more robust methodologies and comprehensive sampling schemes than have previously been employed. The studies of the freshwater sponge microbiome mentioned above are few in number and small in scope, relying primarily on PCR-amplified 16S rRNA gene clone libraries or separation by denaturing gradient gel electrophoresis to examine a limited number of individuals or locations (135, 247–249). A handful of studies have used pyrosequencing of 16S rRNA gene amplicons (11, 12), but to our knowledge only one study has used high-throughput Illumina sequencing of 16S rRNA gene amplicons (48), which is now the benchmark technique for exploratory microbiome research because it offers higher resolution at affordable costs. No study has yet determined if the freshwater sponge microbiome differs from adjacent biofilms, another important consideration in sponge microbiome research given that differences between sponges and water could reflect

differences between planktonic and benthic communities rather than a functionally meaningful association with sponge tissue.

In our study, we used high-throughput Illumina sequencing of 16S rRNA gene amplicons to test the hypothesis that the cosmopolitan freshwater sponge *Ephydatia muelleri* harbors a bacterial community distinct from both the ambient water and adjacent biofilms. We tested our hypothesis with samples of five individuals from each of three separate rivers on Vancouver Island, British Columbia. Our work represents the largest sample of the freshwater sponge microbiome to date and the first account in North America, and demonstrates that sponge-associated microbial communities, independent of individual or sampling location, are indeed distinct from their surrounding environment. We use our preliminary data to offer several hypotheses for the function of these communities, which can inform subsequent testing and refinement towards a more comprehensive evaluation of the ecology and biotechnological potential of the freshwater sponge microbiome.

5.4 RESULTS

5.4.1 Limnological data & sponge identification

We sampled sponges (n=15), water (n=14), and biofilms (n=8) from the Sooke, Nanaimo, and Cowichan Rivers in order to characterize the freshwater sponge microbiome (**Fig. 5.1**). All three streams exhibited similar limnological parameters (**Table 5.1**), and morphological analysis of sponge spicules confirmed that the sponges used in our study were *Ephydatia muelleri* (**Supplementary Fig. 5.1**).

5.4.2 Microbial composition and alpha-diversity

We obtained 2,724 unique amplicon sequence variants (ASVs) from our sequencing efforts, with an average of 18,733±6,691 reads per sample (range: 8,286-36,750). 1,803 ASVs were present in the sponge samples and 2,602 ASVs were present in the adjacent water and biofilms. ASVs were distributed across 24 bacterial phyla, 21 of which were found in sponges. In all samples the most dominant phylum was Proteobacteria; within this phylum, sponges and biofilms were dominated by Alphaproteobacteria whereas water samples were primarily comprised of Betaproteobacteria. The next most abundant phyla in sponge samples were Bacteroidetes (32.3%), Verrucomicrobia (2.9%), Actinobacteria (2.5%), and Cyanobacteria

(1.6%) (**Fig. 5.2**). The same four phyla similarly exceeded 1% abundance in water and biofilm samples, with markedly different abundances between sample types: biofilms contained significantly more Cyanobacteria (18.3±16.7% vs. 1.69±2.98% in sponge and 1.54±1.77% in water; p=0.008) and water contained significantly more Actinobacteria (18.9±10.1% vs. 2.54±1.67% in sponge and 5.45±7.23% in biofilm; p<0.001). Planctomycetes in both water and biofilm samples and Acidobacteria in the biofilm samples also exceeded 1% total abundance.

Rarefaction analysis demonstrated that sequencing depth was likely not sufficient to capture rare members of these communities but was still able to accurately assess trends in diversity (**Supplementary Fig. 5.2**). Based on Chao1 species richness estimates calculated after rarefying samples to the minimum library size, sponges hosted an average of 1,107±769 ASVs per individual, comparable to the paired water samples and adjacent biofilms (**Fig. 5.3a**). In contrast, the microbial diversity (Shannon index) of sponge samples was significantly lower than both water and biofilm samples (**Fig. 5.3b**; ANOVA F=17.0, df=2, p<0.001). Richness and diversity estimates for the sponge samples were also more consistent among individuals and among sampling locations than water samples, which varied significantly among rivers (**Fig. 5.3c,d** and **Supplementary Table 5.1**; Chao1: ANOVA F=38.8, df=2, p<0.001; Shannon: ANOVA F=356.0, df=2, p<0.001).

When we examined differences between sample types at finer taxonomic resolution, we found the relative abundance of 35 bacterial families were significantly different between sample types after correction for false discovery rates (**Table 5.2**). Many of these families were present at low abundances (<0.1%), but among the more common taxa, sponges harbored significantly more *Chitinophagaceae*, *Chryseolinea*, and *Methylophilaceae* than water samples, and significantly fewer *Microbacteriaceae* and *Flavobacteriaceae*. Twenty-six bacterial families were differentially abundant between sponges and biofilms (**Table 5.2**): most notably, sponges harbored more *Comamonadaceae* and *Chitinophagaceae* and were depleted in a variety of Proteobacterial families. *Chitinophagaceae* and *Comamonadaceae* together accounted for almost two-thirds of the sponge communities compared to only one-third of the water communities and 10% of the biofilm communities. The dominance of these two families, which in sponge samples is driven by the genera *Sediminibacterium* and *Comamonas*, largely explains the lower richness and diversity measures for sponge samples. *Microbacteriaceae, Chitinophagaceae, Cryomorphaceae, Methylophilaceae,* and *Rhodospirillaceae* were the only five families with

significantly different abundances between sponges and both water and biofilms. Three bacterial families that were not detected in water or biofilm samples were detected in sponges at extremely low abundances: *Nitrosomonadaceae* (0.003%), *Vibrionaceae* (0.001%), and *Paenibacillaceae* (0.001%).

5.4.3 Beta-diversity

These compositional differences created a strong and significant clustering association that separated sample types (sponge, water, and biofilm), independent of sampling location, in ordination analyses (**Fig. 5.4a**; PERMANOVA F=10.2, df=2, R^2=0.375, p<0.001). There were no significant differences in multivariate dispersion. These clusters were robust to multiple commonly used dissimilarity indices and were consistent when each sampling site was evaluated separately (**Supplementary Fig. 5.3**). Random forest models trained to classify samples based on their community composition were 100% accurate in discriminating sponge samples from water and biofilms, and only mistakenly classified one biofilm sample as water. The most discriminatory taxa in our random forest models based on Gini scores included three ASVs from *Chitinophagaceae* and four ASVs from *Comamonadaceae*, along with *Rhodospirillaceae* and several ASVs from the phylum Actinobacteria (**Supplementary Table 5.2**).

5.4.4 Location-specific variation

We explored variation in the sponge microbiome as a function of location and found a similar and significant clustering association separating sponges from different rivers in ordination analyses (**Fig. 5.4b,** PERMANOVA F=4.5, df=2, R^2=0.43, p<0.001). Random forest models again exhibited perfect accuracy when discriminating sponges based on their location (**Supplementary Table 5.2**). The relative abundance of *Chitinophagaceae* varied significantly among all three streams (**Fig. 5.5**; Sooke-Nanaimo-Cowichan - 9.8%, 33.9%, 57.5%; p < 0.001). The Sooke River sponges were additionally distinguished by significantly more *Cytophagaceae* (16.4%, 3.7%, 0.14%; p<0.001), *Cryomorphaceae* (3.0%, 0.08%, and 0.01%; p=0.001), and *Flavobacteriaceae* (4.2%, 0.8%, 0.2%; p=0.032) (**Fig. 5.5**). Nanaimo sponges contained significantly more *Burkholderiaceae* (2.8%, 6.9%, 0.5%; p<0.001) and Cowichan sponges contained significantly fewer *Microbacteriaceae* (2.6%, 1.5%, 0.1%) and significantly fewer Alphaproteobacteria from a variety of lower taxa (38.6%, 37.7%, 17.9%; p=0.044) (**Fig. 5.5**).

Several of these stream-specific characteristics of the sponge microbiome were not matched by the water samples: for example, the abundance of *Chitinophagaceae* in water samples was equal among all three streams, and the Cowichan River contained significantly more Alphaproteobacteria despite Cowichan River sponges containing significantly fewer (**Fig. 5.5**). When we used random forest models to predict sample affiliations based on both sampling location and sample type, sponge samples were correctly identified by location with perfect accuracy; water and biofilm samples exhibited only slightly less fidelity, with an out-of-bag error rate of 13.5%. The most discriminatory taxa again included *Chitinophagaceae*, *Cytophagaceae, Comamonadaceae*, *Flavobacteriaceae*, , and *Cryomorphaceae* (**Supplementary Table 5.2**).

5.5 DISCUSSION

Our results demonstrate that the microbial community associated with the freshwater sponge *Ephydatia muelleri* in Vancouver Island rivers distinctly differs in structure and composition from the communities found in ambient water, consistent with previous studies of freshwater sponges in Lake Baikal (135, 136, 246, 247) and elsewhere (9, 10, 15–18, 20). The sponge community also differs from biofilms growing on the adjacent substrate, an observation that has been made for marine sponges (252) that has not been shown in freshwater sponges. Because our study is one of only few characterizations of the freshwater sponge microbiome using next-generation sequencing, and the first to use amplicon sequence variants (ASVs), it is difficult to directly compare our measures of species richness and diversity with other reported freshwater sponge microbiomes. In general, our predictions that individual sponges harbor approximately 1,000 unique taxa appear consistent with some estimates (11, 20) slightly higher than another (246), and well within the range of values reported for marine sponges (3, 21). Our observation that the richness and diversity of the sponge microbiome was lower than that of its surrounding freshwater environment is also consistent with studies in both marine and freshwater environments (11, 22, 23).

With few exceptions, the taxonomic composition of the sponge communities in our study largely resembled other previously studied sponges. Proteobacteria, especially Alphaproteobacteria, are universally the most abundant phylum in sponge-associated microbiomes (3, 21), and *E. muelleri* was no exception. Other commonly reported phyla in freshwater sponges include Actinobacteria, Bacteroidetes, Planctomycetes, and Cyanobacteria

88

(3, 11), all of which were present in our samples. Interestingly, although Actinobacteria have been reported at anywhere from 10-36% abundance in several freshwater sponges, including *E. fluviatilis* (248) and *S. lacustris* (137), the sponges in our study appeared resistant to accumulating Actinobacteria and harbored two-fold fewer Actinobacteria than the surrounding water. Their lower abundance in our samples may reflect some unknown species- or location-specific quality or competitive exclusion by other, more abundant taxa. The abundance of Cyanobacteria was also comparatively low and variable between rivers. When we compared cyanobacterial abundance among individual sponges and matched it with photographs of each sponge, we found that sun-lit green-colored sponges contained appreciably more cyanobacteria than shaded, white sponges (**Supplementary Fig. 5.4**). Algal symbionts in freshwater sponges are light-dependent and not obligate (9, 10); cyanobacteria fill a similar ecological niche and the low mean cyanobacterial abundance in our sponges is likely an artifact of random sample choice.

The *E. muelleri* microbiome was primarily distinguished from water and biofilms in our random forest models by a small number of ASVs from the family *Chitinophagaceae* (Phylum: Bacteroidetes) that together comprised almost one-third of the community, four-fold more than in water or biofilms. Most ASVs from *Chitinophagaceae* were associated with the genus *Sediminibacterium*, which is also the most dominant member of microbial communities associated with *Lubomirskia baicalensis* and *Baikalospongia* spp. in Lake Baikal (136). Both genera of Baikal sponges are genetically closely related to *E. muelleri* (255), suggesting that this microbial association may have a shared physiological cause. Representatives from *Chitinophagaceae* are capable of chitin degradation; chitin has been identified as a structural component of the skeleton of *S. lacustris* (256) as well as several other marine and freshwater sponges (reviewed in (257)), and while evidence of chitin in adult *Ephydatia* spp. has not yet been reported (258), samples of *E. fluviatilis* contain a high abundance of chitinases (259). A separate study in Lake Baikal found *Chitinophagaceae* at higher abundances on diseased or dying sponges (246), though the overall abundances were considerably lower than what we report here. The association between *Chitinophagaceae* and freshwater sponges may therefore reflect opportunistic microbial chitin degradation of the sponge skeleton. How this putative microbial chitinase activity affects the physiology of the sponge remains to be explored.

Comamonadaceae (Phylum: Proteobacteria) comprised another one-third of the sponge microbiome. This family, represented by its most abundant genus *Comamonas,* is a group of

betaproteobacterial steroid degraders that have not previously been reported above 3% abundance in freshwater sponges (246). In our study their abundance was only marginally higher in sponges than in water samples, and therefore their high abundance relative to other studies may simply reflect the ambient environment. However, we suspect the association between *Comamonadaceae* and *E. muelleri* encompasses some form of ecological interaction: many unique lipids and steroids have been isolated from freshwater sponges (260) and *Comamonadaceae* were not equally abundant in biofilm samples. Marine sponges are also common hosts of proteobacterial steroid-degraders (261). As with *Chitinophagaceae*, it is unclear if or how the presence of steroid-degrading bacteria affects the sponge, but we hypothesize that the consistent dominance of these two taxa in the *E. muelleri* microbiome represents some selective ecological associations between sponges and these microbes that is stable across varying environments.

In addition to the two dominant bacterial families, several other low-abundance taxa distinguished sponges from water and biofilms. While the ecological function of many of these associations is difficult to predict, the enrichment of these taxa in sponge samples relative to ambient water and biofilms, along with the comparative paucity of several taxa that are highly abundant in water samples, further support our hypothesis that freshwater sponges provide a distinct and selective environment for microbial growth. We note that sponge-enriched taxa encompassed a variety of energetic pathways including autotrophs (such as the purple non-sulfur bacteria *Rhodospirillaceae*), methylotrophs (such as *Methylophilaceae*), and heterotrophs (such as the aerobic heterotroph *Chryseolinea* and the saprophytic *Leptospirillaceae*), and could therefore perform a variety ecological roles both beneficial and detrimental to the sponge. Based on ecological studies of marine sponges, these roles could include a mixture of host-microbe symbioses, transient food sources, microbial degradation of sponge tissue, and potential pathogens (3, 4). We also note that freshwater sponges harbored several groups of Proteobacteria (including *Moraxcellaceae*) and Actinobacteria (especially *Actinomycetales*) that have been the source of bioactives and antimicrobials isolated from marine sponges (47, 262–264), and freshwater sponges may therefore represent a novel source of such compounds. As in marine sponges, these biotechnologically valuable compounds could also help shape the sponge microbiome and confer ecological benefits to the sponge in the form of protection against predators or disease (263).

One additional finding in our work is that among sponges there are site-specific signatures in the microbiome that are not matched by differences in the ambient water, suggesting that these taxa are being differentially recruited to the sponges. This geographic variation is secondary to the observed variation between sample types: random forest models were consistently able to distinguish sponges from water and biofilms based on the same set of taxa, but some of those same taxa also distinguished sponges between streams. Research in marine sponges has consistently shown that within a sponge species, the microbiome is stable across geographic distance (4, 34), but even within conserved microbiomes there can be location-specific signatures driven by variation in light and other environmental factors (266). Even the small differences in physico-chemical properties between our three sampling sites could contribute to the observed site-specific microbial differences, as could variation in light regimes, nutrient inputs, sponge body condition, and other factors we did not measure. This preliminary observation nonetheless offers additional support for ecological factors driving specific sponge-microbe associations and warrants further investigation.

A common question in sponge microbiome research is how these sponge-associated bacteria are acquired by their host. It is possible that some bacteria are housed inside or on the surface of the freshwater sponge gemmule and are thereby vertically transmitted from parent to progeny (267); this mechanism of transfer has been demonstrated for algal symbionts in *S. lacustris* (268). While our data cannot prove or disprove vertical transmission, we instead suspect the sponge-associated microbes in our study are re-acquired from the ambient water every generation. Microbes in the ambient water are considered a seed bank for sponge microbiomes (269), and every bacterial family found in our sponges was also found in the surrounding water save for three families with mean relative abundances less than 0.005%. Rare, sponge-specific taxa in marine sponges were originally attributed to vertical transmission, but recent research has found that with deeper sequencing purportedly "sponge-specific" taxa also appear in bulk water samples (270). Given that our rarefaction curves did not reach saturation, we believe the absence of these three low-abundance taxa in our water samples is similarly due to the limitations of sequencing depth. Sponges can acquire their microbes from the water via microbial chemotaxis towards sponge-derived compounds (271) or passive water flow through the sponge followed by selective enrichment and competitive exclusion (4, 38).

Marine sponge microbiomes continue to be explored for their ecological interest and

biotechnological potential, and here we have shown that the freshwater sponge *E. muelleri* also harbors a unique microbiome that may be worthy of further ecological and biotechnological exploration. Our results demonstrate that the internal and external surfaces of the sponge preferentially accumulate specific microbial taxa but we do not presume the nature of this partnership. For that reason, we have intentionally chosen not to call these microbes symbionts and instead consider them only as sponge-associated microbial communities. Further investigation of the functional activity of these bacteria and their physiological location within the sponge will help elucidate their ecological roles: for example, symbionts reside in the sponge mesohyl (46) whereas archaeocytes and choanocyte chambers are more likely to contain food sources (137). Similarly, isolation of specific microbes or targeted searches for antimicrobial compounds will provide insight on the biotechnological value of these communities. Although freshwater sponges are considerably less numerous and diverse than their marine counterparts, our evidence that freshwater sponges have their own assemblages of associated bacteria raises exciting questions. We suggest freshwater sponge microbiomes represent an important, yet underexplored, component in a comprehensive understanding of sponge ecology and evolution.

5.6 MATERIALS AND METHODS

5.6.1 Sample collection

We collected sponge, water, and biofilm samples from the Sooke, Cowichan, and Nanaimo Rivers in southeastern Vancouver Island, British Columbia, Canada over three days in July 2018 (**Fig. 5.1**). All three rivers are short (<50km) first- through third-order freshwater streams situated within temperate old-growth forests dominated by Douglas fir (*Pseudotsuga menziesii*), Sitka spruce (*Picea sitchensis*), and other conifers. The Cowichan River sits within the Cowichan Valley, where the major industry is agriculture, whereas the Sooke and Nanaimo Rivers begin in unpopulated forestry land and travel through primarily rural areas marked by occasional agriculture, urbanization, and industrial activity. All three rivers are open to recreational use by swimmers and anglers. At our sampling locations, the streams have rocky banks and cobble beds, interspersed with bedrock and boulders that provide substrate for sponges and other freshwater flora.

In each river, we collected two tissue samples approximately 1cm in diameter from five individual sponges ranging from 0-2m in depth. When possible, we attempted to collect samples

working from downstream to upstream to minimize our disturbance to the sponges. One tissue sample designated for microbiome analysis was rinsed gently with distilled water to remove any attached debris and then flash-frozen in a dry ice and isopropanol slurry before being stored at -20°C. We fixed the second tissue sample in 100% ethanol for species identification and stored this sample at 4°C.

To compare the sponge microbiome to its ambient environment, we also collected paired water and biofilm samples for each individual sponge. We used sterile spatulas to scrape biofilm samples from the substrate adjacent to each sponge and then flash-froze the samples as before. We collected a 500ml water sample next to each sponge using Whirl-Pak® bags (Nasco, Canada). Water samples were stored in a cool and dark place for 2-4 hours during transport to a laboratory, where they were vacuum filtered through 0.2µm cellulose acetate (CA) filters (MilliporeSigma, USA). For each stream, we filtered 500ml of distilled water as a negative control prior to filtering the samples. Filters were flash-frozen as before.

At each sampling site, we measured water temperature and pH and collected water samples for chemistry analysis. From a 1 L sample, two 12ml sub-samples were filtered through 0.45µm CA filters and flash-frozen for ammonium, nitrate, and soluble reactive phosphorous (SRP) analysis; one 50ml sub-sample was filtered through a 0.45µm CA filter and stored refrigerated in the dark for dissolved organic and inorganic carbon measurements (DOC, DIC); and 500ml was stored refrigerated and unfiltered to measure total nitrogen (TN) and total dissolved solids (TDS). Water samples were submitted to the Biogeochemical Analytical Services Laboratory (Univ. of Alberta, Edmonton, Canada) within 72 hours for analysis. All samples were analyzed using a Lachat QuickChem QC8500 FIA Automated Ion Analyzer except for the DIC and DOC samples, which were evaluated using a Shimadzu TOC-5000A Total Organic Carbon Analyzer.

5.6.2 Species identification

We confirmed the species identification of each sponge sample using its spicule complement following the procedure described by (272). In brief, ethanol-fixed tissue samples were rinsed with distilled water and then desilicified for 24hr in 70% nitric acid. We rinsed the leftover spicules thoroughly with distilled water, resuspended them in 95% ethanol, and then

visualized wet mounts of the spicules using a light microscope at 200X magnification. Megasclere morphology was compared against (272) and (273) for final identification.

5.6.3 DNA extraction and sequencing

We extracted whole community DNA from the sponge tissue, membrane filters, and biofilm samples using the MP Bio FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) following the manufacturer's instructions, with an added 5-minute incubation at 50°C prior to the final elution to maximize DNA yield. For the extractions, we used 100mg (wet weight) of sponge tissue and aseptically cut the membrane filters into small pieces. All biofilm samples weighed less than 100mg so we extracted from the entire available sample. To achieve a sufficient quantity of biofilm DNA for sequencing, we pooled and concentrated samples from the same river, resulting in final biofilm sample numbers of 4, 3, and 1 for the Sooke, Nanaimo, and Cowichan Rivers, respectively. All DNA samples were submitted to Microbiome Insights (Vancouver, BC) for sequencing, where the V4 region of the bacterial 16S rRNA gene was PCR-amplified using the barcoded primers 515F (5'-TATGGTAATTGTGTGCCAGCMGCCGCGG-TAA-3') and 806R (5'-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3'). Amplicons were diluted to equimolar concentrations and sequenced on an Illumina MiSeq platform using V3 chemistry and paired-end 250bp reads.

5.6.4 16S rRNA gene sequence analysis

16S rRNA sequence data was processed using the package *DADA2* version 1.6.0(152) as implemented in R 3.5.0. We truncated forward and reverse reads at 240bp and 160bp, respectively, and removed low-quality reads using the DADA2 default filtering parameters. Paired-end reads were merged and exact amplicon sequence variants (ASVs) were determined using DADA2's pooled inference procedure. To assign taxonomy, we aligned ASVs against taxa in the RDP reference database (Release 11.5) (154) using the naïve Bayesian classifier method implemented in DADA2 (155). ASVs identifying as chloroplasts or mitochondria were removed. We generated a phylogenetic tree for our data using the package *phangorn* (156) following the procedures described by (157). For our final analyses, we included only ASVs with a length of 250-256bp, and averaged ASV abundances across 1,000 rarefactions to the minimum library size

of 8,000 reads. Our final feature table was imported into the R package *phyloseq* (158) for subsequent analyses.

5.6.5 Statistical analysis

All statistical analyses were performed in R 3.5.0 both 1) across our three sample types, independent of stream and 2) across our three sample types separately for our three different streams. Chao1 predicted species richness and Shannon diversity were calculated using *phyloseq* and significant differences between sample types and streams were evaluated using an ANOVA with Tukey's honest significant difference (HSD) *post hoc* test. We visualized beta-diversity with non-metric multidimensional scaling (NMDS) using the Bray-Curtis dissimilarity index and evaluated significant clustering associations using PERMANOVA with 1,000 permutations, implemented by the 'adonis' function in the package *vegan* (161). We also tested for homogeneity of dispersion within sample groups using a permutational analysis of multivariate dispersion ('permutest' function applied to a 'betadisper' object) in *vegan*. Pairwise comparisons were corrected using the Bonferroni correction.

We identified taxa that were significantly differentially abundant between comparison groups using the package *ALDEx2* (274). We evaluated our data at multiple taxonomic levels using a log-ratio transform of our rarefied feature table centered on the geometric mean and then tested for differences using a one-way ANOVA adjusted with the Benjamini-Hochberg correction. Because *ALDEx2* does not directly perform *post hoc* comparisons, we manually performed pairwise Welch's t-tests in *ALDEx2* using subsets of the transformed feature tables. P-values were adjusted as before, and differences with an adjusted p-value less than 0.05 were considered significant. All taxon relative abundances are reported in percentages as means ± standard deviation.

We used random forest modeling (R package *randomForest* (275)) to identify the most important ASVs distinguishing sponge, water, and biofilm samples and determine how accurately each community could be classified *de novo*. To reduce noise, we removed taxa with an average relative abundance less than 0.01% prior to running the models. Classification was performed by bootstrapping samples across 1,000 trees with sample type as the response variable and ASV relative abundances as predictors. We used the same parameters in two additional models to 1) predict sample location for only the sponge samples and 2) predict both sample type

and location for all samples. Predictors were ranked by their mean decrease in Gini coefficient and model accuracy was determined as out-of-bag error rates.

5.6.6 Data availability

The raw 16S rRNA gene amplicon sequences obtained in this study were deposited as a single project in the NCBI Sequence Read Archive under the accession number PRJNA526747.

5.7 ACKNOWLEDGEMENTS

5.8 TABLES & FIGURES

Table 5.1: Physico-chemical properties of the water at each sampling site.

River	Temp (°C)	pH	NO$_2$+NO$_3$ (µg/L)	NH$_4$ (µg/L)	TN (µg/L)	SRP (µg/L)	DOC (mg/L)	DIC (mg/L)	TDS (mg/L)
Sooke	20	7.0	25	< 3	106	< 1	1.4	3.6	32
Nanaimo	21.5	6.9	25	< 3	74	2	3.6	4.3	23
Cowichan	18	7.1	20	< 3	41	2	1.2	5.2	29

TN: total nitrogen; SRP: soluble reactive phosphorous; DOC: dissolved inorganic carbon; DIC: dissolved inorganic carbon; TDS: total dissolved solids.

Table 5.2: Bacterial families differentially abundant between sample types.

Mean relative abundances are shown (in percentages) for bacterial families that were significantly differentially abundant (p<0.05) among sponge, water, and biofilm samples. Significance was determined using a one-way ANOVA adjusted with the Benjamini-Hochberg correction ("ANOVA p-value") followed by a similarly adjusted *post hoc* pairwise Welch's t-test ("pairwise")[a]. For simplicity, only taxa with a mean relative abundance greater than 0.1% in sponges are shown.

Family	Mean relative abundance			ANOVA p-value	pairwise
	sponge	water	biofilm		
Actinobacteria					
Microbacteriaceae	1.40	**3.92**	0.69	< 0.001	*
Bacteroidetes					
Chitinophagaceae	**33.61**	7.45	5.27	< 0.001	s
Cytophagaceae	6.79	**20.87**	2.16	< 0.001	w
Flavobacteriaceae	1.74	**5.48**	1.23	< 0.001	w
Chryseolinea	1.58	0.07	**2.06**	< 0.001	w
Cryomorphaceae	1.03	**3.80**	0.05	< 0.001	*
Cyanobacteria					
GpVI	0.12	0.02	**1.11**	0.006	b
Proteobacteria					
Comamonadaceae	**29.33**	24.16	6.00	< 0.001	b
Methylophilaceae	**5.16**	0.86	1.54	< 0.001	*
Rhodospirillaceae	**4.64**	0.14	1.05	< 0.001	*
Burkholderiaceae	3.39	**8.21**	0.29	< 0.001	b
Sphingomonadaceae	0.61	0.89	**7.32**	< 0.001	b
Hyphomicrobiaceae	0.39	0.12	**4.81**	0.009	b
Xanthomonadaceae	0.33	0.33	**1.70**	0.022	b
Burkholderiales_incertae_sedis	0.23	0.80	**1.04**	0.040	b
Rhizomicrobium	0.22	0.14	**1.51**	0.030	b
Polyangiaceae	0.21	0.11	**2.37**	0.022	b
Oxalobacteraceae	0.13	0.54	**0.65**	0.008	s
Spirochaetes					
Leptospiraceae	**0.57**	0.00	0.07	0.008	s
Verrucomicrobia					
Verrucomicrobiaceae	0.16	0.56	**2.27**	0.014	b

[a] Pairwise: b, s, and w indicate that biofilms, sponges, and water, respectively, are significantly different in pairwise comparisons against the other two sample types. An asterisk indicates that all three sample types are significantly different from each other.
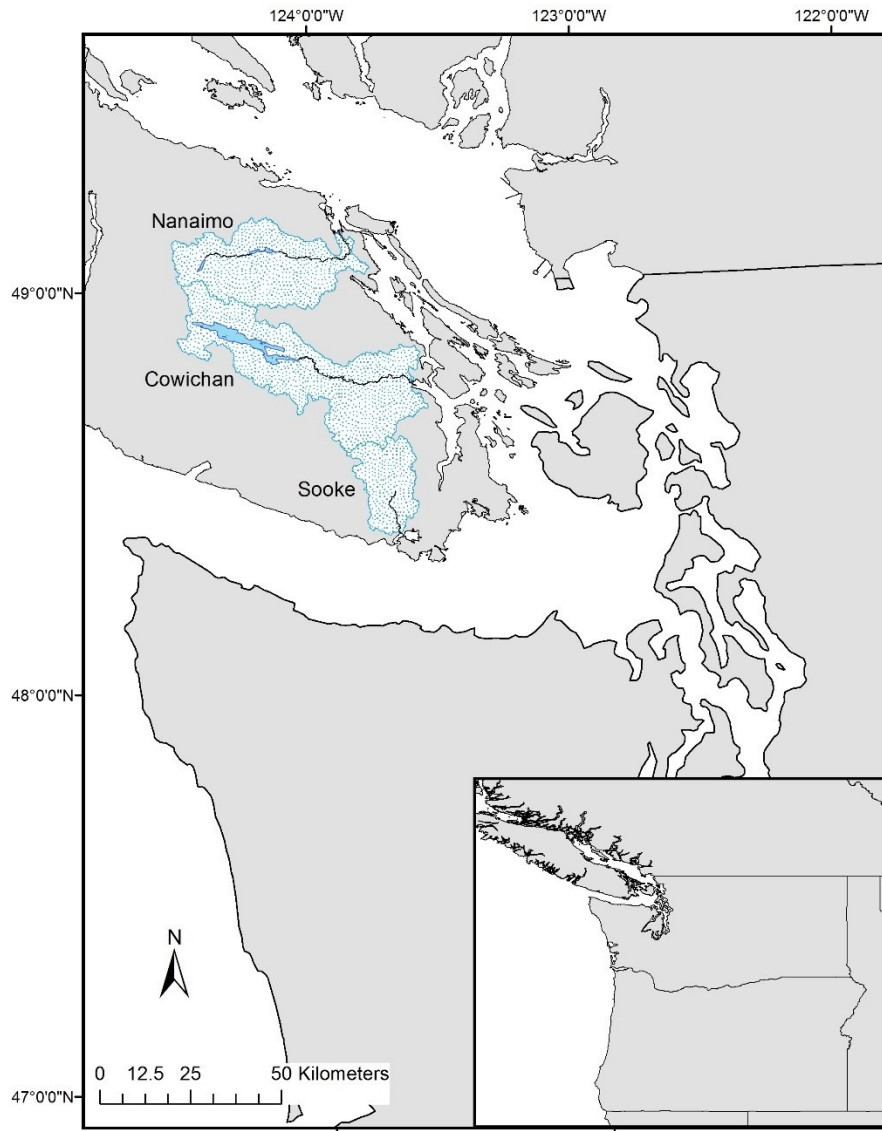
Fig. 5.1: Sampling locations of the freshwater sponge *Ephydatia muelleri*.

Map depicts southern Vancouver Island (British Columbia, Canada), with the inset showing Vancouver Island in relation to the North American Pacific coastline. Samples were collected in July 2018 from suitable habitat in the Sooke, Nanaimo, and Cowichan Rivers. Watersheds are shaded blue.
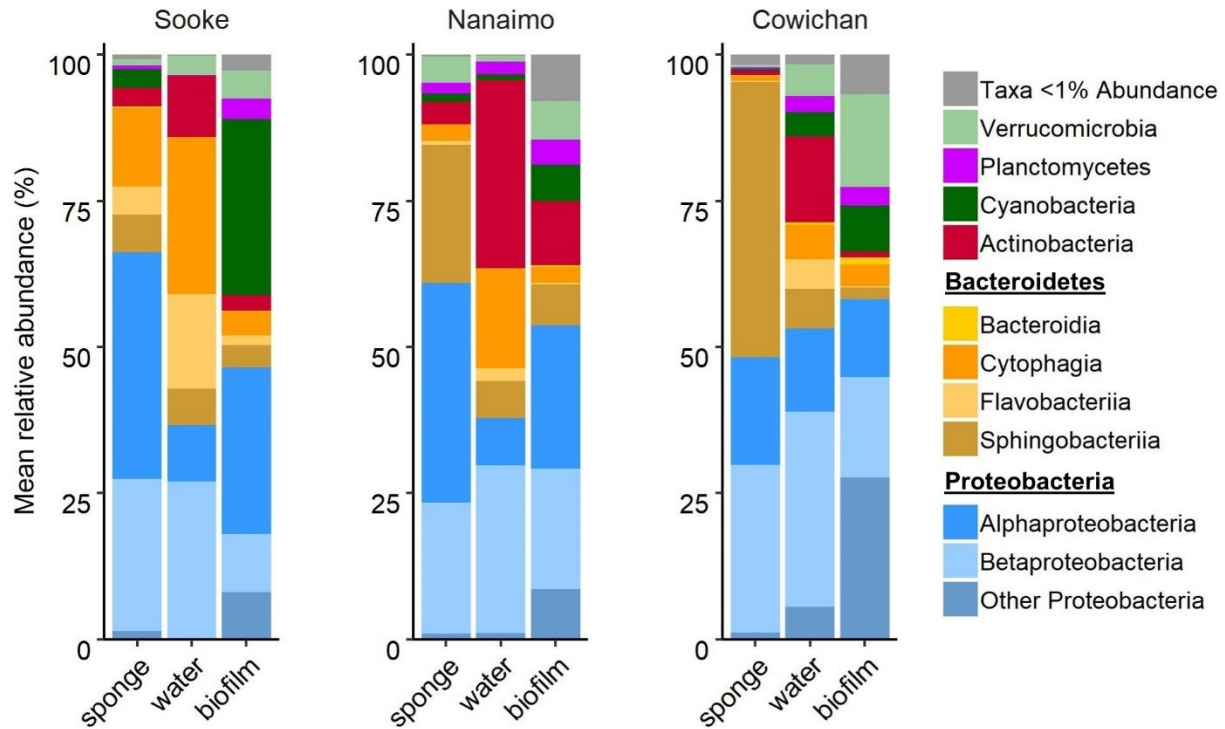
Fig. 5.2: Microbial composition of sponge, water, and biofilm samples.

Average relative abundances of different bacterial phyla comprising each sample type at each of our three sampling locations. The two most abundant phyla are further subdivided into classes: classes within Bacteroidetes are shown in shades of orange and classes within Proteobacteria are shown in shades of blue.
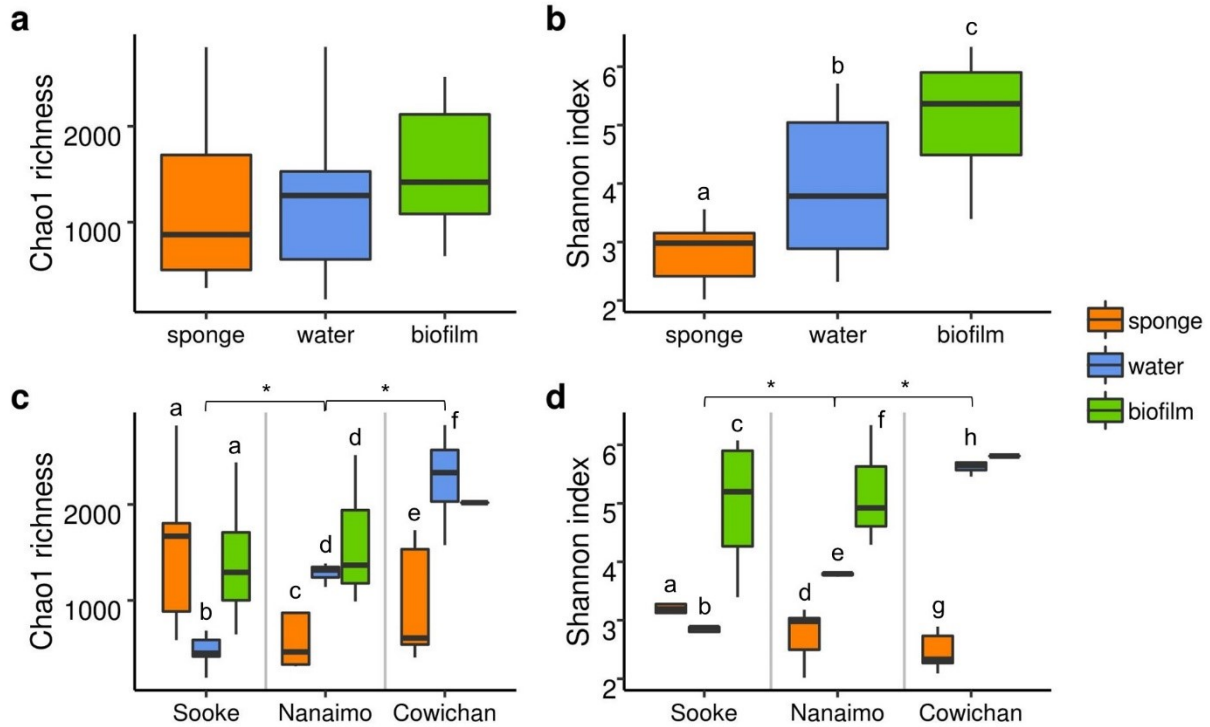
Fig. 5.3: Alpha diversity metrics across sponges, water, and biofilms.

Chao1 predicted richness and Shannon diversity were calculated for each sample type (**a, b**) independently of sampling location and (**c, d**) separately for each sampling location. Letters represent significant pairwise contrasts (p < 0.05) between sample types (sponge, water, biofilm), and, in **c** and **d**, asterisks represent significant pairwise contrasts (p < 0.05) between locations for the same sample type. Pairwise comparisons were performed using Tukey's HSD *post hoc* test. Bars and boxes display medians and interquartile ranges, respectively.
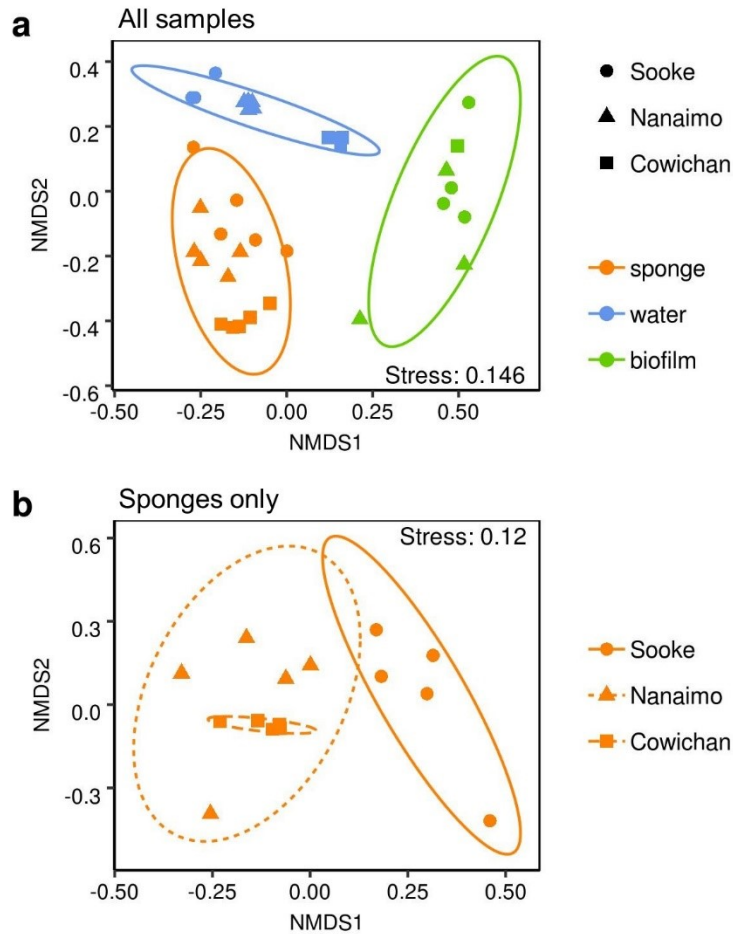
Fig. 5.4: Beta-diversity among sample types and sample locations.

Non-metric multidimensional scaling (NMDS) representations based on the Bray-Curtis distance metric calculated on rarefied data for (**a**) all samples, with ellipses distinguishing samples by sample type; and (**b**) only sponge samples, with ellipses distinguishing sponges by sampling location. Ellipses represent 95% confidence intervals.
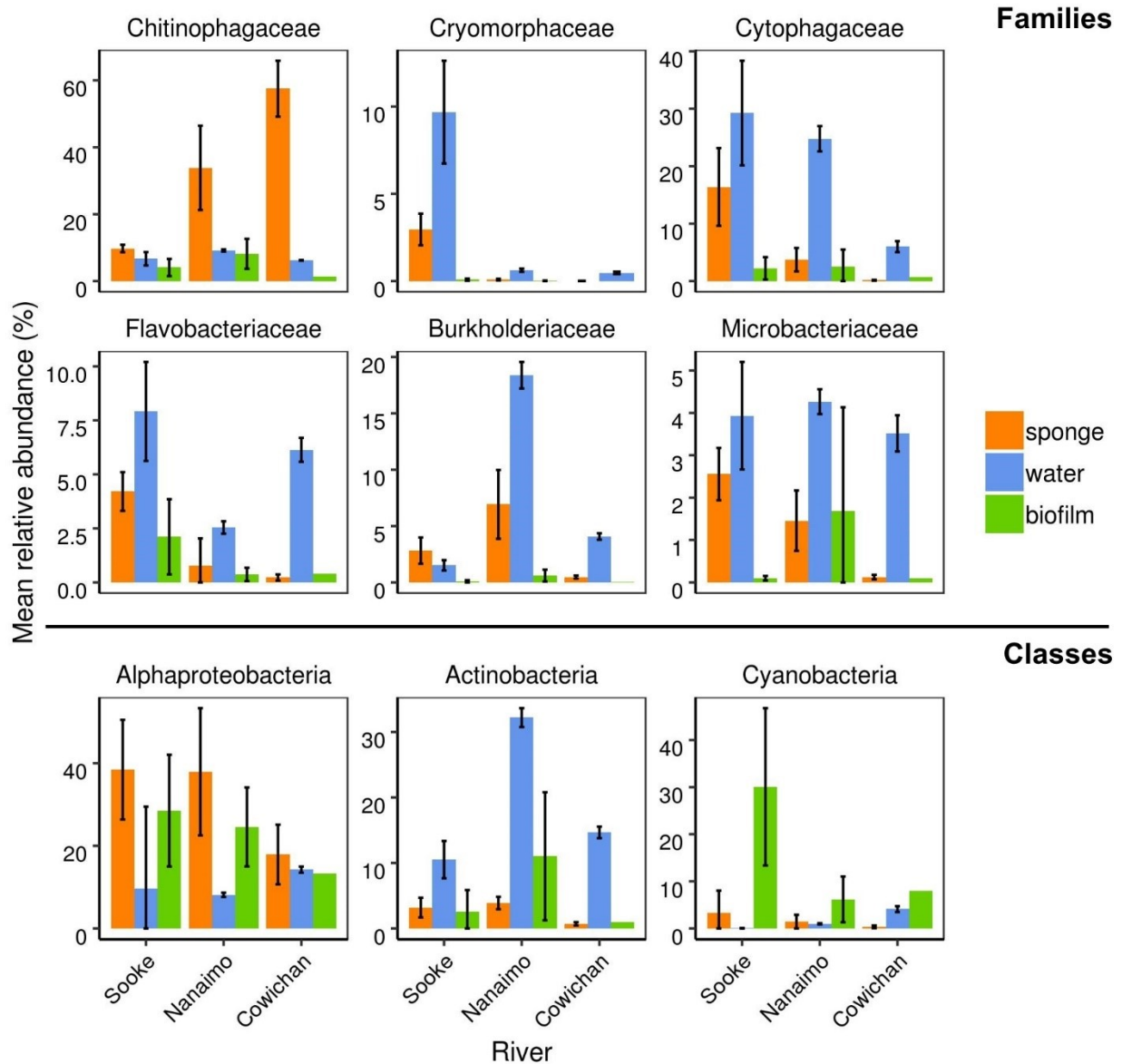
Fig. 5.5: Location-specific variation in sponge samples is not mirrored by water samples. (***Top***) Six bacterial families were significantly differentially abundant among sponge samples taken from different rivers. This variation among rivers was not always matched by equivalent variation in water or biofilms. (***Bottom***) Variation between rivers is shown at the class level for the three most variable higher taxa.

CHAPTER 6
General discussion

6.1 INTRODUCTION

Many of my colleagues questioned how I planned on uniting coyotes and freshwater sponges as part of the same thesis, and almost jokingly asked if I intended to compare the microbiome between the two. The niches these two animals occupy could hardly be more different, and a direct comparison of their microbiomes might only be valuable were it to also include representative organisms from every clade between them on the phylogenetic tree. That was certainly never my intention. Animals and their microbes co-evolved in spatially and temporally variable environments that cannot be captured in traditional laboratory studies, and so using coyotes and sponges as models, I aimed to broadly interrogate host-microbe associations across different or changing natural environments. Because limited information existed about the microbiome of either organism, part of this endeavor necessarily required a descriptive characterization of those microbiomes in the context of the several environmental factors, highlighted in my first chapter, that can structure them.

This process is, as the previous chapters show, a complicated one for free-living organisms. Individual variation in physiology and natural history precludes our ability to perform controlled studies where we manipulate explanatory variables one at a time and observe their effects on the microbiome. Similarly, there are more ways that individuals vary in physiology and life history than we can possibly measure. These hurdles, albeit tall, are not insurmountable, but doing so requires careful experimental design alongside a greater diversity of trans-disciplinary methods than are often found in single pieces of research. In this final chapter I briefly revisit the specific objectives and results of the previous chapters, address the strengths and limitations of each, and discuss their implications for our understanding of host-microbe associations in the face of environmental change. I focus first on each organism separately and conclude with a broader synthesis and some general reflections.

6.2 COYOTES

6.2.1 Synthesis and implications of major findings

The initial question that prompted the work presented in the coyote chapters was how urban habitat use affects the coyote gut microbiome and how those changes may relate to known differences in the diet and health of urban coyotes. This question was addressed directly in Chapter 4 and its answer appears to be multifaceted. In general, urban coyotes ate a greater breadth of carbohydrate-rich, protein-poor anthropogenic food, but different individuals did not necessarily rely on the same types of anthropogenic food: some scavenged leftover meat, while others consumed fruit or fast food. Others still survived on natural prey. Protein-poor diets, independent of habitat use, were most strongly linked to more Bacilli and fewer Fusobacteria, and poorer body condition, in my correlative analysis of diet, microbiome, and health. These same two taxa were respectively three-fold more abundant, and two-fold less abundant, in the fecal microbiomes of urban coyotes, and urban coyotes were less healthy in measures of fat assimilation and immune stress.

It is not altogether surprising that carbohydrate consumption by a carnivore would be linked to lower health via effects on the microbiome. Phylogenetically and physiologically, coyotes are most closely related to wolves (*Canis lupus*) and to other carnivores with a short intestine and small caecum designed for the efficient digestion of meat (142). Microbiomes are intricately tied to host gut physiology (62), and the coyote microbiome correspondingly clusters with other carnivores. Wild wolf diets consist of only 1% carbohydrate (276) and even domestic dogs, which carry additional copies of the starch-degrading amylase gene (277), are still arguably better at digesting meat than kibble and are healthier when fed meat-rich diets (165, 198, 278). As was mentioned in both Chapters 3 and 4, previous studies in coyotes and other organisms have defined "vicious circles" in which the consumption of low-quality food leads to poor body condition and increased susceptibility to disease and parasitism, which further impairs an animal's ability to obtain high-quality food (125, 226). This cycle is more pronounced in urban environments, where anthropogenic food is more readily available. One of the foremost broad-scale contributions of my analyses is a description of how the microbiome participates in this cycle alongside testable hypotheses for the specific mechanisms governing those relationships.

The most unique and unexpected observation in these otherwise straightforward broad connections was the increased heterogeneity in the duodenal microbiome of urban coyotes. The duodenal microbiome is more immediately responsive to specific components of the diet (143), and its increased heterogeneity might intuitively reflect greater dietary breadth. However, the non-mapping of either short- or long-term diet to microbiome divergence suggests that this response in the duodenal microbiome may be more nuanced than a generic statement of dietary breadth can convey. For this reason, I proposed the "prey-switching" hypothesis in Chapter 4, which accounts for weak correlations while still explaining the results observed in both the duodenal and fecal microbiomes. Subsidiary to this hypothesis are the reasons such behavior might impair nutrient assimilation, including a microbiome poorly matched to the current diet and inconsistent diets preventing natural host-microbe co-evolution. Testing this idea will require detailed long-term diet histories and possibly habitat use information from individual coyotes, similar to the types of data presented in the Chicago study that first mentioned this form of prey-switching (210).

Individual identity and lifestyle context also figured importantly in several other results, suggesting that individual behavioral choices may have far-reaching and sometimes unpredictable effects on the microbiome and host health. The strongest selective pressures in the small and large intestinal microbiomes were those conveyed by unmeasured aspects of individual lifestyle context, and the sole aggressive coyote in my sample defied previously established general trends predicting conflict. Other anecdotes for unique individuals abound: one peri-urban coyote was living with an amputated hind leg, which might be expected to foster more reliance on easy-to-obtain anthropogenic food, and yet this individual had more kidney fat than average and a stomach full of deer meat. The urban coyote that had eaten a leather glove, the least digestible food item I recorded, was not as far below average in health measures as its inferred desperation might predict and harbored more Fusobacteria than average. Although these and other individuals may face some of the same selective pressures in their environments, their responses, especially behaviorally, may differ, and the extent of environmental variation experienced by an organism can, in turn, be determined by its behavior (279). The same Chicago study that described prey-switching also noted that some coyotes exploited urban natural lands while others lived in developed areas, and the extent of anthropogenic resource dependency varied not only between the two habitats but also among individuals within each habitat (210).

The behavioral adaptations and lifestyle contexts unique to individual coyotes invariably produce individuals who defy general trends or, more broadly, greater heterogeneity among individuals in lieu of a general trend.

As much as behavioral plasticity may challenge attempts to define linear trends, it may also be part of the process, or the outcome, of urban adaptation. From a co-evolutionary perspective, the massive expansion of truly urban land, and the exploitation of that land by animals able to use it, is remarkably new. Cities have existed for millennia, but only in the past few centuries have they become the metropolises we now think of and only in the past few decades have their proportion of the landscape swelled to the point where they are now considered an ecological concern (100). The prevalence of *E. multilocularis* in North America is likewise a comparatively recent development (186). Evolution has never been an elegant process—the fossil record is replete with evolutionary "failures" and for every beneficial trait natural selection promotes there are countless others that are selected against. Compound this with the heterogeneity of selective pressures in the urban landscape, the diversity of ways in which coyotes respond to them, and the complex nature of host-microbe interactions, and it is easy to see why there may not yet be an easily-defined, universally applicable relationship connecting diet, microbiome composition, health, parasite infection, and potential for conflict in relation to a very new selective environment. My findings don't deny the potential for the microbiome to figure importantly in the ongoing process of urban adaptation, but they do suggest that it will not be a short path to determine what role it plays and how.

6.2.2 Subsidiary findings

Several subsidiary themes permeated my finer-resolution analyses, with promising implications for future research but currently uncertain mechanisms. One such theme was the abundance (or lack thereof) of the class Fusobacteria, which was positively associated with health and protein assimilation, less abundant in the feces of urban coyotes, and conspicuously absent from the sole aggressive coyote in my sample. As the previous chapters mentioned, the beneficial role of Fusobacteria in the microbiome appears to be unique to canines (165), and lower abundances of Fusobacteria have been linked to aggression in dogs (201). Although small populations of Fusobacteria are regularly identified in gut microbiome studies, the physiology and metabolism of these Gram-negative, non-spore-forming, often non-motile anaerobes remain

largely uncharacterized aside from the role one species, *Fusobacterium nucleatum*, may play in human colorectal cancer (200). The majority of ASVs belonging to Fusobacteria in my study were assigned to that same genus, *Fusobacterium*, but the V4 region of the 16S rRNA gene is not long or variable enough to make reliable species-level identifications. My results suggest that Fusobacteria will be an important taxon to consider in future evaluations of urban coyotes, and moreover highlight the value in explicitly discerning the unique role of Fusobacteria in the canine microbiome.

Another important consideration is the balance between the short- and long-term effects of consuming anthropogenic food. Coyotes that had recently consumed anthropogenic food were less healthy, and long-term reliance on anthropogenic food, measured as δ^{13}C, was likewise negatively correlated with health. However, readily identifiable relationships connecting microbiome composition to this more anthropogenic diet, such as increased species richness, only emerged when the analysis was based on recent diet, even though longer-term δ^{15}N signatures could be associated with taxon abundances. As I described briefly in Chapter 3, I hypothesize that this discord arises because anthropogenic food represents a short-term perturbation to a naturally carnivorous microbiome. Between anthropogenic meals, the natural environment created by the coyote gastrointestinal system promotes the redevelopment of a carnivorous microbial ecosystem. Long-term consumption of protein matches a coyote's natural digestive capacity and correspondingly the effects of protein consumption are more consistent across time. Previous work has suggested that the microbiome returns to a "stable state" after short-term perturbations (25), and that more frequent and higher-magnitude perturbations may negatively affect microbiome resilience and host health (140). Teasing apart these nuances between short-and long-term diets will be important in understanding how the microbiome responds to diet to mediate health, as the frequency of prey-switching in urban coyotes could affect microbiome resilience in a manner not easily captured by cross-sectional analysis.

My results lastly hint at the role helminth-microbiome interactions may play in the susceptibility to and spread of *E. multilocularis*. Infected coyotes had higher species richness in their fecal microbiome, regardless of whether they had recently consumed anthropogenic food, suggesting that *E. multilocularis* may interact in some way with the native microbiota. The field of helminth-microbe interactions is still in its infancy, with many interactions appearing to be host-, parasite-, and even population-specific (209), and given the diversity of intestinal parasites

and the breadth of their hosts, it will likely be some time before any conclusive trends can be established. However, the prevalence of *E. multilocularis* in urban coyotes, the recent establishment of the more virulent European strain in North America (280), and the human health risk posed by this parasite (186), all underscore the potential value of better understanding the specific nature of parasite transmission and establishment in urban areas.

6.2.3 Limitations and future directions

The unfortunate aspect of opportunistic sampling is that I could not choose my samples, and consequently the conclusions I make come from a highly unbalanced selection of coyotes. Peri-urban coyotes comprise 85% of my sample and are themselves not an ideal comparison group because they came from suburban areas where they also had access to anthropogenic food. The sample is also unequally distributed across capture months, with most urban coyotes coming from the fall and spring and most peri-urban coyotes from the winter. Results from the health-based analyses presented in Chapter 3 do not significantly change with the exclusion of urban coyotes, and capture month did not explain any significant variation in microbiome composition or body condition, suggesting that many of my conclusions are robust to the unbalanced sample. For several analyses, including anthropogenic food consumption, diet breadth, and body condition, the distinctions that could be drawn between urban and peri-urban coyotes would likely only be further magnified by including a more rural sample group. Some results, however—particularly the evidence for increased duodenal microbiome heterogeneity in urban coyotes—are more strongly influenced by the unbalanced sample. To that end, an additional 17 road-killed or lethally managed urban coyotes have been collected from the City of Edmonton between September 2017 and April 2018, and one additional aggressive coyote has been obtained from the City of Calgary. I intend to process these 18 coyotes using the same methodology described in Chapters 3 and 4, bringing my final sample count to 28 urban coyotes and 65 peri-urban. While still unbalanced, this addition will nonetheless bolster the results discussed in Chapter 4.

An expansion of this data set to include more detailed quantitative information will also help tease apart the nuances connecting habitat use, diet, microbiome, and body condition. Two of my most compelling explanatory variables, the presence of anthropogenic food in stomachs and infection by *E. multilocularis*, were only evaluated as binary variables (presence/absence),

which necessarily masks a large degree of variation among individuals. I was fortunate to explore Kyra Ford's quantitative analysis of 28 stomachs, and from her sub-sample it appears that stomach contents may be able to link diet with microbiome heterogeneity, where coyotes consuming more divergent diets are also those with the most divergent intestinal microbiomes. Processing more stomachs, and perhaps increasing the level of detail with which contents are classified, could help elucidate the exact nature of this relationship. On the parasite front, Deanna Steckler is currently leading a project to quantify the *E. multilocularis* burden in each of these coyotes. Her data will hopefully illuminate more specific relationships among worm burden, microbiome species richness, and physiological health.

Supplementing an already large data set with additional samples, detailed stomach contents, and quantified parasite burdens will create an impressively comprehensive survey of coyotes in and around Edmonton. I have also entertained the idea of genetically assessing familial relatedness among these coyotes using microsatellite loci, which would add yet another layer to this data set but one that is known to have implications for the microbiome in other species (281). More common statistical approaches might still enable direct *a priori* hypothesis-testing in this expanded data set but will inevitably become unwieldy when exploring the nuanced connections among such a diverse suite of covariates. As the breadth and depth of this data continues to grow, more sophisticated analysis techniques, such as structural equation modeling (282), might become a better tool for teasing apart its multidimensional relationships.

No cross-sectional study can perfectly capture longitudinal variation in this large suite of variables, and new samples will be needed to examine the temporal nature of the relationships I discussed. Scats could be collected from both urban and peri-urban habitats over extended periods of time and analyzed for recent diet, microbiome composition, and parasite burden. With genotype data from microsatellite loci, these measures could be tracked over time in individual coyotes, with the expectation for more temporal variability in urban coyotes. These could additionally be paired with stable isotope signatures and measurements of body condition, which can feasibly be taken from live individuals captured in the same area at the end of the study period and genotypically matched to scat samples. Alternatively, these individuals could be radio-collared during the study period to track their movement and habitat use. Although this approach still suffers from its own limitations, including the inability to access the duodenal microbiome and the well-documented shortcomings of scat analysis (193), complementing my

cross-sectional data with a longitudinal experiment could paint a better picture of how within-individual temporal variation disrupts natural host-microbe co-evolution.

6.3 SPONGES

6.3.1 Review and synthesis of major findings

The results of the sponge project are already self-contained in Chapter 5 and need little additional synthesis here; the freshwater sponge *E. muelleri* in three Vancouver Island streams harbors microbiota distinct from the ambient water, with some universal similarities and some characteristics unique to each river. All three streams were limnologically similar by the parameters I measured, making it difficult to describe how environmental change might affect the sponge microbiome, but the observation of stream-specific differences not mirrored in the ambient water nonetheless suggests some role of other physiological or environmental factors that I did not measure. Despite not being able to characterize sponges from definably different nutrient environments, my results still provide a preliminary understanding of the freshwater sponge microbiome that could be used in comparisons against other, more eutrophic lakes or streams that are included in future studies. They also provide initial insights into the ecology and biotechnological potential of freshwater sponge microbiomes.

6.3.2 Limitations and future directions

This study was small in scope, with only five samples from each stream, and the limnological similarity among streams precluded a robust multi-environment comparison. Expanding this analysis to include more samples, especially from a more diverse selection of aquatic habitats, would better explore the among-stream variation I observed. An expanded analysis could additionally hint at the consequences of processes like eutrophication or sediment loading, which are ongoing on Vancouver Island in response to agriculture (128, 129), logging operations (130), and recent wildfires. Coupling a more comprehensive sampling scheme with more detailed geospatial analysis of land use could directly link sponge microbiome composition and water chemistry to overall watershed conditions.

Determining the functional role of these communities will also be important for predicting how the ecosystem services they and their hosts provide may be affected in different streams or environments. Microscopy, including fluorescence *in situ* hybridization (FISH) or

electron microscopy (SEM), could be used to determine the location of these communities within the sponge and thereby provide some clues as to their function. FISH would be especially useful for targeted investigation of the two most abundant bacterial families, *Chitinophagaceae* and *Comamonadaceae*. Metagenomic and metatranscriptomic analysis could additionally elucidate the functional roles of these microbial communities; I currently possess metagenomic data for three sponges collected from the Sooke River in September 2017 that are not represented in this thesis, and metagenomic data from three additional Sooke River sponges that were collected in the summer and used in this study is forthcoming.

6.4 SUMMARY & REFLECTION

Despite the millions of years of evolutionarily history that separate coyotes from freshwater sponges, two overarching and related themes about wildlife microbiome research emerge from the work presented in this thesis: 1) wildlife microbiomes do not respond in the ways we would necessarily predict or expect because 2) there are many additional facets of lifestyle context that affect microbiome composition and are challenging and methodologically intensive to simultaneously assess. In Chapter 4 I briefly referenced the 'Anna Karenina' hypothesis for animal microbiomes, primarily in relation to the increased heterogeneity observed in the duodenal microbiome of urban coyotes. Other aspects of my results only loosely reflected the explicit trend defined by that hypothesis, but the premise is a valuable one to consider in the context of environmental changes that are new in direction or magnitude compared to natural spatiotemporal variation in habitat or resource availability. Drawing from Leo Tolstoy's observation in *Anna Karenina* that "all happy families are alike; each unhappy family is unhappy in its own way," this hypothesis proposes that many different forms of dysbiosis can be induced by the same stressor (229). Functionally, this divergence is believed to arise because the stressor impairs the ability of the host or its microbiome to self-regulate community composition (229). I would argue the same result, and the same effects on host health, can also arise from diversified behavioral responses, and that those two probable causes are not mutually exclusive. Despite the aesthetic appeal of searching for linear trends distinguishing microbiome composition between two different environmental or physiological conditions, these so-called "dispersion effects," and their consequences for health and behavior, should not be discounted.

Chapter 6

Because of the numerous factors that can influence microbiome composition, identifying the mechanisms behind how environmental variation affects the microbiome or promotes among-individual variation requires an exhaustive suite of data. One of the strengths of this thesis is its methodological breadth; while every chapter used 16S rRNA gene amplicon sequencing as its centerpiece for analyzing the microbiome, the supporting data was provided by several trans-disciplinary methodologies borrowing from wildlife ecology, animal physiology, parasitology, and limnology, among others. Even so, the preceding chapters and paragraphs point to the value of having more data that could only be acquired with additional or higher-resolution methods. The effects of having recently consumed anthropogenic food begs the question of what specific types or amounts of anthropogenic food, which would require detailed stomach analysis, and the general heterogeneity of urban coyotes begs the question of how they each exploited the urban landscape, which would require GIS collar data. Assessing how freshwater sponge microbes interact with the sponge would involve microscopy and even *in situ* filtration studies. The integration of wildlife microbiome research and conservation biology will require methodological partnerships that extend beyond just microbiologists and ecologists and speaks to both the ongoing challenges and exciting prospects for evaluating the microbiome of different animals in changing and uncontrolled environments.

Most of the work I performed as part of this thesis is in some way encapsulated in the preceding chapters, which represent more skills and knowledge than I would have expected to obtain in two years. However, there were few trivial discoveries that had no place in the preceding chapters but are still worthy of acknowledgement. Through trial and error, I found that it takes approximately 14 hours to thaw a skinned coyote and 26 for a furred one. In the course of learning how to skin coyotes, I discovered that coyote skins, on average, comprise 12% of a coyote's body weight. I also learned that our Nanodrop Spectrophotometer consistently overestimates DNA concentration by 10% relative to fluorometric quantitation using Qubit. When I attempted to take my sponge samples on an airplane as carry-on luggage, packaged in dry ice, I learned that passengers can only take 1kg of dry ice on a plane, and only if your Styrofoam cooler is further packaged inside a cardboard box. I also learned that airport security does not weigh your dry ice to ensure compliance. The more compelling scientific discoveries outlined in the preceding chapters would not have been possible without the support of countless trivial and often humorous observations like these.

Chapter 6

   With the increasing awareness that no animal is devoid of commensal microbes comes a new appreciation of the role these microbes may play as animals adapt to ongoing and future environmental changes. I have provided preliminary characterizations of the microbiome of two previously unstudied organisms in relation to many, though certainly not all, of the environmental and physiological factors that may shape them, and I compared those host-associated microbiomes across different environments. It is the nature of science for the results of one study to serve as the springboard for the next, and I have generated several testable hypotheses for the role these microbiomes may play, whether to the benefit or detriment of the host, in future environmental changes experienced by those organisms. Although in several places I cautioned that results from one organism cannot be extrapolated to another, many of the mechanisms I proposed can be tested for in other species. The challenge remains to find ways to define causative links in studies that currently rely on correlative relationships, and especially, as I suggested in Chapter 1, to identify direct links between microbiome composition and host fitness. As the cost of sequencing continues to decline and the quality continues to improve in a century where the consequences of global environmental change are quickly becoming an ecological crisis, the intersection of microbiome research and conservation biology has never held greater promise.

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APPENDIX 1

Future work and figures or analyses not presented in other chapters

Future work: Comparing stable isotopes among urban, peri-urban, and rural coyotes

I have exchanged stable isotope data with one of Colleen's former graduate students, Maureen Murray, who performed a similar stable isotope survey of urban coyotes but used a much more rural sample than my "peri-urban" coyotes for her comparison group (124). We would like to explore how assimilated diet, measured using stable isotope signatures, changes along a more continuous gradient from 'rural' to 'urban.' For my urban coyotes, I also have stable isotope data from bone samples, which provide longer-term dietary information than claw samples. I could use this data to explore temporal variation in anthropogenic resource use within the urban individuals in my sample. My preliminary analysis has suggested that some animals become more dependent on anthropogenic resources while some become less dependent, but I'm still thinking of ways to better explore this data.

Future work: A metagenomic comparison of Sooke river sponges in fall and spring

As part of a collaboration with Dr. Jojo Holert at the University of British Columbia, I have access to metagenomic data from three sponges sampled in September 2017 and three sponges sampled in July 2018, all from the Sooke River. Amplicon data from the three sponges collected in July is presented in Chapter 5. I intend to explore this metagenomic data to determine how the function of the sponge microbiome changes across seasons, with the expectation that the fall sponge microbiome is metabolically more dormant, mirroring its host. I will also focus on the abundance of specific metabolic pathways, including carbon fixation and ammonia oxidation, for which the sponge likely depends on microbial services. Specific gene searches will be performed using hidden Markov models in HMMer (233) and more general functional profiles using HUMAnN (283). I could additionally compare the results of taxonomic profiles obtained from 16S rRNA gene amplicons to those obtained from the metagenomes, which might make for an interesting observation alongside the seasonal comparison.

Appendix Table 1.1: Results from sequencing of a mock community.

I sequenced a synthetic mock community alongside my samples as part of both sequencing runs that were performed, to control for possible sequencing error. The synthetic community consisted of 5 species, listed at left. The V4 region of the 16S rRNA gene is not long or variable enough to make reliable species-level identification, so mock community results are shown as genus-level classifications. The five genera represented in the mock community comprised greater than 99.5% of all reads and there was general agreement between the two sequencing runs, suggesting the sequencing process was performed well and was free of contaminants.

		Relative abundances	
Original community	RDP Classification	Sequencing run	
Species	Genus	1	2
Staphylococcus epidermidis	Staphylococcus	59.30	63.47
Proteus vulgaris	Proteus	13.50	11.23
Escherichia coli	Escherichia/Shigella	13.53	12.85
Nitrosomonas europaea	Nitrosomonas	9.59	10.11
Sphingomonas wittichii	Sphingomonas	3.63	2.14
	Cumulative abundance	99.54	99.80
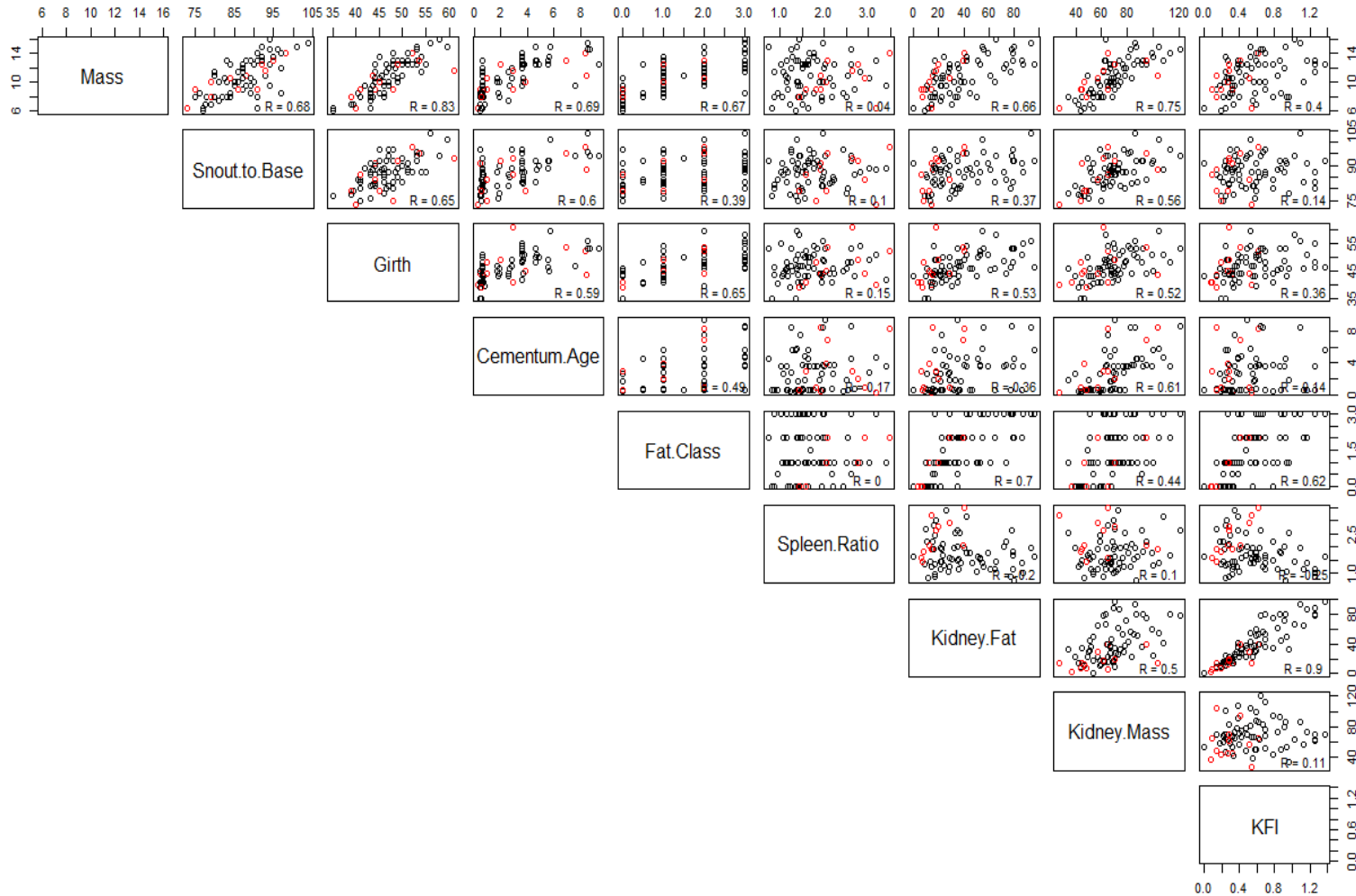
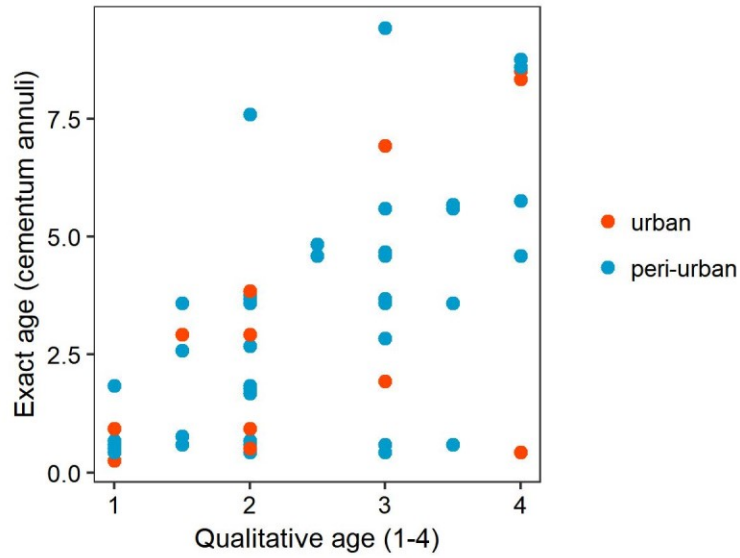**Appendix Fig. 1.1: Relationships among physiological measures in studied coyotes.**

Scatter plot matrix showing the correlations among the physiological metrics measured for 76 coyotes in our sample. Urban coyotes are shown in red. Pearson's correlation coefficient is given in the bottom right of each panel.

Appendix Fig. 1.2: Correlation between estimated age and actual age.

Each coyote was initially aged on a scale from 1 (young) to 4 (old) based on the color and wear of their teeth (214) (x-axis). Coyotes were then aged to the nearest month using cementum annuli (y-axis). In general, visual assessment tooth wear patterns overestimated age.
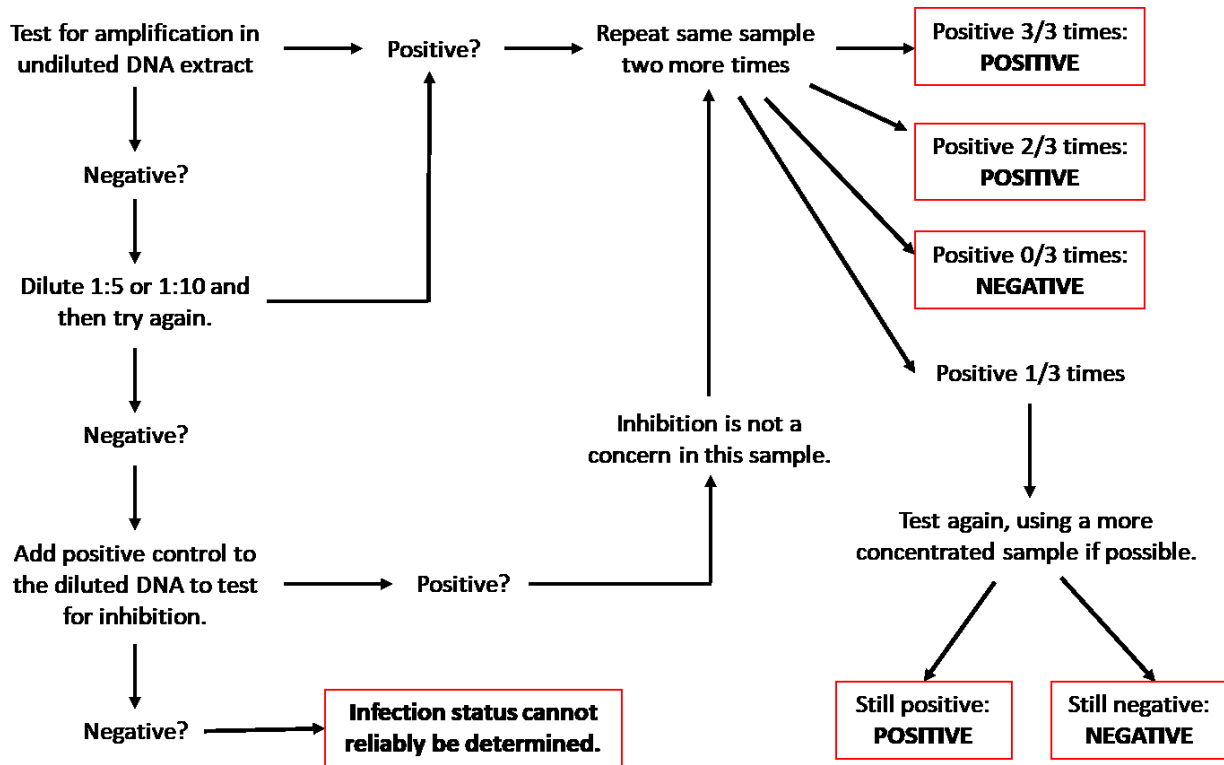
Appendix Fig. 1.3: Workflow for the detection of *E. multilocularis* using PCR.
Schematic showing the method used to determine whether each sample was either positive or negative for *E. multilocularis*. This workflow was performed on DNA extracted from both intestinal samples and feces. Coyotes were considered positive for *E. multilocularis* if one or both of their samples tested positive.

Supplementary Table 2.1: Tukey's honest significant differences (HSD) post hoc test comparing bacterial alpha diversity among sample sites.

| Comparison		ASV Richness			Shannon index		
		t	df	p	t	df	p
Duodenum -	Jejunum	0.36	60	1.000	1.07	60	0.934
	Ileum	2.16	60	0.330	1.68	60	0.632
	Caecum	4.02	60	**0.003**	3.75	60	**0.007**
	Asc. Colon	3.53	60	**0.014**	4.16	60	**0.002**
	Des. Colon	3.14	60	**0.040**	3.79	60	**0.006**
	Feces	3.60	60	**0.011**	3.69	60	**0.008**
Jejunum -	Ileum	2.60	60	0.146	2.83	60	0.087
	Caecum	4.51	60	**0.001**	4.96	60	**< 0.001**
	Asc. Colon	4.00	60	**0.003**	5.38	60	**< 0.001**
	Des. Colon	3.60	60	**0.011**	4.99	60	**< 0.001**
	Feces	4.04	60	**0.003**	4.82	60	**< 0.001**
Ileum -	Caecum	1.91	60	0.482	2.13	60	0.349
	Asc. Colon	1.40	60	0.799	2.55	60	0.159
	Des. Colon	1.00	60	0.952	2.17	60	0.329
	Feces	1.59	60	0.691	2.16	60	0.333
Caecum -	Asc. Colon	0.51	60	0.999	0.42	60	1.000
	Des. Colon	0.91	60	0.969	0.04	60	1.000
	Feces	0.21	60	1.000	0.15	60	1.000
Asc. Colon -	Des. Colon	0.40	60	1.000	0.39	60	1.000
	Feces	0.27	60	1.000	0.25	60	1.000
Des. Colon -	Feces	0.65	60	0.995	0.12	60	1.000

Supplementary Table 2.2: Tests for homogeneity of dispersion among individuals, between segments, and among sites.

Multivariate dispersion was calculated using the 'betadisper' function in *vegan* and significant differences among individuals, between segments, and among intestinal sites were evaluated using a permutational analysis of variance (**a**). Pairwise comparisons were further computed separately for the small and large intestine, first between intestinal sites (**b**) and again between individuals (**c**). In (**c**), pairwise comparisons for small intestinal sites are shown *below* the diagonal and pairwise comparisons for large intestinal sites are shown *above* the diagonal.

a Test for homogeneity of dispersion				**b** Pairwise comparisons by site			
Variable	df	F	p	Comparison			p
All samples				Duodenum	-	Jejunum	0.699
Individual	9	0.762	0.640			Ileum	0.126
Segment	1	0.165	0.699	Jejunum	-	Ileum	0.475
Site	6	0.449	0.855				
Small intestine				Caecum	-	Asc. Colon	0.821
Individual	8	0.674	0.692			Des. Colon	0.587
Site	2	0.745	0.489			Feces	0.769
Large intestine				Asc. Colon	-	Des. Colon	0.775
Individual	9	0.897	0.554			Feces	0.885
Site	3	0.091	0.964	Des. Colon	-	Feces	0.892

c Pairwise comparisons by individual

Individual

| Individual	34	37	38	39	40	42	46	47	63	65
34		0.287	**0.027**	0.772	0.360	0.391	**0.036**	0.502	0.223	0.212
37	0.672		0.923	0.481	0.538	0.628	0.802	0.385	0.645	0.813
38	0.979	0.576		0.098	0.150	0.210	0.454	**0.026**	0.322	0.579
39	0.533	0.701	0.486		0.680	0.698	0.169	0.956	0.460	0.396
40	0.894	0.173	0.913	0.254		0.975	0.349	0.564	0.708	0.532
42	0.265	0.108	0.221	0.651	**0.013**		0.437	0.574	0.756	0.609
46	0.822	0.554	0.787	0.524	0.380	**0.050**		0.055	0.682	0.972
47	0.547	0.707	0.481	0.924	0.204	0.474	0.467		0.349	0.274
63	-	-	-	-	-	-	-	-		0.794
65	0.578	0.764	0.505	0.817	0.102	0.181	0.383	0.902	0.767	
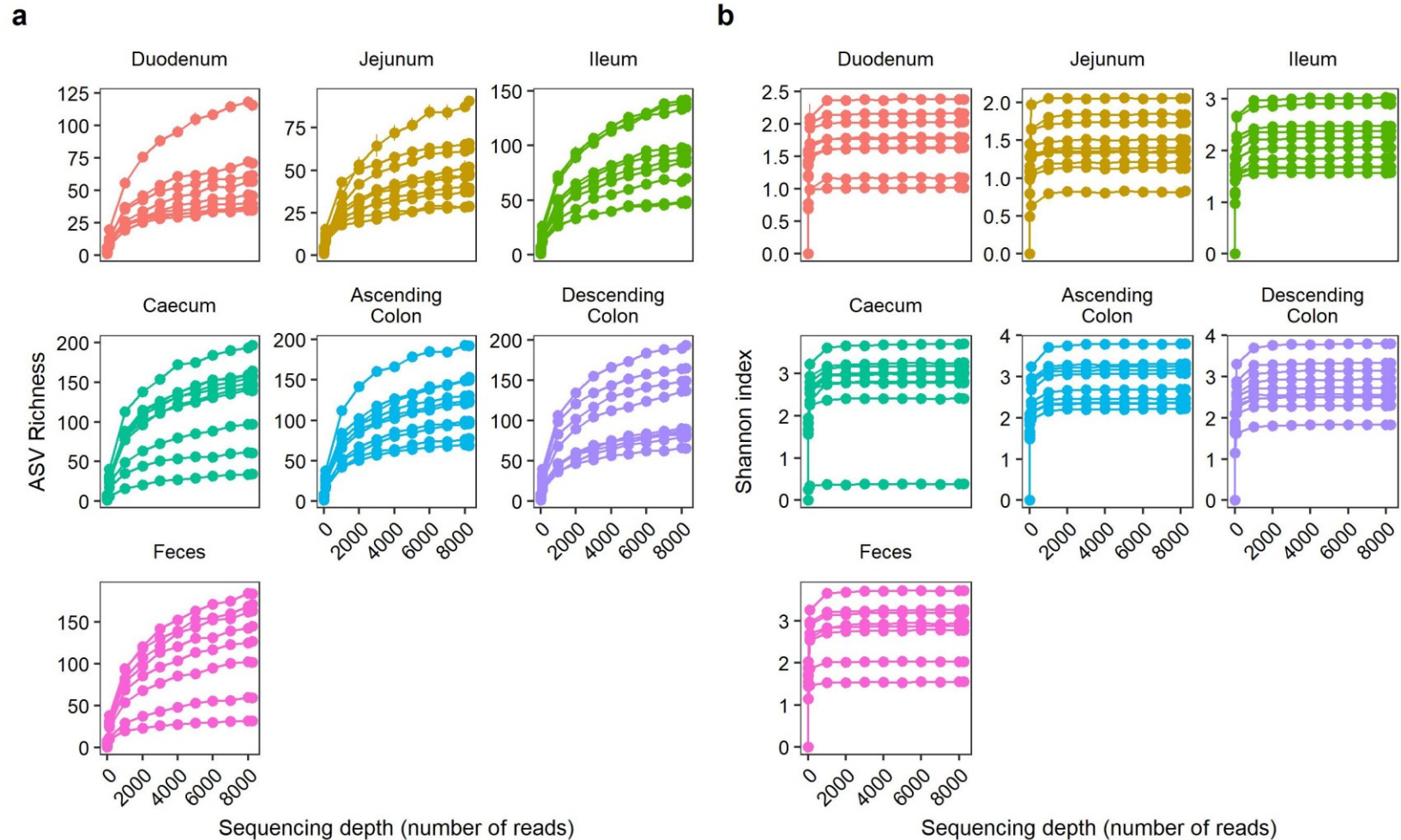
Supplementary Fig. 2.1: Rarefaction curves approach the saturation plateau.

Rarefaction curves for ASV richness (**a**) and the Shannon diversity index (**b**) are shown separately for each intestinal site. Curves approach the saturation plateau for ASV richness and demonstrate complete saturation for the Shannon diversity index, suggesting our sequencing depth was adequate for assessing microbial richness and diversity.
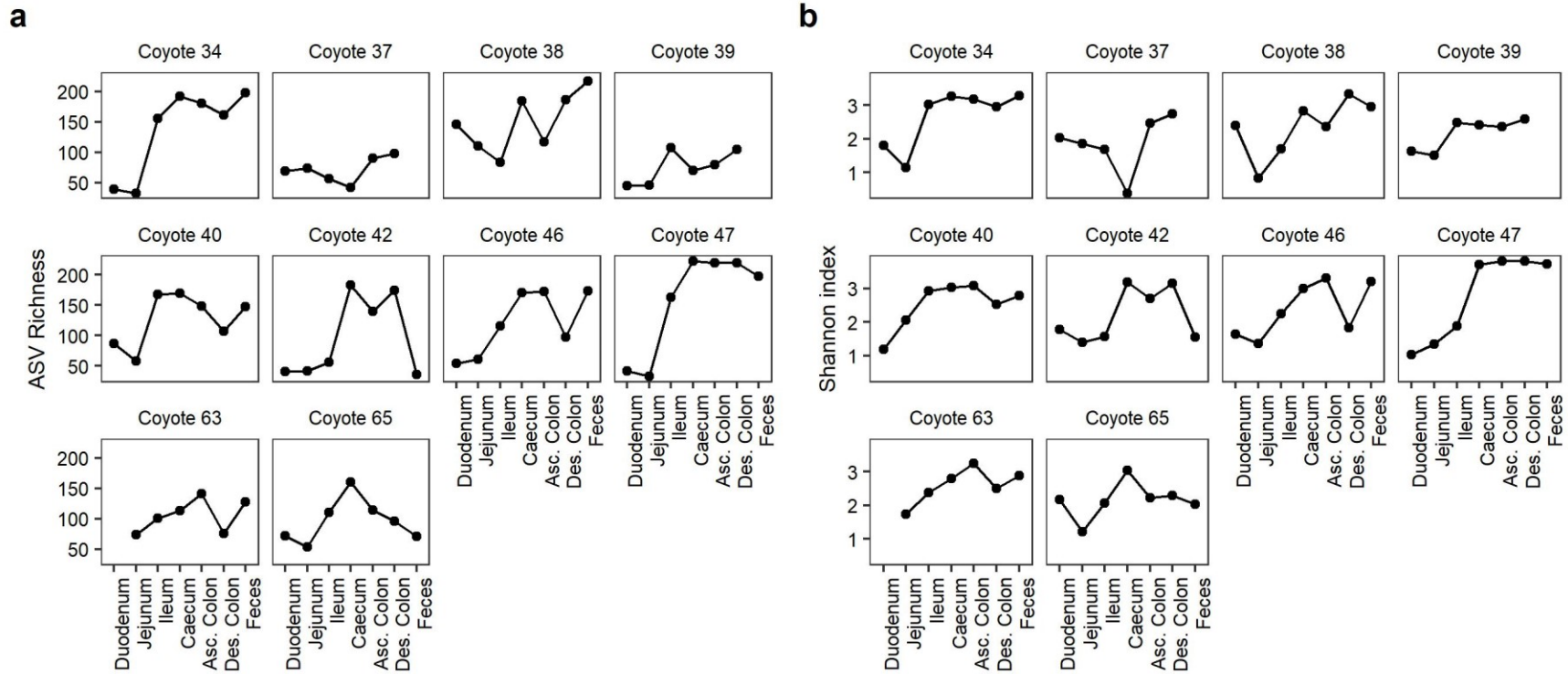
Supplementary Fig. 2.2: Species richness and diversity increase in the large intestine in all individuals.

ASV richness (**a**) and the Shannon diversity index (**b**) plotted along the length of the intestine individually for each coyote. Although there are notable inter-individual differences in diversity profiles, the colonic microbiome is consistently richer and diverse than the small intestine. Coyote numbers refer to their sample identity in our larger sample pool (see Methods).
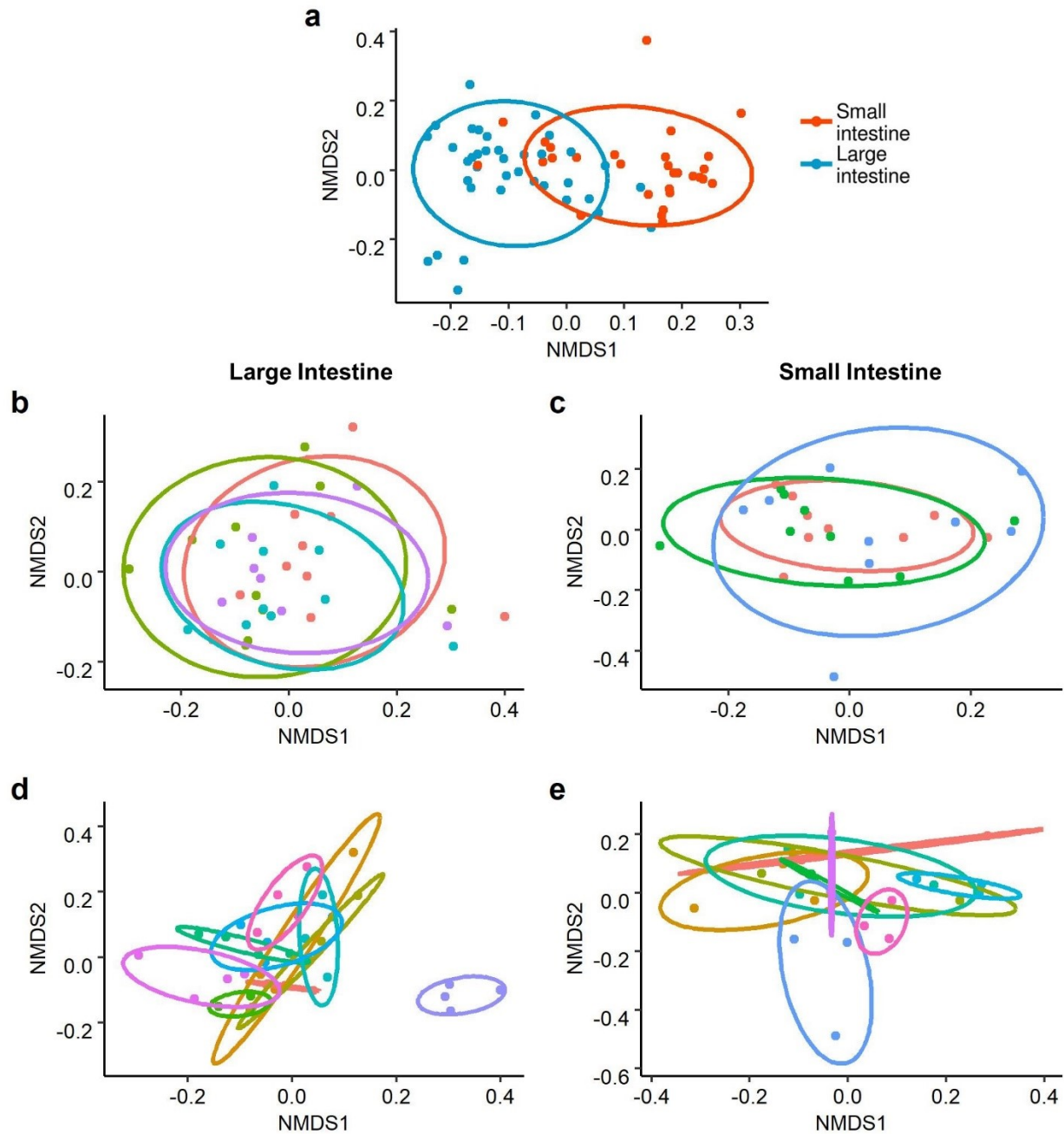
Supplementary Fig. 2.3: Individual identity explains more variation than intestinal site using a phylogenetically informed distance metric.

NMDS ordinations of intestinal samples using the weighted UniFrac distance metric, which additionally accounts for phylogenetic relatedness among taxa. The large and small intestine cluster independently of each other (**a**), but within the small and large intestines, no clear clustering associations distinguish intestinal sites (**b, c**). Instead, individual identity better explains inter-sample variance (**d, e**). Ellipses denote 95% confidence intervals.

APPENDIX 3

Supplemental material for Chapter 3

Supplementary Table 3.1: Top-ranked candidate models predicting Fusobacteria and Bacilli abundance.
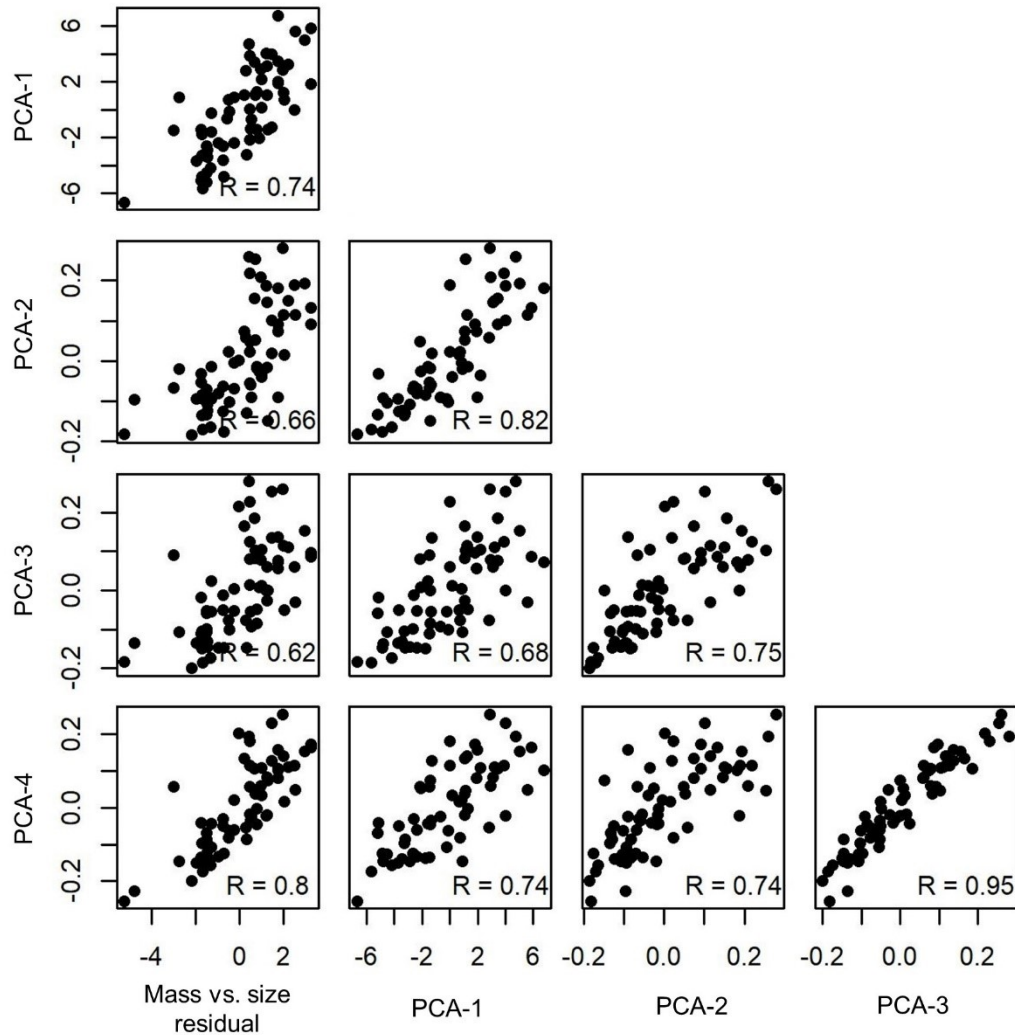
Dependent variables were modeled with a negative binomial distribution and predictors were mean-centered and scaled. Model-averaged coefficients, after adjustment by model weight, are shown at right.

Response variable	Model terms	df	Weight	dAICc	Model-averaged coefficients	
Fusobacteria	δ^{15}N	3	0.213	0	δ^{15}N	0.065
	Age + δ^{15}N	4	0.167	0.49	δ^{13}C	-0.002
	δ^{15}N + E. multi	4	0.091	1.70	E. multi	-0.003
	δ^{15}N + δ^{13}C	4	0.086	1.81	Age	-0.009
Bacilli	Age	3	0.140	0	Age	0.011
	(Intercept)	2	0.099	0.68	E. multi	0.001
	δ^{15}N	3	0.096	0.75	δ^{13}C	-0.001
	Age + δ^{15}N	4	0.077	1.19	δ^{15}N	-0.008
	Age + δ^{13}C	4	0.052	1.96		
	Age + E. multi	4	0.052	1.99		

Supplementary Table 3.2: Top-ranked candidate models predicting health and gut microbiome diversity.

Dependent variables were normally distributed and predictors were mean-centered and scaled. Model-averaged coefficients, after adjustment by model weight, are shown at right. All models predicting health included sex and age as additional covariates to control for the effects of these variables on our health metric.

Response variable	Model terms	df	Weight	ΔAICc	Model-averaged coefficients	
Health	Stomach + δ^{15}N	4	0.084	0	Age	0.305
	Stomach + δ^{15}N + Richness	5	0.070	1.03	δ^{15}N	0.199
	Stomach	3	0.045	1.51	Sex	0.096
	δ^{15}N + Richness	4	0.041	1.53	δ^{13}C	0.008
	Stomach + δ^{15}N + E. multi	5	0.039	1.85	Emulti	-0.014
	Stomach + δ^{13}C + δ^{15}N	5	0.032	1.92	Richness	-0.065
					Stomach	-0.206
Shannon diversity	Stomach	3	0.054	0	Stomach	0.133
	E. multi	3	0.038	0.71	E. multi	0.063
	(Intercept)	2	0.036	0.79	δ^{15}N	0.038
	Stomach + δ^{15}N	4	0.034	0.91	Age	0.026
	Stomach + E. multi	4	0.030	1.18	δ^{13}C	0.005
	Stomach + Age	4	0.030	1.20		
	δ^{15}N	3	0.024	1.64		
	E. multi + δ^{15}N	4	0.023	1.66		
	Stomach + E.multi + Age	5	0.023	1.73		
	Stomach + δ^{13}C	4	0.020	1.94		

Supplementary Fig. 3.1: Multiple composite health metrics are collinear.

We used principal components analysis to a composite index of health and tested the effects of including different variables in the calculation. Relationships among three representative indices are shown, as well as the residuals of a least-squares regression of body mass against body size. Pearson's correlation coefficient is given in the bottom of each panel. All metrics were highly collinear, suggesting downstream analyses were not dependent on the metric used.
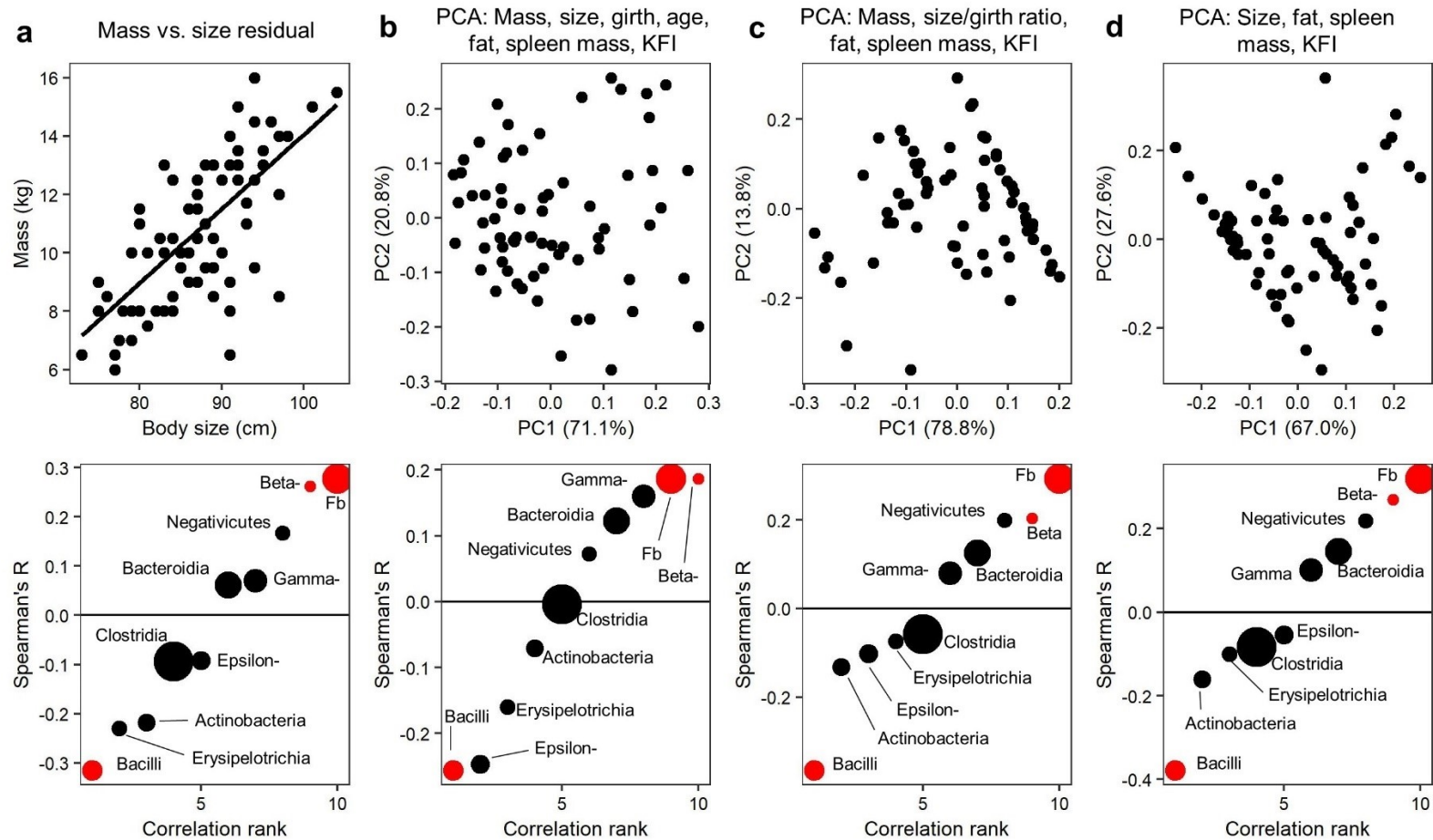
Supplementary Fig. 3.2: All indices of body condition achieve similar results.

Fusobacteria and Betaproteobacteria correlate positively with health, and Bacilli correlate negatively with health, independent of how condition is measured. Top panels show linear regressions or principal coordinate analyses used to calculate health metrics; bottom panels show the corresponding taxon correlations with those metrics. See Fig. 3.3 for a detailed explanation. Proteobacterial classes are denoted only by their Greek letter prefix and Fusobacteria are shown as Fb.
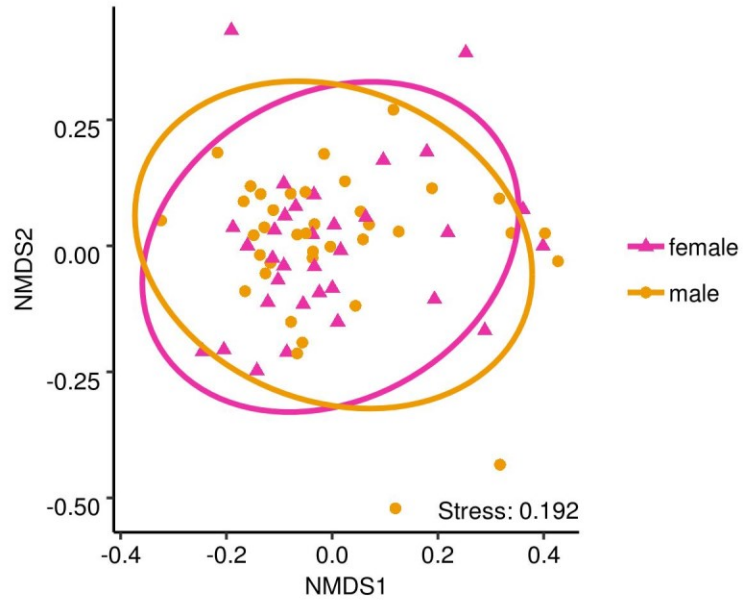
Supplementary Fig. 3.3: Sex does not explain community-level variation in microbiome composition.

NMDS ordination using the Bray-Curtis distance, with individuals colored by sex.
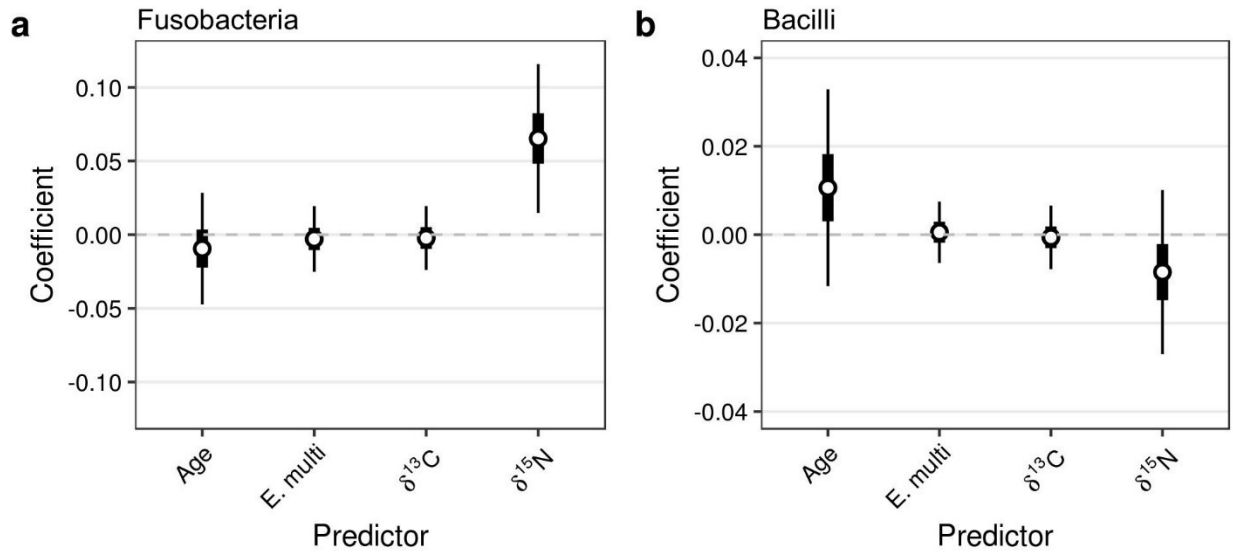
Supplementary Fig. 3.4: Abundances of Fusobacteria and Bacilli are most strongly predicted by coyote age and protein consumption.

We used generalized linear models with a negative binomial distribution to determine which covariates most strongly predicted the abundance of (**a**) Fusobacteria and (**b**) Bacilli. Predictors were mean-centered and scaled. Only coefficients present in the top-ranked models (ΔAICc < 2) are shown. Thick and thin bars indicate 50% and 95% confidence intervals, respectively.
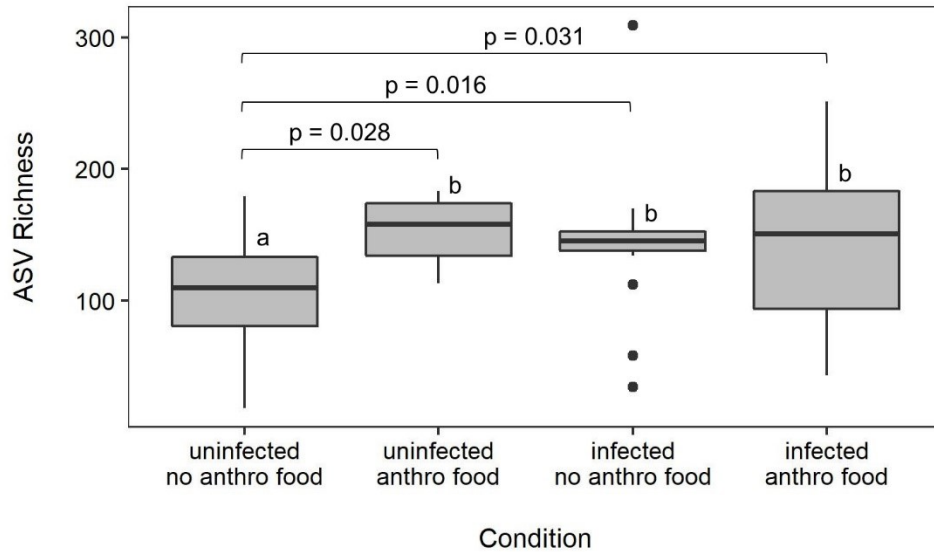
Supplementary Fig. 3.5: Pairwise comparisons for ASV richness across four possible conditions of infection status and stomach contents.

We partitioned our samples into four groups based on their *E. multilocularis* infection status ("infected" or "uninfected") and the presence of anthropogenic food in their stomach ("anthro food" or "no anthro food") and compared fecal microbiome species richness across all four groups with an ANOVA followed by Tukey's honestly significant differences *post hoc* test. P-values distinguishing significantly different groups ($p < 0.05$) are shown.

APPENDIX 4

Supplemental material for Chapter 4

Supplementary Table 4.1: Representative stomach contents from urban and peri-urban coyotes.

A representative list of the types of food items that could be found in coyote stomachs. Urban coyotes consumed some prey but also a wide variety of fruit and anthropogenic material. Peri-urban coyotes were more likely to consume large mammals and prey but still occasionally acquired anthropogenic food items.

urban	peri-urban
apple chunks	apple chunks
apple seeds	deer meat
blue crumbly plastic	unidentifiable animal fat
part of a yogurt tube	beaver
pine needles	duck
cherry pits	mouse
Tim Horton's wrapper	procupine quill
mouse	cherry pits
apple seeds	
pineapple	

Appendix 4

Supplementary Table 4.2: Alpha- and beta-diversity of urban and peri-urban coyotes compared to published data for carnivores, herbivores, and omnivores.
Published data for carnivores, omnivores, and herbivores taken from (55). PERMANOVA was performed using 1,000 permutations and p-values are shown both before and after Bonferroni correction.

		Alpha diversity	
Group	n	Richness	Shannon
urban	11	142	2.87
peri-urban	60	123	2.68
carnivore	7	202	3.67
omnivore	12	294	3.47
herbivore	20	446	4.82

Pairwise comparison			Tukey's HSD Richness p	Shannon p	PERMANOVA F	R^2	p	p (adj.)
urban	vs	carnivore	0.775	0.355	0.65	0.039	0.738	1.000
		omnivore	**0.009**	0.505	4.44	0.174	0.004	**0.040**
		herbivore	**< 0.001**	**< 0.001**	13.30	0.314	0.001	**0.010**
		peri-urban	0.985	0.966	2.59	0.036	0.022	0.220
peri-urban	vs	carnivore	0.362	0.051	2.52	0.037	0.030	0.300
		omnivore	**< 0.001**	**0.049**	14.55	0.172	0.001	**0.010**
		herbivore	**< 0.001**	**< 0.001**	33.12	0.298	0.001	**0.010**
carnivore	vs	omnivore	0.394	0.990	3.14	0.156	0.009	0.090
		herbivore	**< 0.001**	**0.032**	9.38	0.273	0.001	**0.010**
omnivore	vs	herbivore	**0.002**	**0.001**	4.36	0.127	0.002	**0.020**

Supplementary Table 4.3: Results of Welch's t-tests on physiological variables.

Measure	Mean		Welch's t-test		
	urban	peri-urban	t	df	p
Mass (kg)	10.35	10.85	0.70	16.7	0.492
Body size (cm)	86.08	87.31	0.49	13.5	0.629
Girth (cm)	46.23	46.87	0.34	15.2	0.737
Age (yr)	3.21	2.82	-0.41	13.8	0.690
KFI	0.298	0.573	4.38	31.1	**< 0.001**
Adj. spleen mass	2.31	1.70	-3.01	14.5	**0.009**

* KFI = kidney fat index; Adj. spleen mass = the absolute spleen mass (in grams) divided by total body mass (in kg).

Supplementary Fig. 4.1: Map depicting coyote capture locations in and near Edmonton, Alberta, Canada.

Eleven coyotes were collected from the Edmonton area ("urban") and the remaining 65 came from forested areas near Leduc and Beaumont, south of Edmonton. Sample areas are boxed in red. Image is taken from Google Earth © 2018.
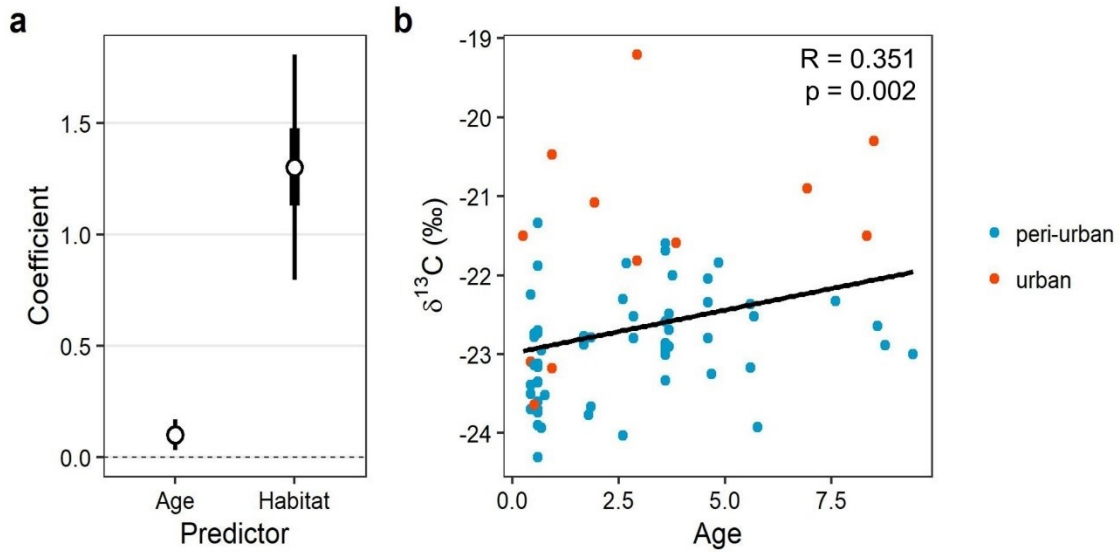
Supplementary Fig. 4.2: δ¹³C increases with both age and urban habitat use.

The absolute value of $\delta^{13}C$ signatures were used as the dependent variable in a gamma-distributed generalized linear model with an inverse link and habitat use, age, and other physiological measures (mass, size) as predictors. (**a**) The top-ranked model, based on AICc score, included both urban habitat use and increasing age as predictors of higher $\delta^{13}C$. (**b**) Scatter plot showing Spearman's correlation for the relationship between age and $\delta^{13}C$.
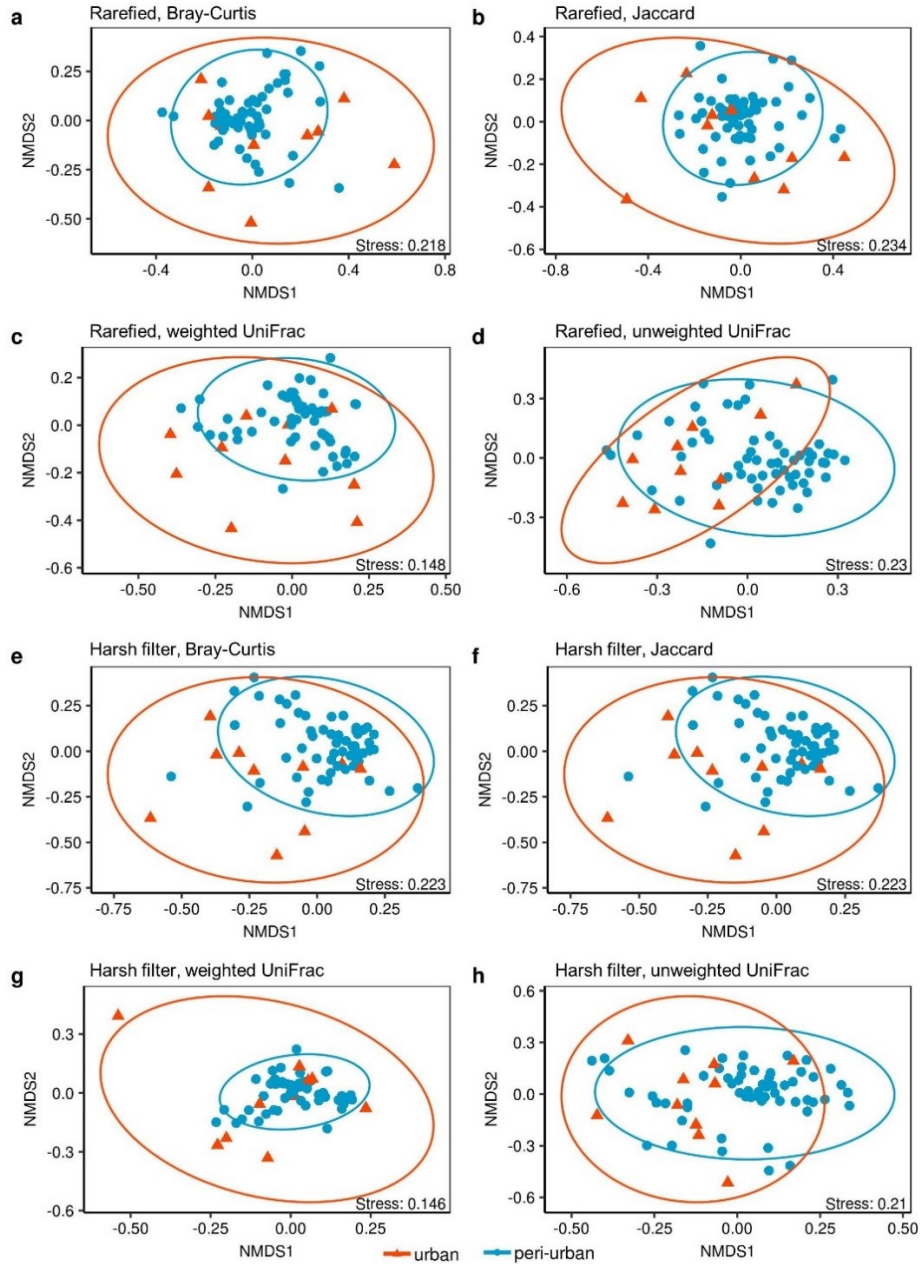
Supplementary Fig. 4.3: Intestinal dispersion effect is robust to multiple indices.
The increased multivariate dispersion observed in urban samples using rarefied data and the
Bray-Curtis distance (**a**) was also observed using the Jaccard index (**b**) and weighted UniFrac
distance (**c**). No effect was observed using unweighted UniFrac (**d**); however, this metric
considers phylogenetic similarity without abundance information, and the range of phyla present
in the gut is obligately narrow. The same urban dispersion effect was present across all four
dissimilarity indices even when only ASVs with more than 3 reads in at least 5% of samples
were retained, eliminating the effect of rare taxa (**e-h**).
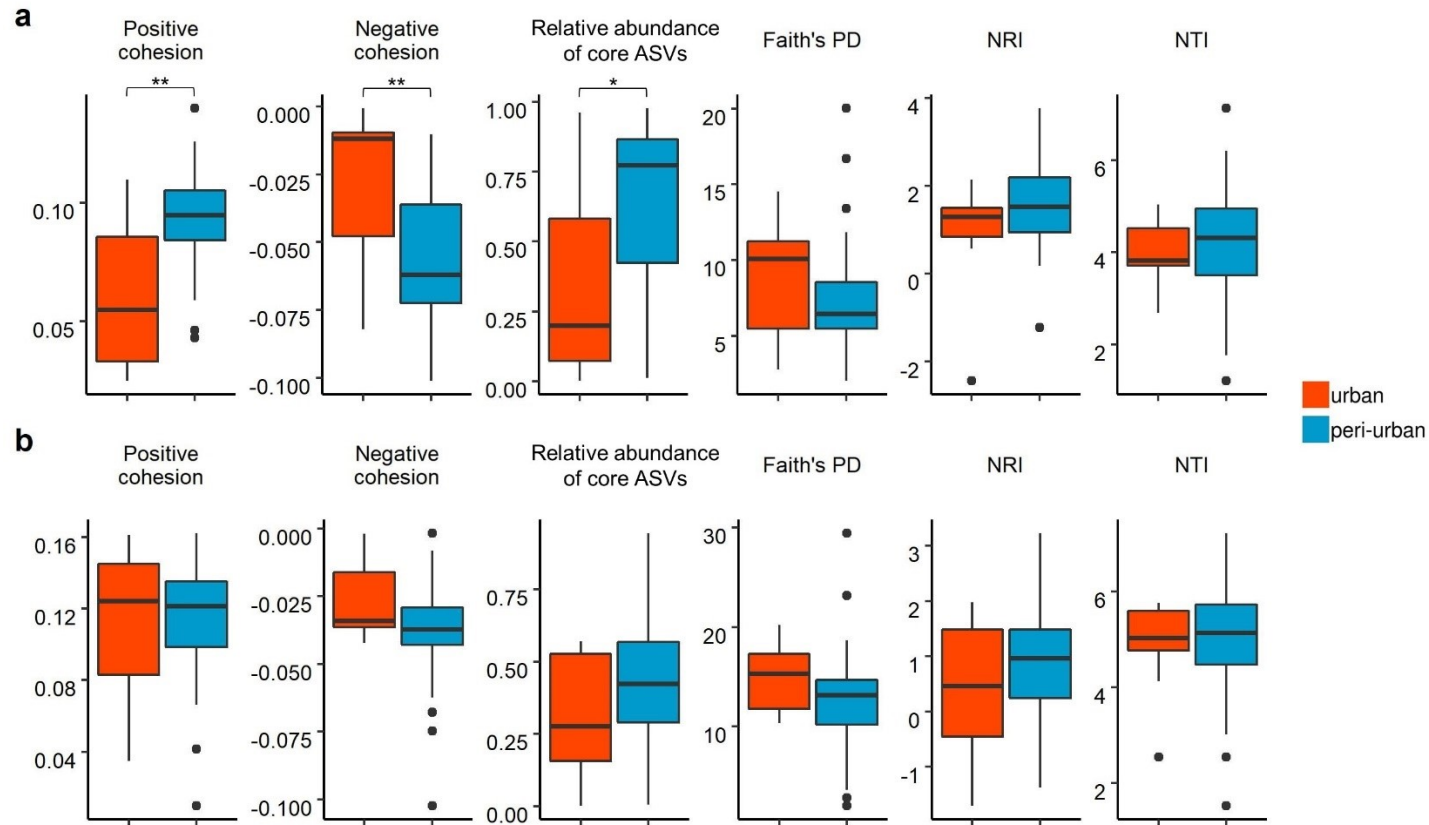
Supplementary Fig. 4.4: Additional measures of diversity evaluated for duodenal and fecal samples.

For each sample we calculated cohesion (219), which has both positive and negative components, to quantify interconnectedness among taxa. We also separately determined a 'core' microbiome for duodenal and fecal samples, defined as ASVs present with at least 0.2% relative abundance in 50% of samples, and calculated the total relative abundance of this core in each sample. We lastly calculated three measures of phylogenetic diversity: Faith's phylogenetic diversity (PD), net relatedness index (NRI), and nearest taxon index (NTI), which assess phylogenetic breadth in a community(284). Results are shown for both duodenal samples (**a**) and fecal samples (**b**). Asterisks indicate significant differences evaluated using the Kruskal-Wallis test (* $p < 0.05$, ** $p < 0.01$).
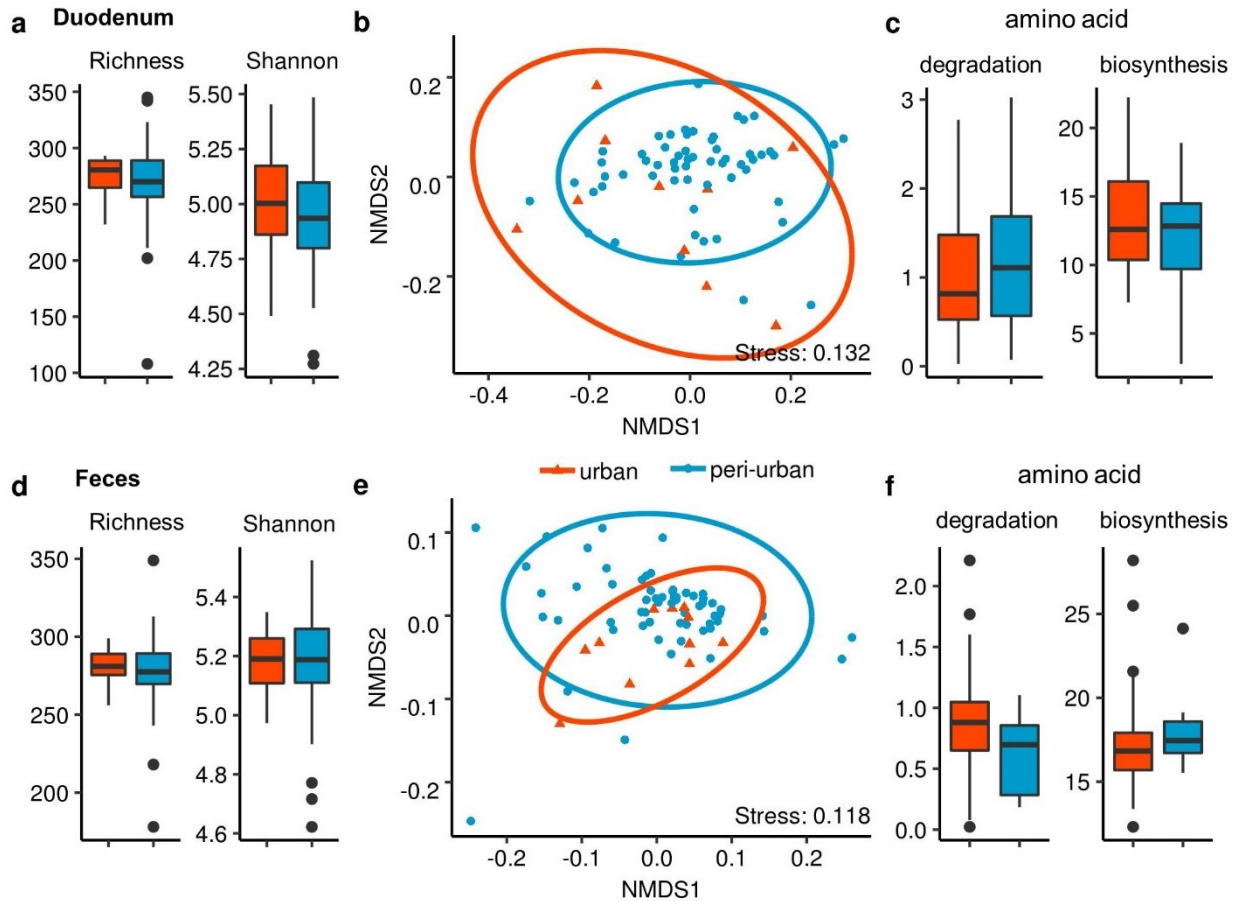
Supplementary Fig. 4.5: Functional alpha-diversity, beta-diversity, and abundance of amino acid degradation and biosynthetic enzymes predicted using PICRUSt.
Metabolic information for each coyote sample was predicted from 16S amplicon profiles using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt). Duodenal samples showed no change in functional alpha (**a**) or beta (**b**) diversity between urban and peri-urban individuals, nor were there any differences in the abundance of enzymes involved in amino acid metabolism (**c**), which are often cited in comparisons of carnivores and herbivores. Fecal samples (**d-f**) similarly showed no significant changes in metabolic profile.
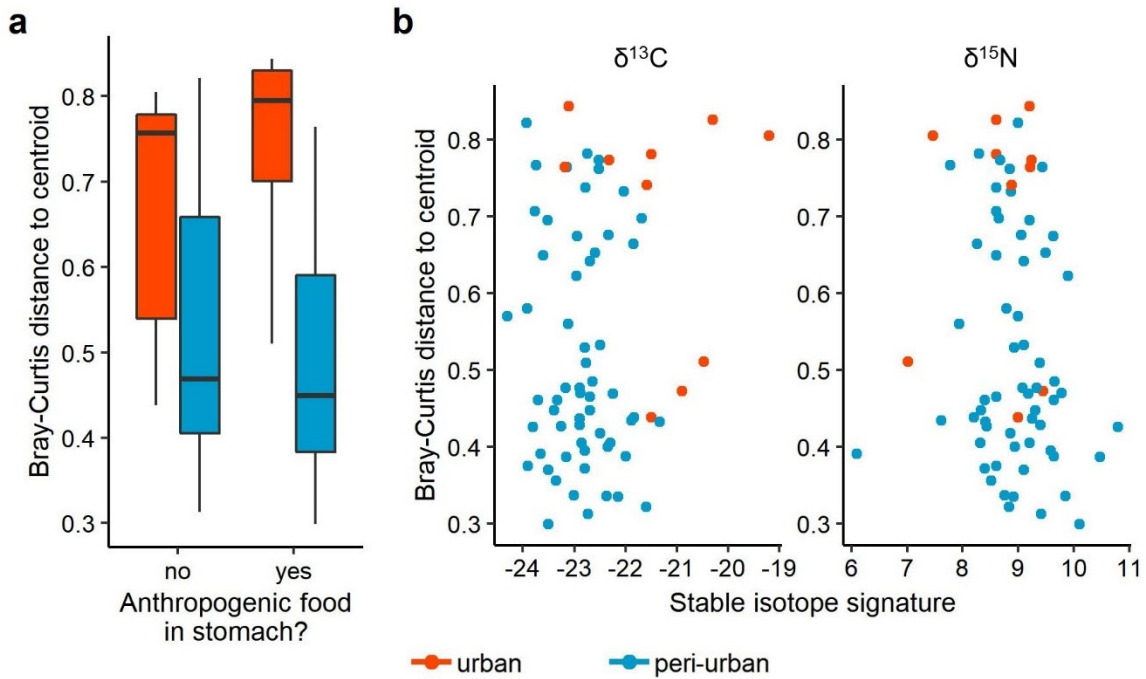
Supplementary Fig. 4.6: Duodenal microbiome divergence does not correlate with recent or assimilated diet.

The distance from each duodenal microbiome sample to the centroid in an NMDS ordination (**Fig. 4.2c**) is not associated with the presence of anthropogenic food in the stomach (**a**), shown separately for urban and peri-urban coyotes. There is similarly no association with either stable isotope signature (**b**).
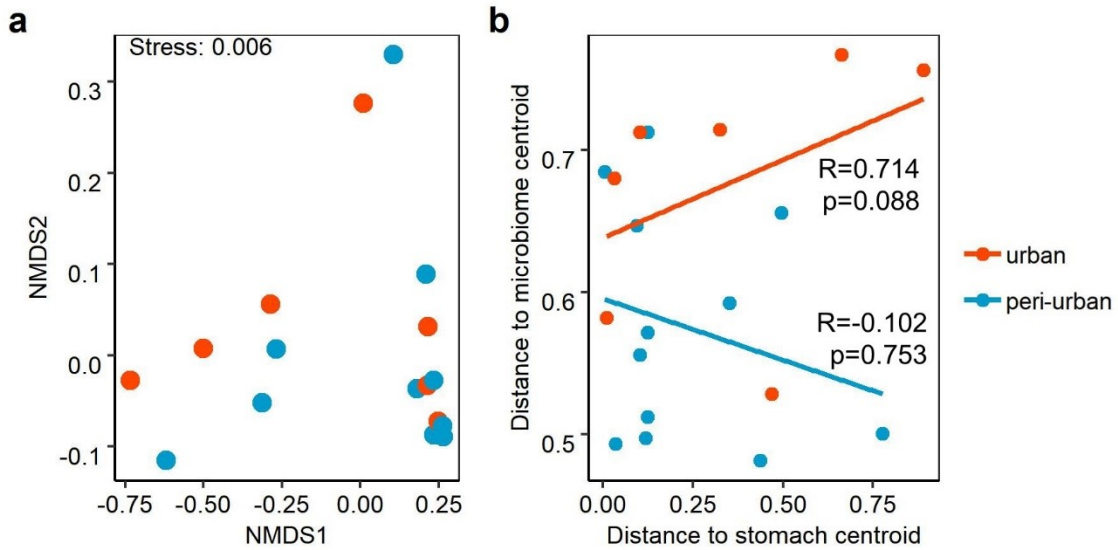
Supplementary Fig. 4.7: Diet divergence correlates with duodenal microbiome divergence in urban coyotes.

For all non-empty urban stomachs and a subset of 15 peri-urban stomachs, stomach contents were quantified by relative volume as either prey, anthropogenic food, or other fruit/vegetation. Stomachs were then ordinated using NMDS and the Bray-Curtis dissimilarity index (**a**). The distance from each point to the overall centroid was then correlated with the corresponding distance from each duodenal sample to its microbiome centroid (**Fig. 4.2c**) using Spearman's correlation separately for urban and peri-urban coyotes (**b**).
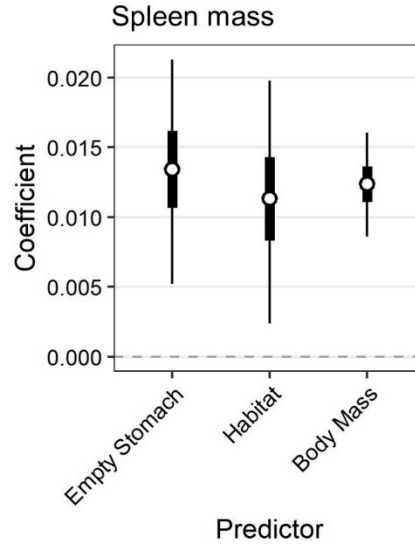
Supplementary Fig. 4.8: Empty stomachs and urban habitat use both predict spleen mass.
Spleen mass was used as a dependent variable in a gamma-distributed generalized linear model
with an inverse link and both dietary information and habitat use included as predictors,
controlling for variation in body mass. The best model, chosen based on the lowest AICc score,
included urban habitat use and whether the stomach was empty.

APPENDIX 5

Supplemental material for Chapter 5

Supplementary Table 5.1: Pairwise ANOVA statistics of bacterial alpha diversity among sample types and sampling locations.

	Tukey's HSD *post hoc* test					
Pairwise comparison	Chao1 index			Shannon index		
All samples	t	df	p	t	df	p
sponge - water	0.587	34	0.828	3.268	34	**0.007**
sponge - biofilm	1.386	34	0.360	5.750	34	**< 0.001**
water - biofilm	0.876	34	0.659	2.953	34	**0.015**
Sooke River						
sponge - water	2.565	11	**0.063**	1.172	11	0.493
sponge -biofilm	0.304	11	0.951	3.779	11	**0.008**
water - biofilm	2.115	11	0.132	4.884	11	**0.001**
Nanaimo River						
sponge - water	1.305	10	0.424	2.951	10	**0.035**
sponge - biofilm	1.922	10	0.183	5.983	10	**< 0.001**
water - biofilm	0.792	10	0.716	3.428	10	**0.016**
Cowichan River*						
sponge - water	-3.334	7	**0.013**	-17.752	7	**< 0.001**
water - biofilm	-	-	-	-	-	-
sponge - biofilm	-	-	-	-	-	-
Sponges						
Sooke - Nanaimo	1.585	12	0.289	2.291	12	0.096
Sooke - Cowichan	1.248	12	0.449	3.539	12	**0.011**
Cowichan - Nanaimo	0.337	12	0.940	1.248	12	0.449
Water						
Sooke - Nanaimo	4.252	11	**0.004**	10.135	11	**< 0.001**
Sooke - Cowichan	8.808	11	**< 0.001**	26.547	11	**< 0.001**
Nanaimo - Cowichan	4.800	11	**0.001**	16.992	11	**< 0.001**
Biofilms*						
Sooke - Nanaimo	-0.349	5	0.741	-0.242	5	0.818
Sooke - Cowichan	-	-	-	-	-	-
Nanaimo - Cowichan	-	-	-	-	-	-

* Due to limited DNA yield, there was only one biofilm sample from the Cowichan River. Pairwise comparisons between alpha diversity of sponges and water in the Cowichan River, and of biofilms in the Sooke and Nanaimo River, were therefore evaluated using Student's t-test.

Supplementary Table 5.2: Discriminatory taxa in random forest models trained to classify samples based on type (sponge, water, biofilm) or location (Sooke, Nanaimo, Cowichan). Models were run to classify 1) all samples based on sample type; 2) sponge samples based on sample location; and 3) all samples based on both sample type and location. Out-of-bag error rates are listed for each model. The top 20 ASVs in each model are ranked by their mean decrease in the Gini coefficient and identified at the family level. Taxa without family-level identifications in the RDP database are listed as unclassified ("uncl.") members of the higher taxa to which they belong.

| All samples - classified by type		Sponges - classified by location		All samples - classified by type + location	
out-of-bag error: 2.7%		out-of-bag error: 0%		out-of-bag error: 13.5%	
Gini	Family	Gini	Family	Gini	Family
0.695	Uncl. Actinomycetales	0.174	Comamonadaceae	0.350	Cryomorphaceae
0.653	Chitinophagaceae	0.163	Cryomorphaceae	0.336	Flavobacteriaceae
0.597	Uncl. Actinomycetales	0.157	Acetobacteraceae	0.274	Cytophagaceae
0.493	Uncl. Actinomycetales	0.143	Comamonadaceae	0.246	Microbacteriaceae
0.472	Caulobacteraceae	0.142	Tepidisphaeraceae	0.242	Cytophagaceae
0.438	Chitinophagaceae	0.127	Chitinophagaceae	0.240	Chitinophagaceae
0.420	Comamonadaceae	0.126	Burkholderiaceae	0.237	Uncl. Bacteroidetes
0.413	Rhodospirillaceae	0.120	Burkholderiaceae	0.236	Uncl. Parcubacteria
0.398	Comamonadaceae	0.120	Chitinophagaceae	0.235	Burkholderiaceae
0.360	Uncl. Actinomycetales	0.116	Uncl. Actinomycetales	0.232	Comamonadaceae
0.356	Rhodospirillaceae	0.116	Uncl. Actinomycetales	0.229	Chitinophagaceae
0.355	Burkholderiaceae	0.114	Comamonadaceae	0.227	Flavobacteriaceae
0.330	Uncl. Rhodospiralles	0.112	Chitinophagaceae	0.210	Chitinophagaceae
0.325	Burkholderiaceae	0.108	Unknown	0.209	Acetobacteraceae
0.293	Flavobacteriaceae	0.108	Uncl. Actinomycetales	0.209	Uncl. Proteobacteria
0.286	Chitinophagaceae	0.105	Methylocystaceae	0.209	Uncl. Actinomycetales
0.273	Unknown	0.104	Burkholderiaceae	0.205	Flavobacteriaceae
0.270	Comamonadaceae	0.103	Chitinophagaceae	0.205	Methylophilaceae
0.256	Uncl. Verrucomicrobia	0.103	Chitinophagaceae	0.200	Uncl. Actinomycetales
0.242	Comamonadaceae	0.103	Flavobacteriaceae	0.197	Unknown

Appendix 5
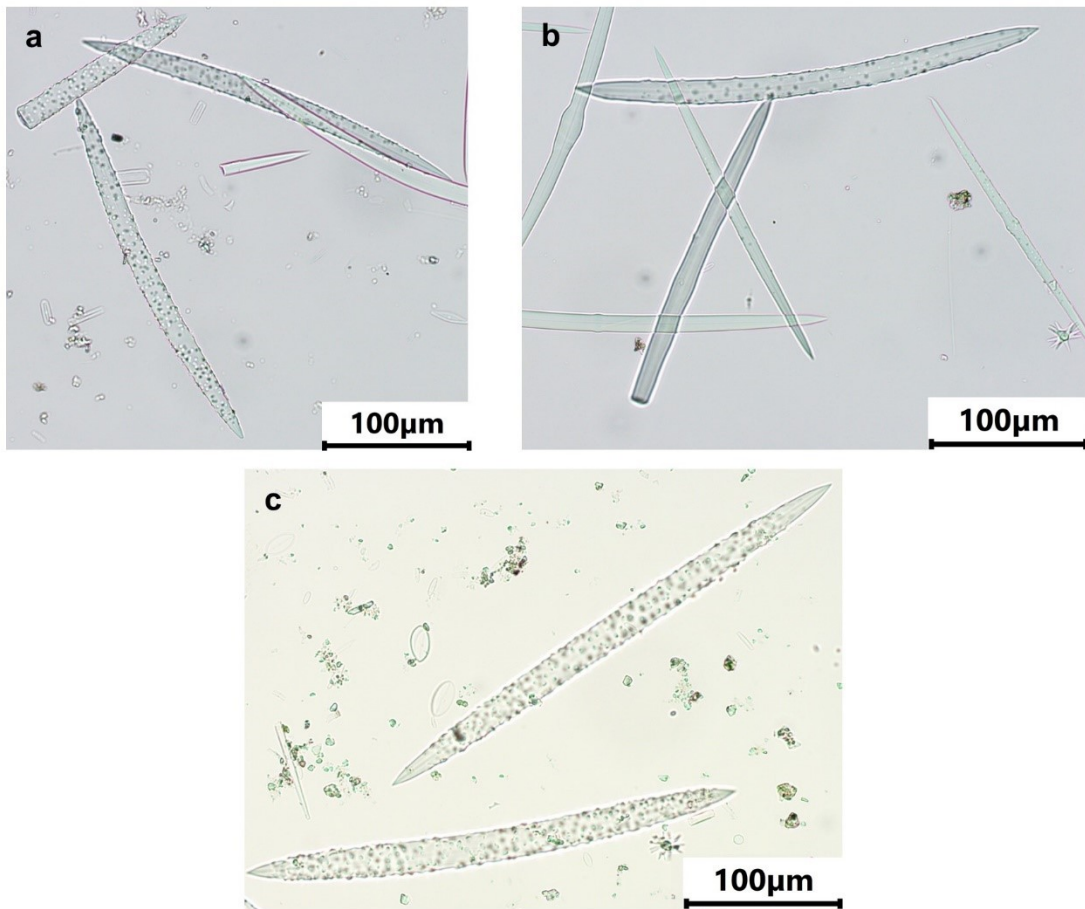
Supplementary Fig. 5.1: Spicule morphology confirming *E. muelleri* identification.
Representative images displaying spicule morphology of samples from the (**a**) Sooke, (**b**)
Nanaimo, and (**c**) Cowichan Rivers are shown. *E. muelleri* is distinguished by slightly curved
megascleres approximately 300μm in length and covered in numerous small spines.
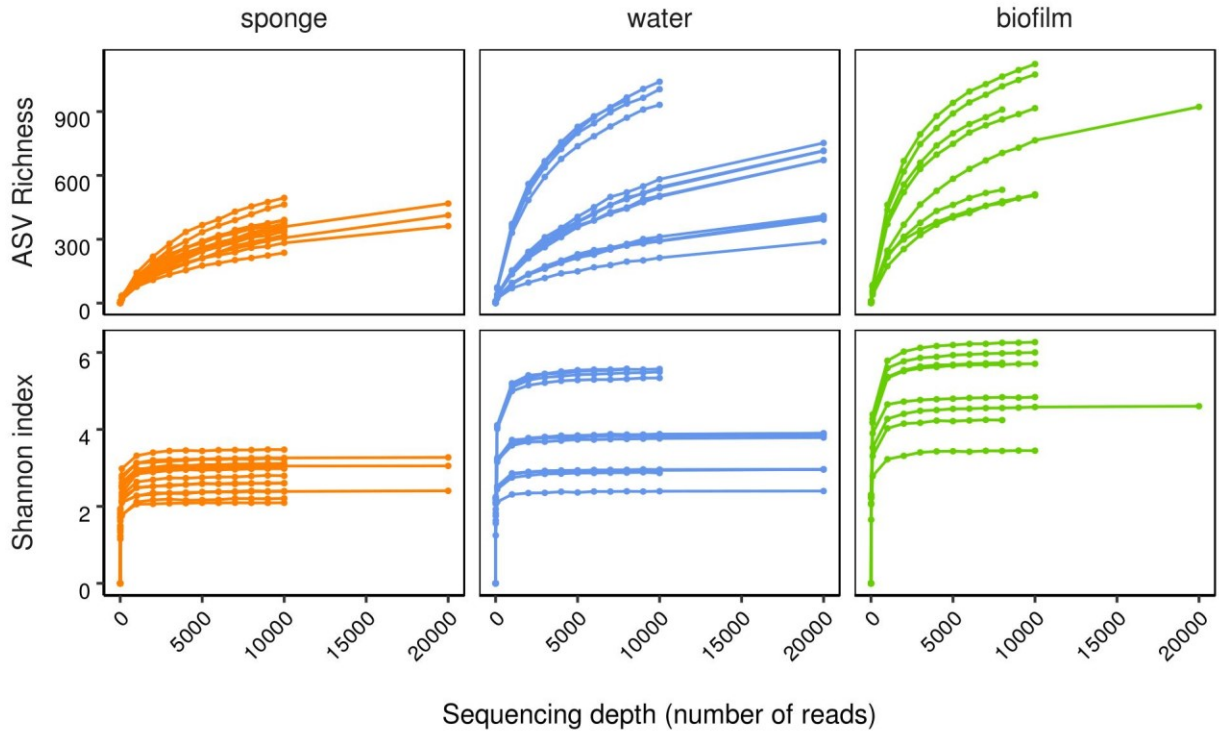
Supplementary Fig. 5.2: Rarefaction curves for ASV richness and the Shannon index.
Rarefaction curves were used to assess sequencing effort. (***Top***) ASV richness does not appear to approach an asymptote for any sample type, suggesting our sequencing depth was insufficient for capturing rare members of the community. (***Bottom***). The asymptotic appearance of the Shannon index rarefaction curves suggests that we were able to accurately assess diversity.
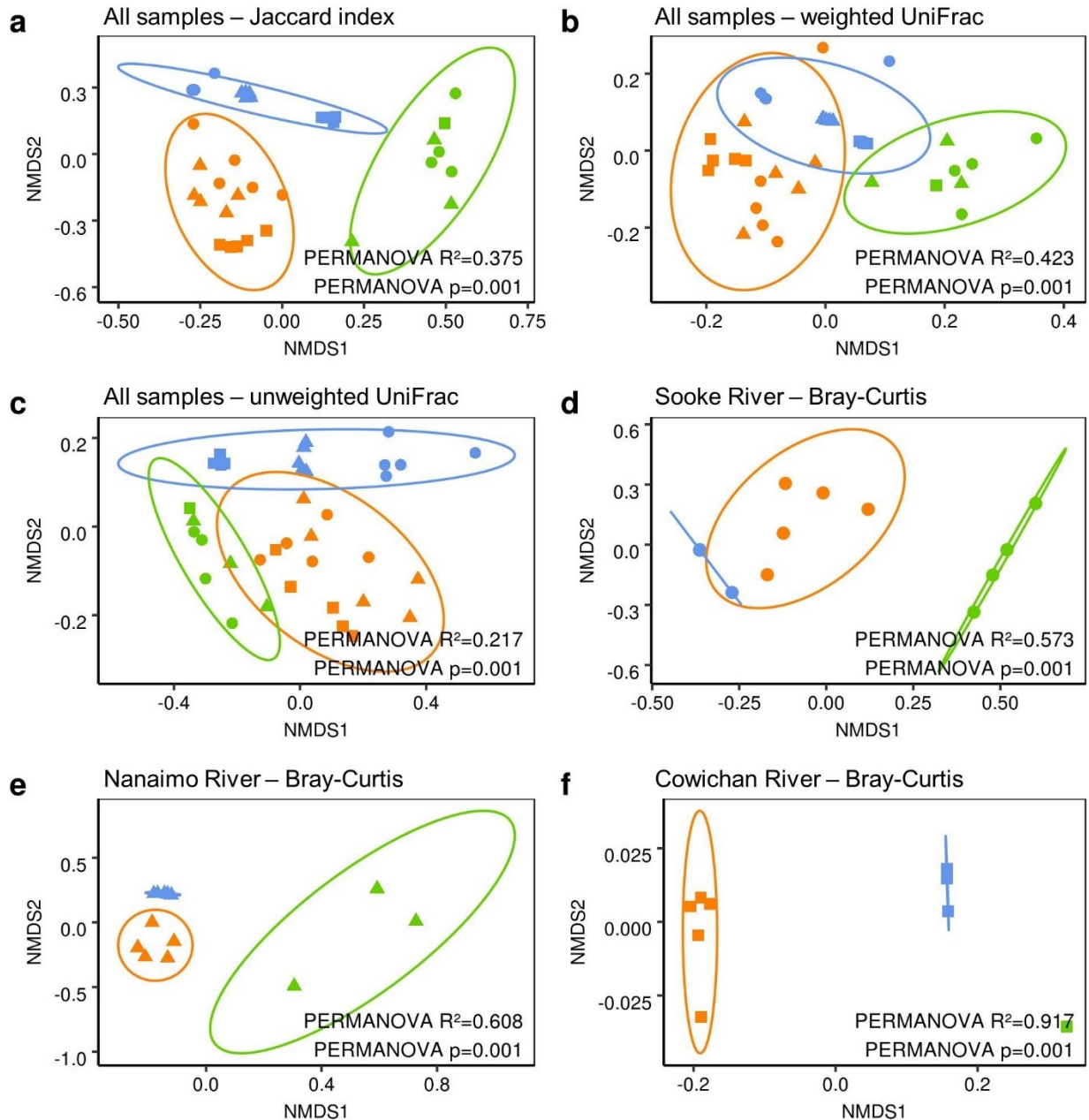
Supplementary Fig. 5.3: Robust community-level differences separate sponges, water, and biofilms.

Significant clustering associations separating sponges, water, and biofilms in non-metric multidimensional scaling (NMDS) analyses, displayed in Fig. 4a using the Bray-Curtis distance metric, are robust to the choice of dissimilarity index (**a-c**). These associations are also true when streams are evaluated independently (**d-f**), shown here using Bray-Curtis distance.
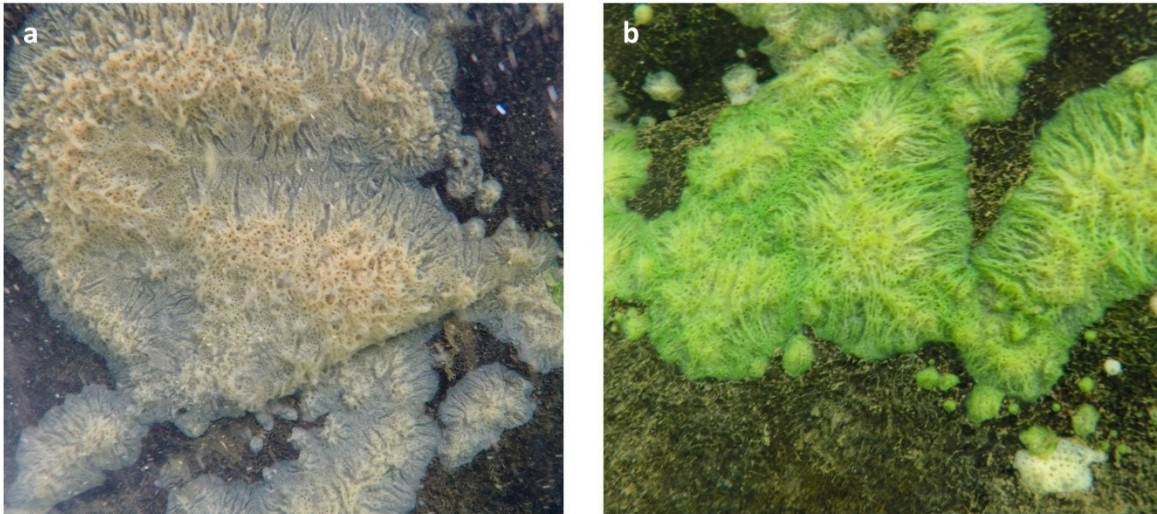
Supplementary Fig. 5.4: Sponge exposure to light influences colonization by photosynthetic microbes.

Two representative sponges from the Nanaimo River, (**a**) one growing on substrate that received no sunlight and (**b**) the other growing on substrate that was partially lit. The relative abundance of Cyanobacteria in these two sponges was 0.12% and 3.69%, respectively, and there may be additional differences in the abundance of eukaryotic algae associated with each sponge that further contribute to the different coloration.