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Patterns in richness and community structure: From bacteria to apex predators

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PATTERNS IN RICHNESS AND COMMUNITY STRUCTURE: FROM BACTERIA TO APEX PREDATORS

by

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ABSTRACT

Patterns of community structure and richness provide context for studies from microbial ecology, global macroecology, languages, to Bayesian statistics. Diversity patterns for animals on land and their predictor variables are well studied. However, diversity patterns for bacterial communities and marine macroorganisms are not well studied or understood. Here I examine diversity patterns in caves, on the external surface of Chiroptera (bats), and in marine ecosystems. At the local to regional scale we investigate factors that drive bacterial community patterns in richness and composition in lava cave microbial mats and microbes on bats. Lastly, out of the cave and into the surface world, a global picture emerges of factors that drive community structure and richness from bacteria to apex predators in marine environments. I hypothesize that for cave microbial mats found in lava caves, local factors (i.e. sample site temperature and relative humidity) are important factors for determining community structure and richness. For bacteria on bats, a mix of local factors (bat species, bat body mass, location of capture) and regional factors (net primary productivity (NPP), annual mean rainfall) explain richness and structure of the microbial communities. In addition, the predictor variables for richness and community structure will vary with spatial scale (local to regional to landscape). In the global marine data set, richness and community structure will be dependent on net primary productivity, temperature, thermal lifestyle, and foraging behavior. At small scales, temperature and NPP will be

variable in their predicting power, while at large scales they will be positivity correlated with species richness. Local factors likely drive the larger scale patterns in community structure and richness.

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Introduction

Significance and intellectual merit

Understanding species diversity patterns is important for understanding how species disperse, how life history can determine species ranges, helping to inform conservation strategies (Kaschner, 2004, Paradinas, et al. 2015), to understanding the diversity and extinction risk of human languages (Moore, et al. 2002; Sutherland, 2003), which show mammal richness correlated with human language richness. Exactly which factors help to drive richness and community structure are complicated by local and global variables, spatial autocorrelation, and the importance how predictor variables change with spatial scale. In addition, there are ill-defined species ranges, many unknown variables, and predictor variables that are cofounding, such as temperature and net primary productivity. This work focuses on untangling factors that help to govern richness and community structures across different spatial scales and body masses. In this work we are using a Bayesian framework for understanding and determining factors that shape community structure and richness.

Background

Biogeography is a science that attempts to answer the question of Why do organism live where they do? Biogeographers seek to explain and model patterns of richness and diversity across distances (Pasternak, et al. 2013) and time (Bisset, et al. 2010; Bahl, et al. 2011). Determining what factors contribute to community structure and their richness may shed light on our understanding of why organisms occur where they do. Microbial biogeography, while relatively new, is important for locating rare and unique Actinobacteria (Riquelme, et al. 2015a) and Cyanobacteria, which are major sources of novel drugs (Wang, et al. 2015).

Microbial

Caves are excellent places to study biogeographic patterns due to cave environments generally being more stable than surface environments. In addition, many caves are aphotic, thermostable, and often have very low connectivity to the surface. The arid-land caves from this study are characterized by being oligotrophic, having relatively constant temperature and high relative humidity, and in many cases, having no running water. A review of the global cave literature (Lee, et al. 2012) showed that the most common cave bacteria phyla were (in order of most abundance): *Proteobacteria*, *Chlorobi*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, *Nitrospirae* and *Chloroflexi*. In lava cave microbial mats the rare OTUs helped to structure the ß-diversity between caves and islands (Riquelme, et al. 2015b), while locally, the Azores microbial mat community structures were influenced by commonly shared OTUs. Between two different island arcs, geographic location and local host rock geochemistry were the most important drivers of community structure (Hathaway, et al., 2014). In the Lava Beds National Monument caves Nitrospirae distributions of OTUs shift by cave (Figure 1) showing a change driven by biotic and abiotic factors.

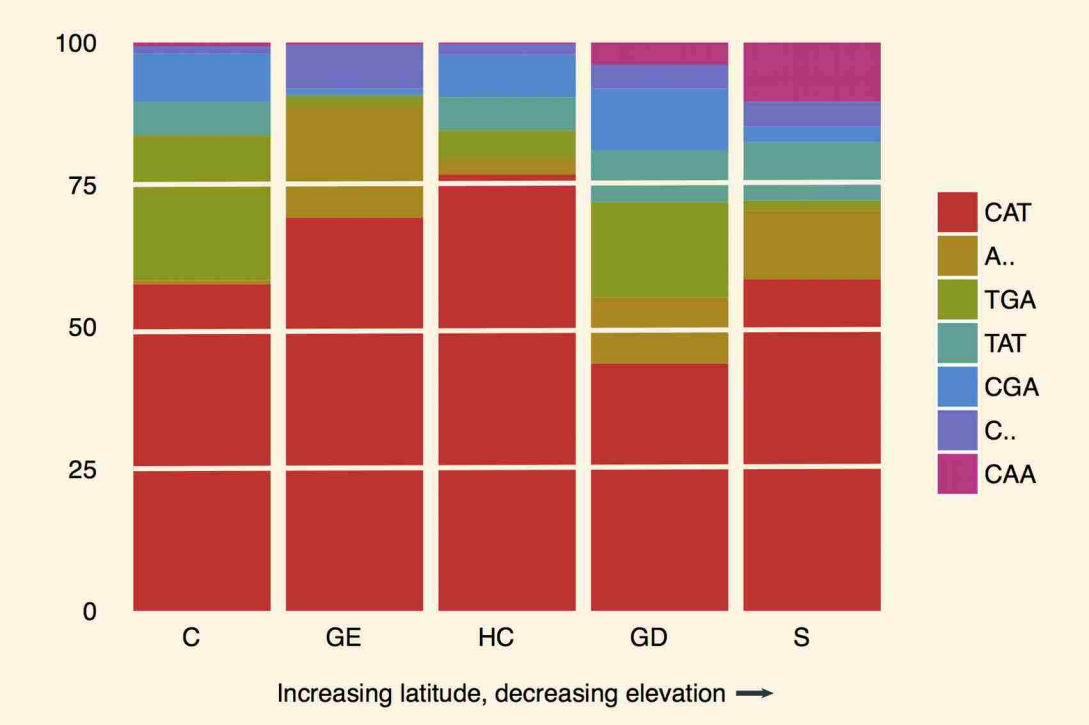

Figure 1. Distribution of Nitrospirae oligotypes by cave name along a latitudinal and elevation gradient in Lave Beds National Monument. Each band of color represents a unique oligotype of Nitrospirae.

Microbial-Macroecology

Recently, large-scale microbial data allowed microbial biogeographers to provide the missing link between microbes and macroecology (Barberan, et al. 2014). With the advent of cheaper next generation sequencing techniques, researchers are examining regional and global patterns of microbial diversity (Ladau, et al. 2013; Selama, et al. 2013; Zinger, et al. 2011). At the intersection of microbial and macroecology from local to region scales are

microbiome studies of animals. Two species of fish raised in the laboratory had skin microbiomes that were different from the surrounding bacterioplankton in the tank (Chiarello, et al. 2015). A humpback whale study that consisted of 57 skins samples and four seawater samples showed that the humpback whale skin microbiome is different from the surrounding sea water (Apprill, et al. 2014). In addition, the microbiome varied predictably by geographic region and metabolic state of the whale. A study of 337 samples from five body sites on 48 dolphins and 18 sea lions (Bik, et al., 2016) found that diet, host species, and phylogeny drove the gut microbiome patterns and that dolphins harbored a unique, rare biosphere that dominates their microbiome. In this dissertation research , factors that predict bat richness (Figure 2) area also predictors for external bat bacteria.

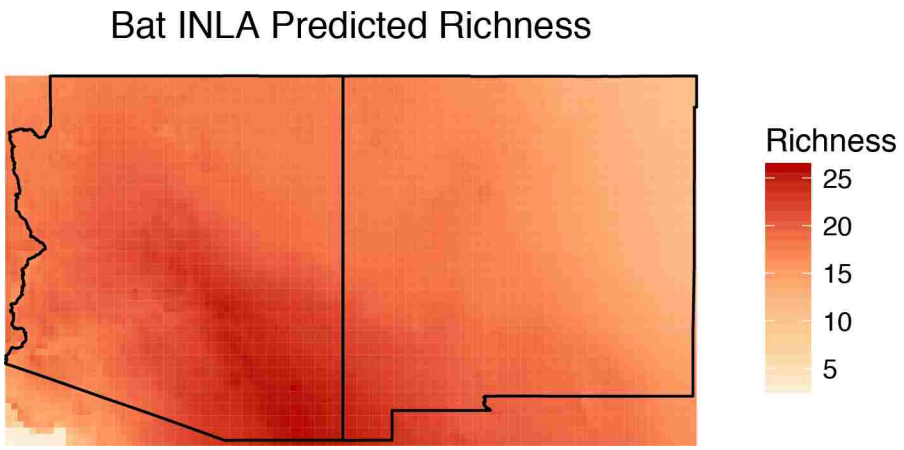

Figure 2. Integrated nested Laplace approximation model of bat species richness (Winter, unpublished) in Arizona and New Mexico. Bat species richness is best predicted by elevation, landscape complexity, NPP, and temperature. The external bat bacteria follow similar trends in richness (Winter, et al., in preparation).

Macroecology

On the global scale, patterns of community structure and richness provide information on anthropomorphic impact on global ecosystems, help establish more sustainable fisheries, and determine which factors control species distributions. The global marine system is particularly important for its role in nutrient cycling and carbon storage potential. Recently, with the advances in next generation sequencing, there exists an

abundance of publicly available data sets that can be used to model the global distribution of marine bacteria. Marine bacteria have peak diversity in temperate latitudes in both the boreal and austral winters (Ladau, et al. 2013). Marine bacteria have a higher diversity in areas that are highly impacted by humans following a similar relationship to macroorganisms (Tittensor, et al. 2010). Zinger et al. (2011) used 509 samples from sea surface to ocean abyssal plains to determine the diversity of marine bacteria. This study found that across all samples, they shared less then 10% of their taxa (Zinger, et al. 2011).

Marine macroorganisms share many predicator variables with microbes, but display different patterns of community structure and richness. Two broad patterns emerged (Figure 3) for marine species: coastal species had peak diversity in the Western Pacific and ocean taxa peaked across mid-latitudes. In contrast to marine bacteria, which follow anti-tropical patterns (Ladau, et al. 2013, Milici, et al. 2016). Ocean surface temperature was consistently predictive of ocean taxa richness.

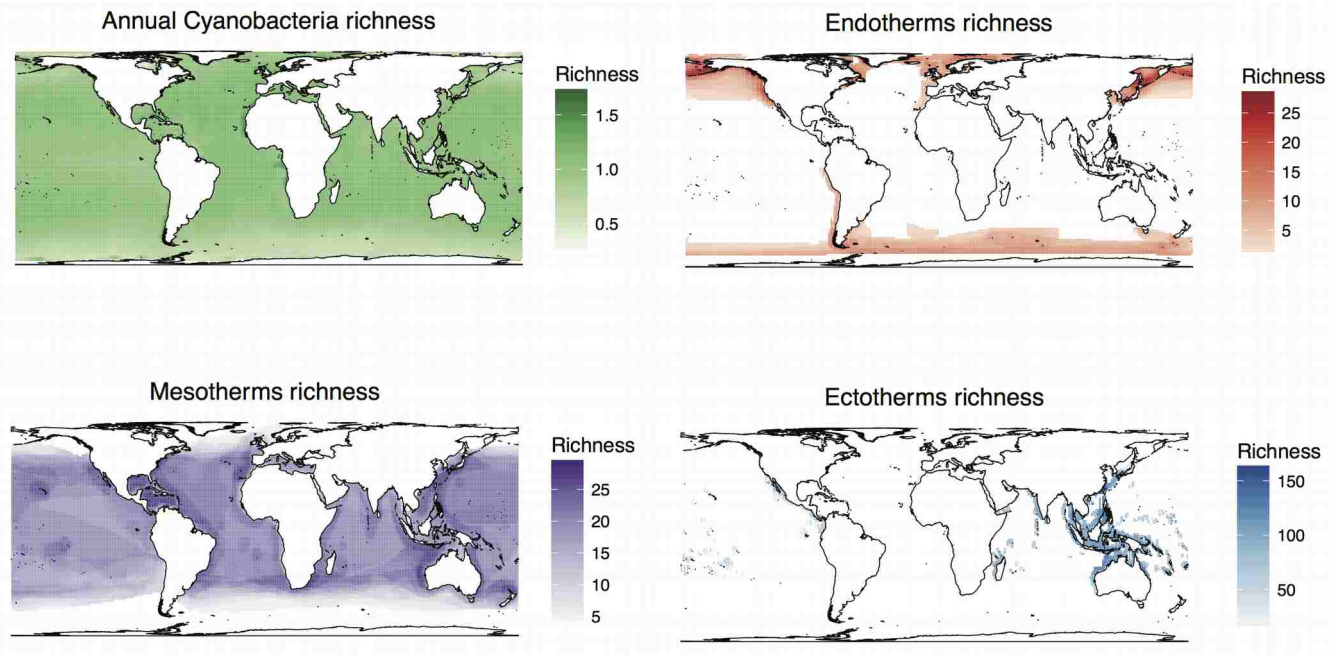

Figure 3. Global distribution of communities reflects environmental preferences, evolutionary history, thermal lifestyles, and dispersal capabilities (Grady, et al. in preparation).

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Comparison of Lava Cave Bacterial Mat Communities to Overlying Surface Soil Bacterial Communities from Lava Beds National Monument, USA

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Short title: Surface vs. Lava Cave Mat Bacterial Communities

Keywords: cave, soil, microbial mat, bacterial diversity, *Actinobacteria*, Acidobacteria, *Nitrospiraea*, *Proteobacteria*, mat color, lava caves, biogeography

Abstract

Lava caves around the world often support extensive microbial mats on ceilings and walls in a range of colors. Little is known about lava cave microbial diversity and how these subsurface mats differ from microbial communities in overlying surface soils. We generated and analyzed bacterial 16S rDNA from 454 pyrosequencing from three colors of microbial mats (tan, white, and yellow) from seven lava caves in Lava Beds National Monument, CA, USA, and compared them with surface soils overlying each cave. *Actinobacteria* dominated

in all samples, with 39% (caves) and 21% (surface soils). *Proteobacteria* made up 30% of phyla from caves and 36% from surface soil with *Gamma*- 20% and *Alpha*- 10% in the caves and *Gamma*- 18% with *Alpha*-17% in soil. Other major phyla in caves were *Nitrospirae* (7%) followed by Minor Phyla (7%), compared to surface soils with *Bacteriodetes* (8%) and Minor phyla (8%). A very high proportion (53.33%) of the most abundant sequences could not be identified to genus, indicating a high degree of novelty. Surface soil samples had more OTUs and greater diversity indices than cave samples. The same phyla were represented in both soils and cave microbial mats, but the overlap was only 11.2% at the operational taxonomic unit (OTU). Although surface soil microbes immigrate into underlying caves, the environment selects for microbes able to live in the cave habitats, resulting in very different cave microbial communities. In terms of species richness, diversity by mat color differed, but not significantly. Number of entrances per cave, distance from an entrance, cave length, and temperature also contributed to observed differences in diversity. With high levels of novel microbes, caves may represent excellent habitats for the isolation of new bioactive compounds. This study is the first comprehensive comparisons of bacterial communities in lava caves with the overlying soil community.

Introduction

Most life on Earth is microbial and in the aphotic subsurface [1]. Caves can provide a natural way to access subsurface environments ranging from very deep limestone caves (Krubera Cave in the Western Caucasus is more than 2,190 m deep [2]), to shallow caves, such as lava caves that have an overburden of up to 10 m [3]. Discovery of extensive lava flows and lava caves on Mars [4] supports the concept that Earth's lava caves may serve as a model for the study of life on other planets (astrobiology) [5,6,7]. Lava caves provide protection from ionizing radiation and would retain liquid water longer than the surface.

Lava caves, formed during active lava flows, contain diverse microbial mats that range in size from extensive mats covering walls and ceilings to small, scattered colonies (Fig 1, Fig 2).

Coverage is more extensive in moist lava caves in Hawai`i and the Azores than in arid caves in Hawai`i and New Mexico. Mat colors include white, yellow, tan, gold, orange, and pink, with shades in between [7-11]. Lava caves become colonized as soon as they cool down, and caves in lava flows in Hawai`i show diverse mat structure in tens of years.

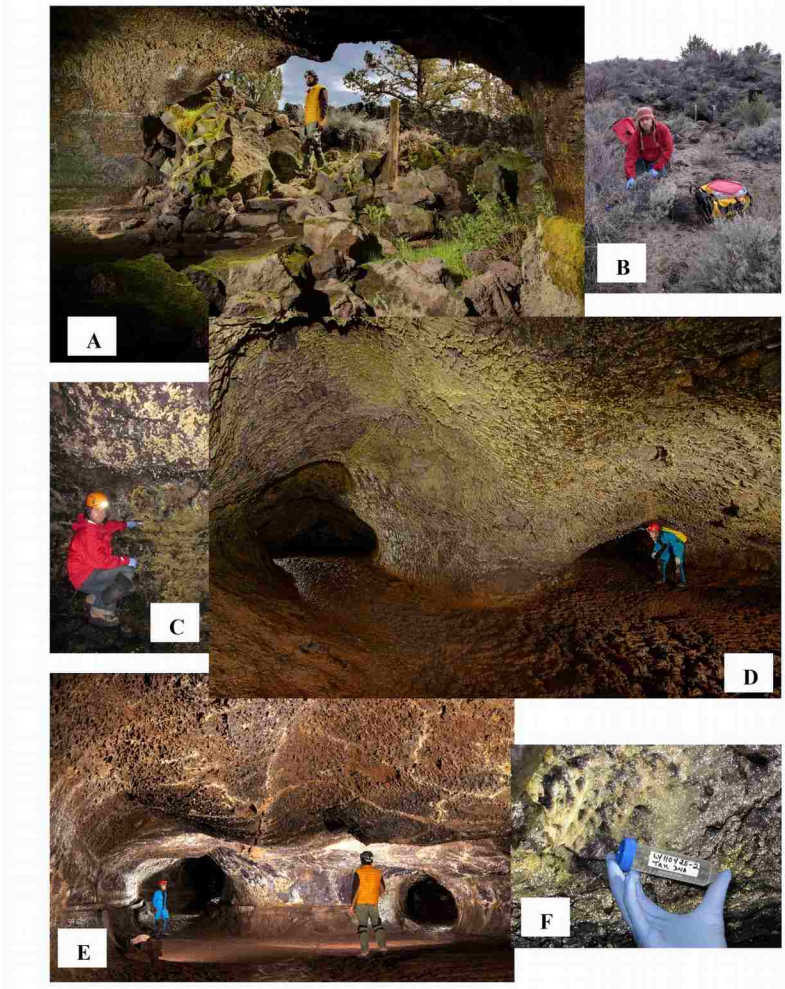


Fig 1. Overview of some of the lava cave sampling sites and caves, plus a view of the surface terrain at Lava Beds National Monument, CA (LABE). (A) Entrance to Valentine Cave. (B)

Surface samples taken above Hopkins Chocolate Cave. (C) Yellow microbial mat sampling site in Valentine Cave. (D) Extensive yellow microbial mats on walls of Hopkins Chocolate Cave. (E) Passage in Valentine Cave showing less microbial mat coverage near the entrance. (F) Tan microbial mat sample taken in L-V460 Cave. Photos copyright Kenneth Ingham (A, D, E) and Diana Northup (B, C, F).

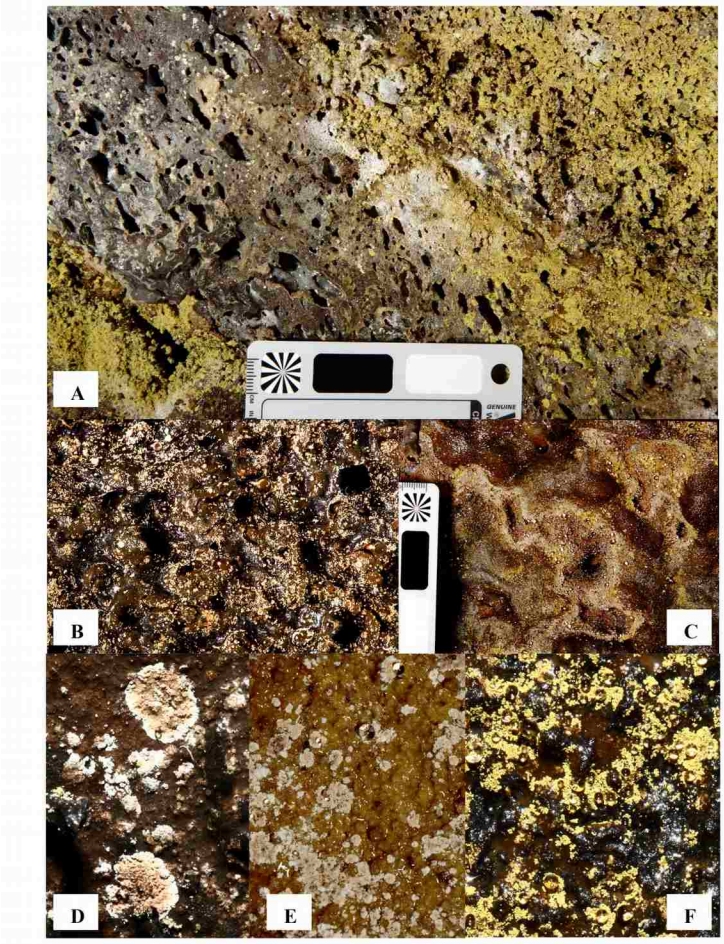


Fig 2. Microbial mat and colony morphology.

(A) Overview of predominantly yellow and white microbial mats, some separate and some intermixed. (B) Overview of tan and white microbial mats. (C) Intermixed tan, white, and yellow microbial mats. Close ups of (D) tan and white colony morphology, (E) tan colonies,

and (F) yellow colonies.

Despite their extensive nature, little is known about microbial mat diversity (reviewed in [11,12]). Studies of microbial diversity [7-15] in lava caves lag behind such studies in karst caves. Stoner & Howarth [16] first described the mats or "slimes" in Hawaiian lava caves using culture-dependent methods for isolation of chemoheterotropic microorganisms and reported on the presence of fungi and aerobic bacteria. They suggested that white and brown slimes may be important sites for nutrient cycling in caves, particularly nitrogen.

Lava caves are extreme environments, simplified by the lack of photosynthesis in the deep or dark zone of the cave, resulting in extremely oligotrophic conditions. The simplified nature of caves makes them a model natural laboratory to study factors controlling biological diversification [17,18]. The isolation of most caves limits the ability of organisms to migrate, resulting in high levels of endemism among troglobionts and stygobionts as the norm [19]. Culver *et al.* [20] found that about 30% of cave-adapted invertebrate species in U.S. caves are found in only a single cave. The results of Hathaway *et al.* [21] show that the trend can be extended to bacterial diversity in Azorean and Hawaiian lava cave microbial mats. The authors were far from sampling total diversity, but less than 5% of the OTUs found in lava caves occur in other caves or in other volcanic environments. Sequences were more likely to be related to samples from the same cave or the same island than between islands. If microbial distribution is ubiquitous, then they would expect a higher percentage of shared OTUs between the two island archipelagos.

Biospeleologists originally thought that cave microbes were simply a subset of surface microbes washed into underlying caves [22,23]. Ortiz *et al.* [24,25] recently published what we believe to be the first comparisons of cave microbial diversity with the overlying soil microbial community. Their study focused on bacterial diversity across carbonate speleothem surfaces sampled by swabbing from Kartchner Cavern in Arizona. Comparison of bacterial

taxonomic profiles to soil samples revealed major differences and only a 16% overlap between cave speleothem and soil OTUs [24]. Their later study [25] explored the differences between the cave microbial communities on speleothems and other habitats using metagenomics. Wu *et al.* [26] compared bacterial communities from rock walls, aquatic sediment, and sinkhole soil from a small limestone cave in China. Communities on rock walls were more diverse than surface or aquatic sediments.

Our study is the first and most comprehensive, comparing lava cave bacterial communities to bacterial communities from the overlying surface soils of each cave. We also examined a range of environmental, geographical, and chemical factors that may contribute to bacterial diversity in microbial mats across a range of colors (tan, white, and yellow) from lava caves in Lava Beds National Monument, California, USA.

Materials and Methods

Field Studies Ethics Statement

All sampling was done under Permit LABE-2011-SCI-0007 to Northup issued by the National Park Service. Lava Beds National Monument is a federally-protected area under the National Park Service, Department of the Interior. No protected species were sampled.

Sampling Sites

Lava Beds National Monument (LABE) is located in northern California close to the borders of Oregon and Nevada [27]. The Monument covers 190 km^2 on the NE flank of the Medicine Lake Volcano. Two-thirds of the lava came from the Mammoth and Modoc craters over the last two million plus years and as recently as 1,100 years ago. Flows are largely of basalt with smaller amounts of silica-rich basaltic andesite [28].

LABE has the largest number of lava caves in North America, with 778 known [27]. Twenty-five of the lava caves have signed entrances and developed trails for ease of visitation.

The area is a high-elevation (1219-1737 m above sea level), semi-arid desert with average yearly precipitation of 375 mm. Temperature ranges from an average low of -5.4 °C in January to an average high of 22.3 °C in July and August. Some of the lava caves contain perennial ice.

We worked with LABE personnel to select seven lava caves for sampling to cover a range of parameters: amount of human visitation, elevation, length, number of entrances, and age of the lava flow, which determines the age of the cave. During sampling we characterized color of the microbial mats (tan, white, and yellow) and distance from the nearest entrance, along with temperature, humidity, RH, and pH when suitable water pools or dripping water were available near mats. In addition to the cave samples, a sample of soil overlying each cave entrance (e.g. Fig 1(B)) was collected for comparison. Each sample was photo-documented at the collection site (Fig 1(F)).

Temperature, RH, and pH Measurements

Temperature (web bulb and dry bulb in order to obtain an approximate RH) was taken in April, 2011 and in September, 2012 with an IMC temperature probe (http://www.imcinstruments.com/), which was calibrated at frequent intervals in the cave to improve accuracy. Wet bulb readings were obtained with the IMC probe sheathed with wicking soaked in deionized water before each reading. For some RH samples a portable Kestrel 3000 wind meter (https://kestrelmeters.com/products/kestrel-3000-wind-meter) was used, which was calibrated at the beginning of each cave. A Javascript program (http://home.fuse.net/clymer/water/wet.html) used dry and wet bulb temperatures to approximate relative humidity. Readings for pH were taken with a Twin Cardy pH meter (Spectrum Technologies, Inc., http://www.specmeters.com/nutrient-management/ph-and-ec-meters/ph/cardy-twin-ph-meter/), calibrated with pH 7 buffer.

Sample Collections for DNA and Scanning Electron Microscopy (SEM)

Sampling took place in April 2011 and additional sampling (GEM2, HCC1, SC2) was done in August 2012 to increase the number of white mat samples. A range of microbial mats with different colors (yellow, white, and tan) was sampled from each cave. Samples for DNA extraction were collected aseptically with a flame-sterilized cold chisel into a sterile 50 cc Falcon tube. Soil samples were collected from above each cave entrance by removing any surface plant detritus and scooping the top 2 cm of soil into a sterile 50 cc Falcon tube. All samples for DNA were covered within hours with sucrose lysis buffer [29] to release and stabilize the DNA. All samples were brought to the Northup Lab at the University of New Mexico for further processing and analysis within seven days. We collected surface soils from above seven caves, and had seven samples of white and tan mats and nine samples from yellow mats, with at least one of each color from each cave.

Samples for SEM from rock chips were mounted directly onto SEM stubs with super glue and placed in a carrying case for transport. A microbial mat sample (L-V460-110425-6), consisting of chips of the wall rock with white to pale yellow colonies, was taken approximately 200 m into Cave L-V460 at the bottom of a pillar. One of the two yellow microbial mat samples (HC110423-5) analyzed with SEM was a rock chip with yellow colonies from the floor of Hopkins Chocolate Cave, approximately 36 m into the cave. The second yellow mat sample (S-L280-110427-3) was taken approximately 60 m into Cave S-L280 and 1 m above the floor and 1 m below the ceiling.

Water Chemistry Analysis

Dissolved organic carbon (DOC) in infiltrating water was collected and passed through a 0.45 μm filter and preserved with phenyl mercuric acetate (PMA) in the field. Samples for nutrient analysis were preserved with 6N hydrochloric acid in the field, as described in [30]. Organic carbon water samples were analyzed using the persulfate digestion method as described [31] on a Shimadzu TOC-5050A instrument (Shimadzu Corporation, Kyoto,

Japan). Amounts of chloride, nitrite, nitrate, phosphate, and sulfate were analyzed using a Dionex Ion Chromatograph DX-100 (Dionex, Sunnyvale, CA, USA) as described [32].

Molecular Phylogeny

DNA extraction, sequencing, and sequence analysis

DNA was extracted from triplicate samples of rock chips with microbial mats from each cave by mat color and from the surface soil samples using the MoBio Power Soil™ DNA extraction kit following manufacturer's protocol except we used bead beating rather than vortexing, which the Northup Lab finds to be more effective at releasing DNA from Gram positive cells.

Polymerase Chain Reaction (PCR)

PCR was performed to verify the quality and quantity of the DNA prior to sequencing. One hundred twenty five to three hundred ng of purified DNA was used to amplify the 16S rRNA gene from environmental DNA by PCR with universal primers, p46 forward (5'-GCYTAAYACATGCAAGTCG-3') and p1409 reverse (5'-GTGACGGGRGTGGTGTRCAA-3'; [33] and AmpliTaq LD (Applied Biosystems) with an MJ thermal cycler using: 4 min denaturation at 94°C followed by 35 cycles of 45 sec annealing at 55°C, extension for 2 min at 72°C, denaturation for 30 sec at 94°C, with a final 45 sec 55°C and a 20 min 72°C extension step after cycling was complete.

Sequencing and phylogenetic analysis

Samples were analyzed with next-generation sequencing of the 16S SSU gene bacterial V1-3 region (primer 27F) using Roche FLX and Titanium 454 technology conducted by MR DNA, Shallowater, TX (http://www.mrdnalab.com/). The shorter, but more numerous sequences generated by pyrosequencing give us a much more comprehensive view of the diversity present than from clone libraries. MR DNA designed tagged primer constructs, unique to each sample location, for post sequencing sample identification. In addition to the

sample tag, the primer constructs incorporated primer sequences specific to Bacteria, as well as 454 A and B adaptors for emulsion PCR and sequencing.

All 454 data were processed in QIIME 1.9.1 [34]. Quality control and trimming of the 454 dataset were done using the split_libraries.py command with a lower length (-l) of 100 bp and an upper length (-L) of 500. A quality score (-s) of 30 was chosen. Removal of erroneous sequences (denoising) and otu clustering were done using pick_de_novo_otus.py pipeline with the sumaclust option [35]. The sumaclust algorithm is mainly useful to detect the 'erroneous' sequences created during amplification and sequencing protocols. OTUs were clustered at the 97% similarity level using sumaclust. The pick_de_novo command also picks the representative set and assigns taxonomy using uclust [36] against the greengenes 13.8 database [37]. The pipeline also aligns and builds a phylogenetic tree using pynast [38] and fasttree [39] from the representative sequence set. Chimera checking was done using USEARCH to detect artifacts created during sequencing.

Diversity by phyla with the *Proteobacteria* separated out by class was compared for all samples. The L2 phyla data were reduced to 9 groups, including Unassigned Phyla and a group we called Minor Phyla. The Minor Phyla are entries that had less than 1000 OTU across all samples. Of a total of 140,848 OTU, 26,609 were from the surface samples, and 38,214 from tan, 36,014 from white, and 40,011 from yellow mats. The process was repeated at the L6 genus level resulting in nine groups, an unassigned group, and Minor Genera with 471 taxa. The percent unclassified samples at the different taxonomic levels were as follows: Phyla 2.38%, Class 18.6%, Order 35.25%, Family 55.49%, and Genus 53.33% (move to discussion). Good's coverage showed that we were successful in getting nearly all of the diversity from our samples. Values ranged from 99.11% to 87.13% with an average value of 94.98%.

Sequences submitted to the NCBI GenBank database (www.ncbi.nlm.nih.gov/genbank/)

were assigned Accession Numbers JX694094-JX702544, and the three additional white samples KP705489-KP706447.

Statistical Analysis

Community dissimilarity was visualized using the phyloseq package [40] and ggplot2 [41] in R [42]. Alpha diversity was analyzed using observed OTUs, Shannon and Simpson indices in the phyloseq package. Observed OTUs is simply the raw number of OTUs present in each sample of quality controlled and clustered sequences as described above. Beta diversity was analyzed using non-metric dimensional scaling (NMDS) with the Brays-Curtis distance using the vegan package [43] in R. The Brays-Curtis distance was picked because it is invariant to changes in units, unaffected by additions and removals of species, and NMDS recognizes differences in total abundances when relative abundances are similar. The ordination of the taxa and environmental parameters was done using custom R scripts by Umer Zeeshan Ijaz available at: http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/ecological.html. Briefly the scripts use vegan and ggplot to find the taxa and environmental parameters that best correlate with community similarity based on ADONIS and Pearson correlation. Differences in the abundance of taxa were characterized using the DESeq2 package [44] with parameters fitType= “local”. An adjusted p-value threshold of 0.1 was used to calculate log2 fold changes between surface soils and cave microbial mats. Phylogenetic tree analysis was carried out in the phyloseq package. The data were subset by phyla that were differentially abundant as determined by DeSeq2. Tree files with tips label were written out using write.tree(phy_tree(phyloseq_obj),file="phylum_name.newick"). The tree, tip labels, and traits (cave or surface) were loaded into Interactive Tree of Life v3 (http://itol.embl.de/) [45] for visualization. Traits were assigned to the tree tips as relative abundance of the OTUs present in the cave. The tree trait was tested using the phylotools package [46] in R. Pagel’s Lambda was calculated for each tree. The values for Lamda range from 0 to 1. Values near 0 indicate little phylogenetic signal in the trait data given the original tree and a high lambda

value indicates relatively more phylogenetic signal in the trait data.

Results

Environmental Variables

The environmental variables (Table 1) age of lava flow, elevation of entrance, number of entrances, length of cave, distance of sample from entrance, latitude of entrance, number of branching points in cave, temperature at the sampling site, and relative humidity at the sampling site were tested against total richness of the cave microbial mats. The variables that had the highest positive correlation with richness were (from 0.10 to 0.46): distance from entrance, number of cave entrances, temperature, and length of cave. The remaining variables correlated with richness between -0.04 to 0.07. (S1 Fig.).

Table 1. A. Characteristics of the seven study sites in LABE. Visit= number of visitors, grouped into High (open to the public) and Low (closed to visitation). Lava Age= Age of lava flow. Surface describes surface vegetation, where Sagebrush = Big sagebrush-antelope bitterbrush scrubland and Juniper = Juniper-mountain mahogany scrubland. Elevation is the elevation in m of the entrance a.s.l. # Entr= number of accessible or functional entrances into the cave. Branches (Nodes) is an indication of the geometric complexity of the cave by counting the number of passage branch points. Length is the mapped length of the cave in m.

B. Samples: C = Catacombs; GE and GEM = G-L350; GD = Golden Dome; HC and HCC = Hopkins Chocolate; L = L-V460; S and SC = S-L280; and V = Valentine. Color: T=Tan, Y=Yellow, W=White. Distance is the distance in m from the nearest entrance to the sampling site. Temperature, RH, and pH data were collected in April 2011 and Aug 2012. (nd=not determined)

A.

Cave	Surface Soil	Visit	Lava Age	Surface	Elev	# Entr	Branches (Nodes)	L
Catacombs	C15	High	32,000	Sagebru	1,524 m	1	32	2104

				sh				m
Golden Dome	GD25	High	32,000	Sagebrush	1,491 m	2	7	679 m
GE-L350	GE13	Low	32,000	Sagebrush	1,513 m	1	9	461 m
Hopkins Chocolate (HCC)	HC15	High	32,000	Sagebrush	1,503 m	4	4	428 m
L-V460	L14	Low	10,000	Juniper	1,360 m	2	5	698 m
S-L280	S10	Low	32,000	Sagebrush	1,386 m	2	5	686 m
Valentine	V16	High	10,000	Juniper	1,376 m	1	12	498 m

B.

Cave	Sample	Color	Distance from entrance	T° C	%RH	pH
Catacombs	C1	T	51 m	13.6	61	nd
Catacombs	C2	Y	51 m	13.6	61	nd
Catacombs	C9	W	76 m	15.7	45	nd
Golden Dome	GD1	Y	278 m	9.3	99	8.13
Golden Dome	GD2	T	276.5 m	9.2	100	7.96
Golden Dome	GD3	W	276.5 m	9.2	100	7.96
Golden Dome	GD16	Y	278 m	9.3	99	8.13
GE-L350	GE1	T	64 m	9.9	87	7.45
GE-L350	GE2	Y	64 m	9.9	87	7.45
GE-L350	GEM2	W	67 m	7.1	86.6	nd
HCC	HC1	Y	37 m	11.5	89	nd
HCC	HC6	T	107 m	8.9	100	7.98
HCC	HC7	Y	107 m	8.9	100	7.98
HCC	HCC1	W	105 m	8.9	100	7.97
L-V460	L1	Y	200 m	9.3	96.3	7.31
L-V460	L3	W	200 m	9.3	96.3	7.31
L-V460	L9	T	87 m	9.8	92.7	nd
S-L280	S2	Y	255 m	16.1	82.1	nd
S-L280	SC2	W	135 m	16.1	82.1	nd
S-L280	S4	T	145 m	13.1	70.6	nd
Valentine	V1	Y	135 m	11.2	100	7.45
Valentine	V2	T	135 m	11.2	100	7.45
Valentine	V13	W	170 m	11.3	100	7.75

Chemical analysis of water samples collected from each cave with available standing water is

shown in Table 2. Chlorine levels averaged (5.086 ppm), slightly higher than the EPA Maximum Contaminant Level Goals for drinking water of 4 ppm [47] EPA levels for nitrate (1 ppm) and nitrate (10 ppm) were not exceeded in most cave water samples. There are no phosphate level standards set by the EPA, but our cave samples were all low. Sulfate water standards are 250 ppm, and ours were all very low. Bromide for all samples was below the detection limit.

Table 2. Water chemistry of cave water samples from Lava Beds National Monument. bdl = below detection limits.

Cave Sample	ppm	ppm	ppm	ppm	ppm
(F)	Chloride	Nitrite	Nitrate	Phosphate	Sulfate
CAT7	12.571	bdl	0.6039	bdl	0.6103
CAT13	2.184	0.9866	0.5924	bdl	0.3251
GD20	3.567	0.5526	0.7286	bdl	bdl
GE-L350-GE26	9.203	bdl	0.8325	bdl	0.4704
GE-L350-GE10	5.128	0.3320	0.9140	bdl	0.4329
HC3	2.116	bdl	0.6606	0.6715	0.5391
HC10	1.821	bdl	1.5608	bdl	1.1231
L-V460-LY4	4.493	0.5778	2.7485	0.5371	0.5851
L-V460-LY12	2.031	bdl	0.7116	0.5571	0.5505
S-L280-SV6	3.702	0.4489	8.0036	0.4697	2.3381
VAL3	9.132	0.3113	0.5483	bdl	0.5910

Dissolved organic carbon (DOC) in ppm is shown in Fig 3 for lava caves in the Azores, Hawai`i, and LABE caves. The NM samples are from Lechuguilla Cave, and Carlsbad Cavern, both carbonate caves in Carlsba (CAVE)d Caverns National Park, NM. All of the lava caves show a comparable range of levels of DOC. Lowest DOC values are from Lechuguilla Cave, ranging from about 4 to 7.5 ppm. LABE samples were the lowest and the highest among lava cave samples, ranging from about 4-18 ppm DOC.

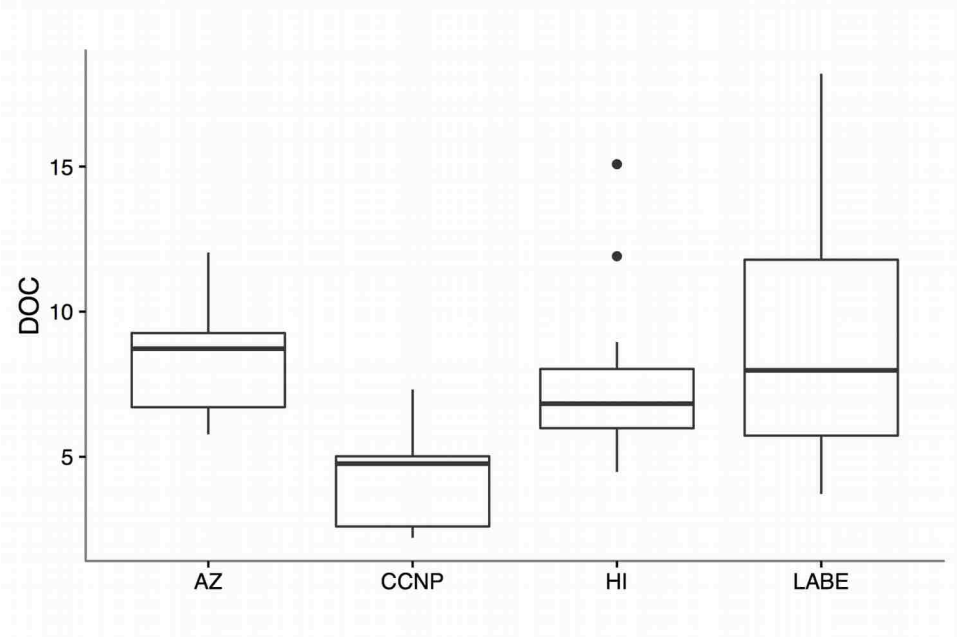


Fig 3. Boxplot of DOC (ppm) in cave water from lava caves in the Azores, carbonate caves from CAVE, NM, lava caves from Hawai`i and from LABE, CA.

Alpha Diversity

Measures of alpha diversity among surface soil samples and cave samples by color of mat (S2 Fig.) show species richness (Observed and Chao1 [48]) and relative abundance (Shannon and Simpson's). Simpson is less influenced by singletons (i.e. rare taxa) than Shannon's Index. The means are very different, with much greater bacterial diversity in soil than cave. Soil bacterial diversity is more evenly distributed among OTUs. Cave samples are less evenly distributed, with a wide distribution of Simpson's values with many outliers in contrast to soil samples. In-cave variation is much higher than surface soil variation. In terms of species richness in differently colored mats, tan samples have higher diversity, followed by yellow and then white. In terms of Shannon's and Simpson's indices the three colors of microbial mats are not that different. Fig 4 shows the difference between cave microbial mats and surface soils in respect to total richness of each sample (observed OTUs) and Shannon Index. In all cases surface soils have more OTUs and higher Shannon indices (indicating higher

diversity).

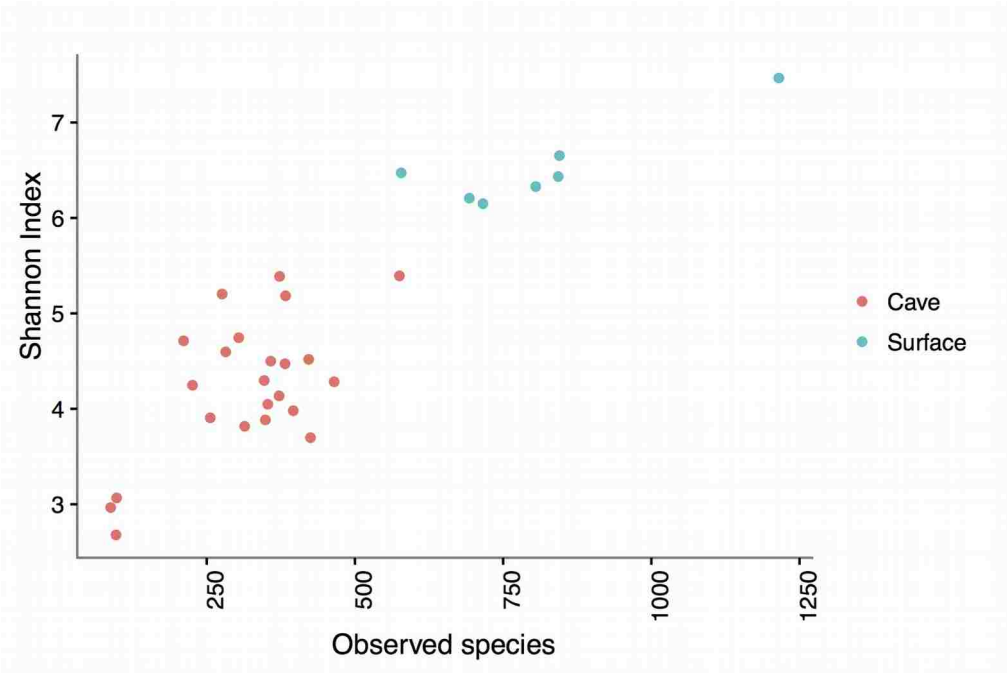


Fig 4. Alpha diversity: Shannon index plotted against observed species for LABE surface soils and microbial mats.

Molecular Phylogeny by Phylum and Proteobacteria Class

Composition by the top 5 phylum and 4 Proteobacterial classes, plus Minor Phyla based on OTU are presented in Fig 5 for surface soil samples (5A), by cave (5B), and by color of microbial mat (5C White; 5D Yellow; 5E Tan). Comparisons of surface soil samples with the underlying cave samples show some differences. Most notable is the reduction in *Actinobacteria* in surface soil samples (21%) versus cave samples (39%), the reduction in the *Nitrospirae* (3%) in surface vs. (7% in cave samples), and the increase in *Alphaproteobacteria* in the surface soil samples (avg. 17%) compared to the cave samples (10%). Smaller, but significant differences that decrease from surface to cave are seen with the *Bacteroidetes* (surface 8% and cave 2%); *Gemmatimonadetes* (avg. surface 3 % and cave <1%); *Planctomycetes* (surface 2% and cave 1%). Among the other *Proteobacteria*, only the

Gammaproteobacteria increase between cave (20%) and surface (18%), while *Betaproteobacteria* (surface 4% and cave 3%) and *Deltaproteobacteria* (surface 4% and cave 1%) both decline in cave samples. None of the phyla differed substantially by mat color. Some differences were observed by mat color in the *Actinobacteria* (tan: 37%, white: 39%, yellow: 44%), *Gammaproteobacteria* (tan: 19%, white: 24%, yellow: 20%), *Nitrospirae* (tan: 11%, white: 4%, yellow: 7%), and *Betaproteobacteria* (tan: 3%, white: 6%, yellow: 2%).

DESeq2 (differential analysis of count data) between all surface samples and all cave samples showed strong evidence for differential abundance of OTUs between surface soils and cave microbial mats (Fig 6). Eighteen phyla stood out as being significantly differential over or under represented between cave microbial mats and surface soils. The major phyla and *Proteobacteria* class that are significantly higher in the cave than in surface soil are the *Actinobacteria* (p>0.00000) and *Nitrospirae* (p=0.00232). Major groups with greater representation in surface soils than the cave are *Gammaproteobacteria* (p>0.00000), *Verrucomicrobia* (p=0.00001), *Bacteriodetes* (p=0.00021), and *Alphaproteobacteria* (p=0.02373). Of the minor phyla, *GAL15* (p=0.00273), *WS3* (p=0.03444), and *SBR1093* (p=0.04227) have higher numbers in the cave samples compared to surface soils. The groups with higher amounts in surface soils than cave samples are *TM7* (p>0.00000), *OD1* (p>0.00000), *Armatimonadetes* (p>0.00000), FBP (p=1e−05), *Cyanobacteria* p=0.00438), *Fibrobacteres* (p=0.00698), and *Elusimicrobia* (p=0.04176). The remaining two minor phyla are approaching significant differences; *Planctomycetes* (p=0.05177) and *NC10* (p=0.05655).

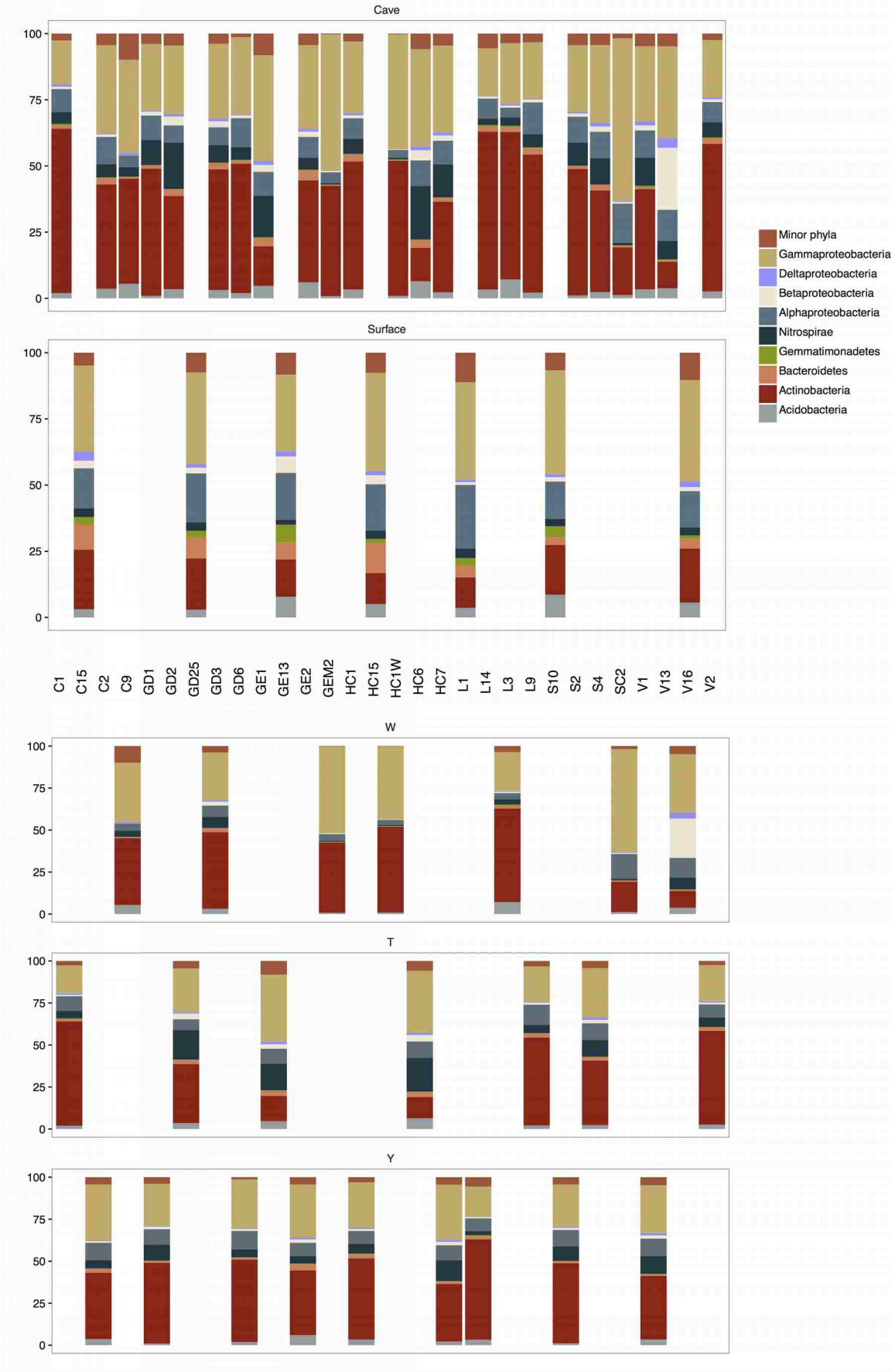

Cave
Surface
W
T
Y
100
75
50
25
0
Minor phyla
Gammaproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Nitrospirae
Gemmatimonadetes
Bacteroidetes
Actinobacteria
Acidobacteria
C1
C15
C2
C9
GD1
GD2
GD25
GD3
GD6
GE1
GE13
GE2
GEM2
HC1
HC15
HC1W
HC6
HC7
L1
L14
L3
L9
S10
S2
S4
SC2
V1
V13
V16
V2

Fig 5. Comparison of phyla and class of *Proteobacteria* in A) cave microbial mats, B) surface soils (C15-V16), and microbial mats by color: C) White = C9-V13, D) Yellow= C2-V1, E) Tan = C1-V2 . See Fig 1 legend for cave names.

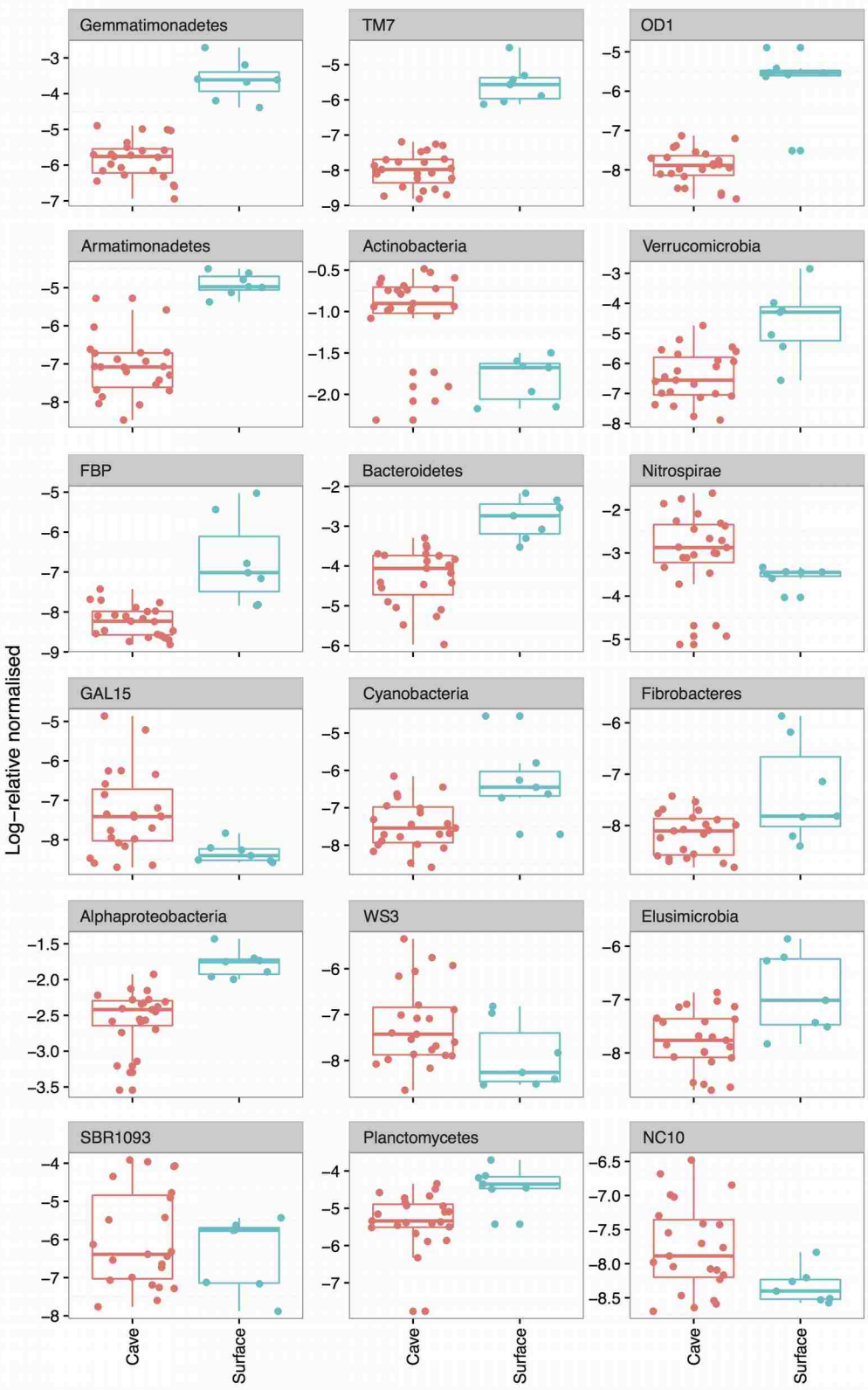

Gemmatimonadetes
TM7
OD1
Armatimonadetes
Actinobacteria
Verrucomicrobia
FBP
Bacteroidetes
Nitrospirae
GAL15
Cyanobacteria
Fibrobacteres
Alphaproteobacteria
WS3
Elusimicrobia
SBR1093
Planctomycetes
NC10
Log−relative normalised
Cave
Surface

Fig 6. Differential proportion plot of phyla and *Proteobacteria* class that were statistically different between LABE surface soils and lava cave microbial mats. The band is the median, and the box delineates the upper and lower quartile. The whiskers show the maximum and minimum values. All data points are shown.

A phylogenetic tree of the *Nitrospirae* (Fig 7) shows the proportion of OTUs between cave and surface samples colored by family. The *Nitrospirae* vary by family in terms of whether they are primarily found in the cave vs. surface samples. The major *Nitrospirae* in our samples are found in three families, the *Nitrospiraceae*, the *Leptospirillaceae*, and a candidate family, *0319-6A21*. Pagel's Lambda, the measure of the strength of a trait on a tree, for relative abundance of OTUs in the cave microbial mat was 0.84. The majority of the *0319-6A21* family are found in the cave microbial mats and the remaining families both in the surface soil and the cave microbial mats. A similar tree was constructed for *Actinobacteria* (S3 Fig.) however the Pagel's Lambda test was 0.26 which is a weak signal for differences between cave and surface.

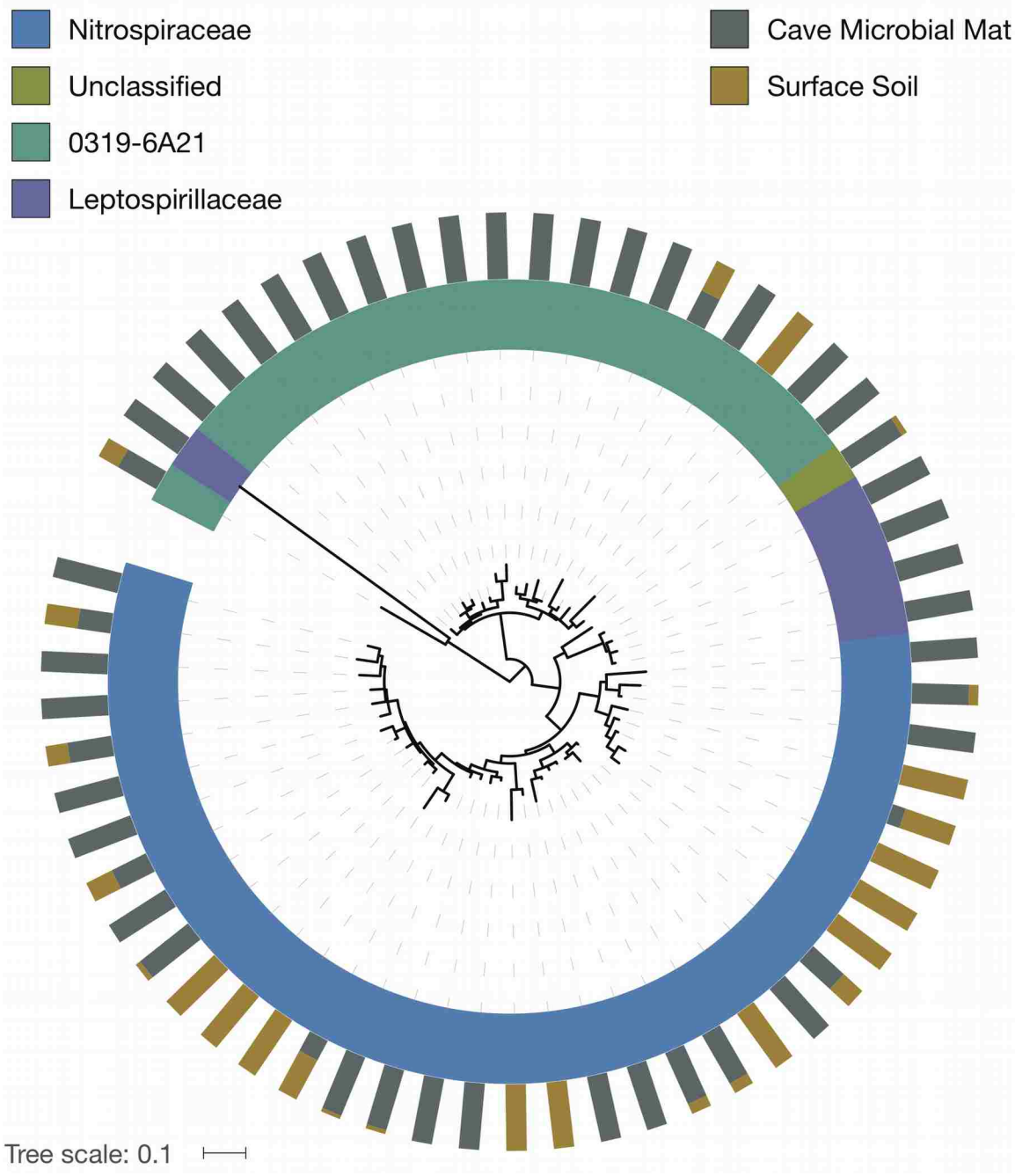


Fig 7. Phylogenetic tree of *Nitrospirae* by LABE lava cave and surface soil. Approximate maximum likelihood tree.

Non-metric dimensional scaling (NMDS) in Fig 8 shows a separation of the cave microbial

mats samples from the surface soil samples, indicating that the two sets of samples are not similar to each other. The surface soil samples cluster tightly in the far right, while the cave spread out to the left. To fill out the dataset with one white sample per cave, we had LABE personnel obtain additional samples from GE-L350, Hopkins Chocolate, and S-L280 Caves in 2012. These three samples (GEM2, HC1, SC2) have very low numbers of OTUs and diversity compared to the other samples collected in 2011, although they have comparable overall sequence numbers as some of the original samples (e.g. GE2). Tan and yellow mats cluster together, while white mat samples are widely spread out and group into three clusters. Within cave similarity is greater than between cave similarity, probably due to the three mat colors sampled in each cave, and suggests the necessity for multiple samples to cover the diversity comprehensively.

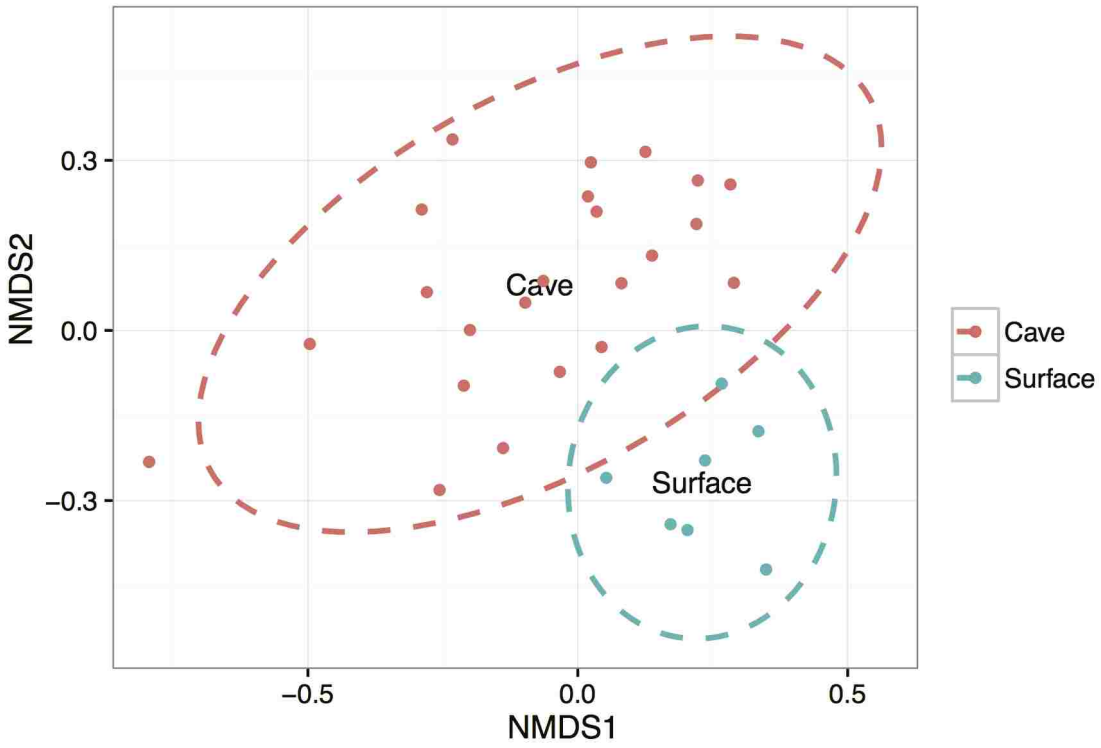
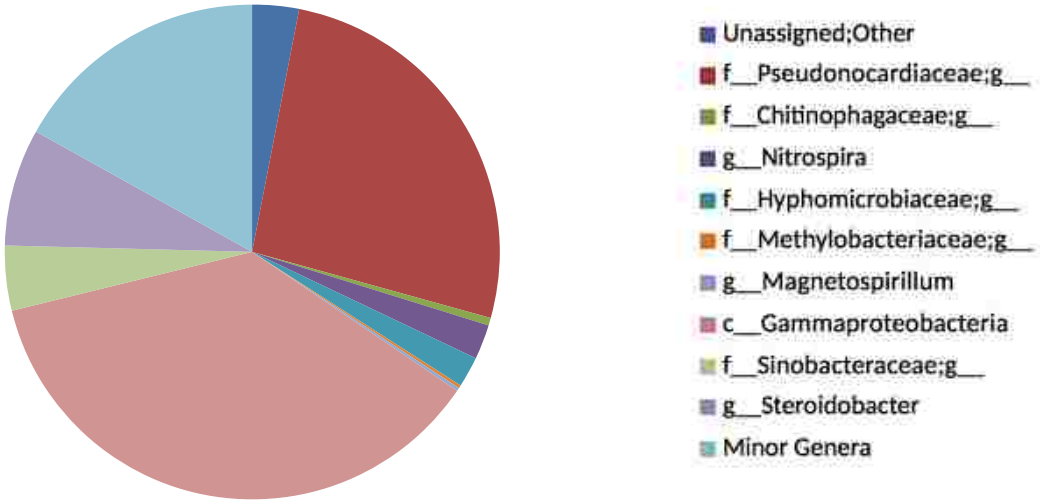


Fig 8. NMDS (Non-Metric Dimensional Scaling) separates out lava cave mat communities from the overlying surface soils. Circles show the 95% confidence interval.

Molecular Phylogeny by Genus

The percentage of OTUs by genus is presented in Fig 9 for surface soil and cave mat samples. Results from the analysis of diversity by genera are interesting in several respects. First of all, a large percentage of OTUs are novel. Of the nine major genera, only four are identified to genus level, one to class, and four to family. There is also a group called Unassigned, which could not even be classified to Bacteria. These unassigned OTU may represent archaeal DNA that amplified with our primers or may be organisms that are particularly difficult to classify through 454 sequencing, like members of the *Verrucomicrobia*. Bergmann *et al.* [49] used barcoded pyrosequencing with surface and subsurface soils to reduce primer bias, and found that *Verrucomicrobia* are ubiquitous and were often the dominant phylum in their samples. In our study, no individual *Verrucomicrobia* genus met our criteria for inclusion in Fig 9.

Surface



Cave

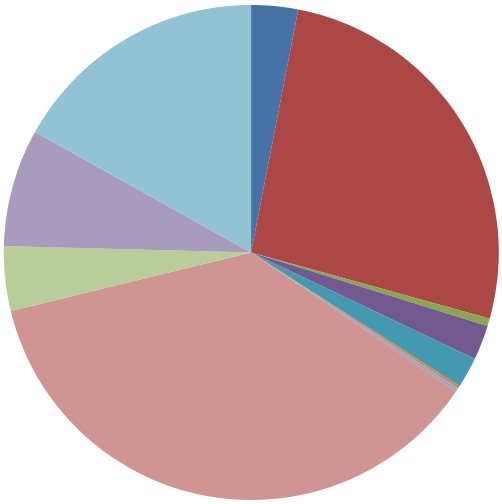


Fig 9. Percent OTUs by lowest level of classification (family or genus) in all surface soils and combined cave microbial mats.

The other major observation from the comparison of soil and cave samples at the genus level is the occurrence of many OTUs that were present in smaller numbers (genera that were present in three or fewer samples with less than 100 sequences), which we grouped together into the category Minor Genera. The genus level diversity in the Minor Genera in surface samples represents 35% of the diversity in surface soils samples and 20% of the diversity in cave samples.

A major difference between surface and cave samples is the proportion of the family *Pseudonocardiacae* present with 14% of surface soil OTUs vs. 30% of cave OTUs. Other major genus level OTUs fell within the *Acidobacteria*, *Nitrospiraceae*, and

Bacteriodetes. Within the *Acidobacteria* there are three groups, all belonging to the candidate class *Chloroacidobacteria*. There are two orders *PK29* and *PK10* that cannot be identified further. The *Nitrospiraceae* is classified to the genus *Nitrospira*. The *Bacteroidetes* group is identified to the family *Chitinophagaceae*. *Chitinophagaceae* are elevated in soils where insect chitin is higher than in the cave, a defining characteristic of this family [50], although new isolates from oligotrophic lake waters lacked this ability [51].

The remaining major bacterial OTU groups are all members of the *Proteobaceria*, with three from the *Alphaproteobacteria* and three *Gammaproteobacteria*. The *Alphaproteobacteria* OTUs are two members of the order *Rhizospiriales,* one family *Hyphomicrobiaceae* and one *Methylobacteriaceae*, and one *Rhodospirillaes* identified to the genus *Magnetospirillum.* Together the *Rhizospiriales* account for only 2% of cave species but 5% of surface species, probably associated with the rhizosphere. The *Gammaproteobacteria* include the class *Gammaproteobacteria* and two members of the *Sinobacteraceae* with one family, *Sinobacteraceae*, and the genus *Steroidobacteria*. The type species is *Steroidobacteria denitricans*, a nitrate oxidizer [52]. There are only two described species.

Scanning Electron Microscopy

The three samples (one white, two yellow) examined with scanning electron microscopy (SEM) revealed extensive microbial morphologies present with similarities and differences observed across the three samples. The white sample from Cave L-V460 contained the most unusual morphologies, which were filaments covered in curly putative pili/fimbrae with spheroids emerging from the tips (Fig 10A-C). The spheroids range in size from 0.8 to 1.2 µm in diameter, and in Fig 10B, one can observe what appears to be a "neck" on one of the spheroids. Some of the "fuzzy" filaments appeared to be segmented (Fig 10B). Some parts of the sample also contained smooth, long filaments, while other areas had small spheroids (0.8 µm in diameter) emerging from the biofilm (not pictured). A somewhat similar morphology (fuzzy filaments with spheroids protruding from the ends) was observed in a yellow sample

from Hopkins Chocolate Cave (Fig 10D). This sample also had many colonies covered by, or partially emerging from, a lawn of biofilm (not pictured). The second yellow sample, from Cave S-L280, had extensive biofilm that appeared to bury filaments, and was dotted with large colony masses. We interpret the fuzzy areas along the margins of the colonies (Fig 10E) as being biofilm. Many strands of beads-on-a-string morphologies are seen on the colonies and biofilm (Fig 10F).

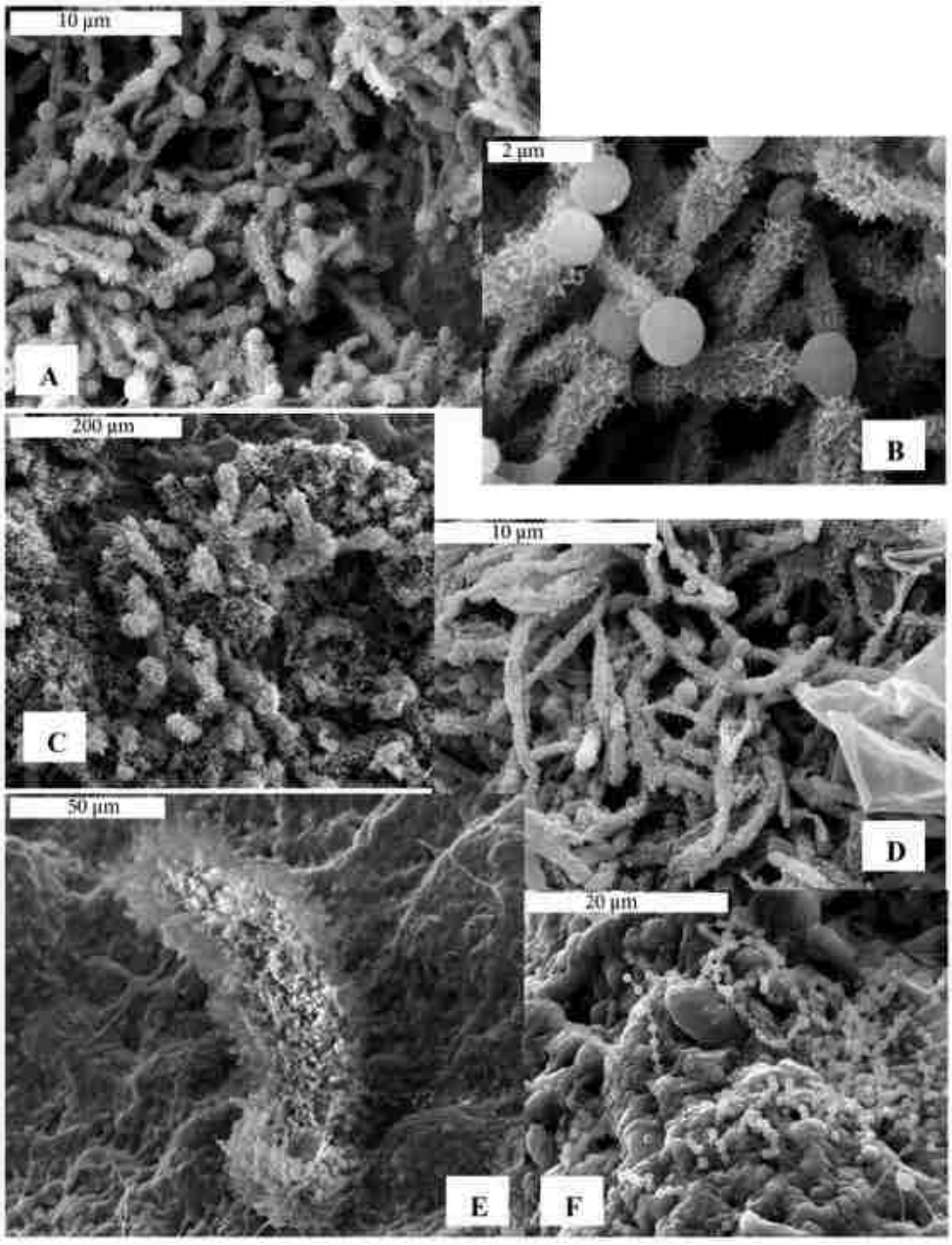


Fig 10. Scanning electron micrographs of cave white and yellow microbial mats. A. White microbial mat from Cave L-V460 showing filaments covered with putative pili/fimbrae with spheroid shapes emerging from the ends. B. Close-up of these morphologies from A. C.

Overview of a field of these morphologies in the same white microbial mat from Cave L-V460. D. Yellow microbial mat from Hopkins Chocolate Cave showing similar morphologies to those seen in images A-C, and including some biofilm and smooth filaments. E. Overview of a yellow colony from Cave S-L280, showing extensive biofilm in the background and on colony edges. Beads-on-a-string morphologies are observed lying on the background biofilm. F. Close-up of beads-on-a-string morphology on biofilm from Cave S-L280 yellow microbial mat.

Electron dispersive spectroscopy revealed the expected high carbon peak from the biofilm and microorganisms present, plus aluminum and silica peaks, possibly suggesting the presence of clays in all three samples.

Discussion

The general belief for decades has been that cave microorganisms are a subset of the microbial community found in surface environments overlying the cave [53], eking out a minimal chemoheterotrophic existence in the cave. Our study is the first to test the hypothesis that bacterial communities in the soil overlying lava caves are substantially different from the bacterial communities found in the cave microbial mats. Also, our study is the most robust study to date of lava cave microbial diversity, with three colors of microbial mats (tan, white, and yellow) from each of seven different caves in Lava Beds National Monument, CA, USA. The percent unclassified samples at the different taxonomic levels were as follows: Phyla 2.38%, Class 18.6%, Order 35.25%, Family 55.49%, and Genus 53.33%. Good's coverage showed that we were successful in getting nearly all of the diversity from our samples. Values ranged from 99.11% to 87.13% with an average value of 94.98%.

The soil clearly can be a source of bacteria for colonization of the underlying caves; however, the actual overlap in OTU in our study is only 11.12%. Microbes in caves enter from the

surface through drip waters, air currents, gravity, floodwater or animals, but quickly adapt or die under the selection pressure of oligotrophic and mineral conditions of lava caves as evidenced by the low level of overlap between cave and surface OTUs. It appears that over time, these communities become very different as the bacteria adapt to the cave and those that can't adapt die off. Most microorganisms in subsurface environments grow as biofilms or individual or groups of cells or microbial mats attached to rock surfaces.

Cave and surface soil sample bacterial communities differ in major ways

To understand the microbial diversity in lava caves, a good knowledge of the microorganisms present on the surface on volcanic rock deposits and in soils is important. *Acidobacteria*, *Alpha-* and *Gammaproteobacteria*, *Actinobacteria*, and *Cyanobacteria* predominate in surface volcanic deposits in Hawai`i, with composition controlled by local differences in environments and the type of volcanic deposits [54]. Janssen [55] reviewed studies of the microbial diversity of soils from many environments found in clone libraries. Across many habitats, soils are dominated by *Proteobacteria*, averaging 39% of soil bacteria, and by *Acidobacteria*, *Actinobacteria*, *Verrucomicrobiota*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, *Firmicutes*, and Other/Unknown. Our soils are fairly similar, dominated by *Actinobacteria* and *Proteobacteria* (together about 60-75% of the total diversity), then lesser amounts of *Nitrospirae*, *Verrucomicrobia*, *Chloroflexi*, *Gemmatimondetes*, and *Bacteriodetes*, lacking only a significant amount of *Planctomycetes* and *Firmicutes*. These findings show the need for a careful comparison of microbial populations from soils that overlie caves that are available to colonize underlying caves, especially when those caves are relatively shallow like lava caves. Although the cave populations will diverge quickly from infiltrating surface microorganisms, an analysis of surface populations provides knowledge of the possible colonizers of caves.

Surface soil sample phyla or class (*Proteobacteria*) that are the most different from the cave phyla or class (Fig 6) are the *Actinobacteria* (21% in soil samples; 39% in cave samples),

Bacteroidetes (8% in soil samples; 2% in cave samples), *Alphaproteobacteria* (17% in soil samples; 10% in cave samples), the *Gammaproteobacteria* (18% in soil samples; 20% in cave samples), *Nitrospirae* (3% in soil samples; 7% in cave samples). The proportion of minor phyla is nearly equal, with 8% in soil samples vs. 7% from cave samples.

Northup *et al.* [14] compared bacterial phyla from lava caves in their study with other recent studies (2006-2010) in carbonate systems and found a great deal of overlap at the phylum level. They noted that the more recent the study the more bacterial phyla were reported, probably a reflection of improved sequencing technology and lower cost of analysis. The comparison suggests that caves in general contain a core set of bacterial phyla. Six of 11 studies found *Actinobacteria*, *Proteobacteria* (*Alpha-, Beta-, Delta-, Gamma-*), *Acidobacteria*, *Verrucomicrobia*, *Planctomycetes*, *Nitrospirae*, and *Bacteroidetes*. Three studies of sulfur-based caves Frassi Caves, Movile Cave (reviewed in [14]), and several other sulfur caves [56] lacked *Actinobacteria*. These sulfur caves are also the only ones with *Epsilonproteobacteria*.

A comparison of bacterial communities in surface soils overlying each of our study caves with those from cave microbial mats (Table 3) shows high overlap at the phylum level. Of the 16 Phyla and *Proteobacteria* classes considered core in the Northup *et al.* [14] review, our LABE study found an overlapping core of nine phyla and four classes of *Proteobacteria*, eliminating *TM7*, *Chlamydia*, *OP10*, and *Firmicutes*. Some of the differences between caves sites are likely due to the use of clone libraries in the early NM, HI, and Azores analysis, and only White and Yellow mats, compared to 454 with LABE and all three mat colors. A newer study from 14 caves across two islands in the Azores by Riquelme *et al.* [57] was also done using clone libraries, but included white, yellow, and tan mats Table 3). Tan mats had the least diversity, which is different from our LABE study. They did not report *Verrucomicrobia* or *Planctomycetes*, leaving seven phyla or *Proteobacteria* classes in common dominating across all studies and mat color, supporting a worldwide biogeographic

core biome in lava cave microbial mats.

Table 3. Distribution of the major phyla and class of Proteobacteria from LABE soil and caves compared with the summary from Northup *et al.* [14] from lava caves in New Mexico, Hawai`i, and the Azores by color of microbial mat, and Azores [57]. All studies include white and yellow mats, but only our study of LABE mats and the second Azores study included White, yellow, and tan microbial mats as indicated by letter. See Table 1 for LABE cave names and Northup *et al.* [14] and Riquelme et al. [57] for other cave names. X = present in surface soil; Act=*Actinobacteria*; P= *Proteobacteria* are separated into *alpha-, beta-, delta-,* and *gamma-*; Acid= *Acidobacteria*; Chlf= *Chloroflexi*; Nit=Nitrospiraceae; Ver= *Verrucomicrobia*; Gem= *Gemmatimonadetes*; Plc= *Planctomycetes*; Bct= *Bacteroidetes*; Chl= *Chlamydia*; Frm= *Firmicutes*. Candidate Phyla are *TM7* and *OP10*.

Location																
CA WYT	Act	αP	βP	γP	δP	Acid	Chlf	TM7	Nit	Ver	Gem	Plc	Bct	Chl	OP10	Frm
SOIL																
CAT	X	X	X	X	X	X	X		X	X	X	X	X			
GD	X	X	X	X	X	X	X		X	X	X	X	X			
GE	X	X	X	X	X	X	X		X	X	X	X	X			
HC	X	X	X	X	X	X	X		X	X	X	X	X			
L	X	X	X	X	X	X	X		X	X	X	X	X			
S	X	X	X	X	X	X	X		X	X	X	X	X			
V	X	X	X	X	X	X	X		X	X	X	X	X			
CAT	WYT	WYT	WYT	WYT	WYT	WYT	WYT		WYT	WYT	WYT	WYT	WYT			
GD	WYT	WYT	WYT	WYT	WYT	WYT	WYT		WYT	WYT	WYT	WYT	WYT			
GE	WYT	WYT	WYT	WYT	WYT	WYT	WYT		WYT	WYT	WYT	WYT	WYT			
HC	WYT	WYT	WYT	WYT	WYT	WYT	WYT		WYT	WYT	WYT	WYT	WYT			
L	WYT	WYT	WYT	WYT	WYT	WYT	WYT		WYT	WYT	WYT	WYT	WYT			X
S	WYT	WYT	WYT	WYT	WYT	WYT	WYT		WYT	WYT	WYT	WYT	WYT			
V	WYT	WYT	WYT	WYT	WYT	WYT	WYT		WYT	WYT	WYT	WYT	WYT			
NM WY	Act	αP	βP	γP	δP	Acid	Chlf	TM7	Nit	Ver	Gem	Plc	Bct	Chl	OP10	Frm
4W	WY	WY	WY	WY	Y	WY	Y		WY	W	WY	Y	WY			

EM2	Y	Y	Y	Y	Y	Y	Y		Y	Y	Y	Y	Y			Y
WM3	W Y	W Y	W	W Y	Y	W Y	Y				WY					Y
EM4	W Y	W Y	W Y	W Y	Y	W Y	W	Y	Y	Y	WY	Y	W Y			Y
HI WY	Act	αP	βP	γP	δP	Ac	Cf	T M7	Nit	Ver	Ge m	Plc	Bct	Ch l	OP 10	Fr m
BP	W Y	W Y	W Y	W Y	W Y	W Y	W Y		W Y	Y	Y				WY	
Ep	Y	W Y	W Y	W Y	W Y	W Y	Y		W Y			Y		W	WY	Y
Ka	W Y	Y	Y	W Y	W	W Y	W Y	W	W Y	W Y		Y	W	W Y	Y	
KK	W Y	Y	W	W Y	W Y	W		W	W				W	Y		Y
Azores WY []	Act	αP	βP	γP	δP	Aci d	Chl f	T M7	Nit	Ve	Ge m	Plc	Bct	Ch l	OP 10	Fr m
BO	W Y	W Y	W Y	W Y	W Y	W Y			W Y		Y					
Ba	W Y	W Y	W Y	W Y	W Y	W Y	W		W Y		Y	Y	Y			W
Ac	W Y	W Y	W Y	W Y	W Y	W Y	W		W Y		W		Y			W
Pr	W Y	W Y	W Y	W Y	W Y	W Y	W Y		W Y	W Y	WY	W Y	W Y	W	WY	
Azores WYT [57]	Act	αP	βP	γP	δP	Aci d	Chl f	T M7	Nit	Ver	Ge m	Plc	Bct	Ch l	OP 10	Fr m
GL	W YT	W YT		W YT	W Y	W Y	Y		W Y		Y					Y
GM	W Y	W YT	W YT	Y	W YT	W YT	Y		W YT		WY					
GRF	W YT	W YT	W Y	W Y	W Y	W Y	W T		W YT		W					
GT	W YT	W Y	W Y	W Y	W Y	W Y	W		W Y		W		W			
GT2	W YT	W Y	W Y	W YT	W Y	W Y	Y		W Y				W			
AC	W YT	W Y	Y	W YT	W Y	W Y			Y		WY		Y			
GA	W YT	W	Y	W T	W Y	W YT			W YT				Y			
GAS	W YT	W YT	W	W	W Y	W T	W		W YT		W		Y			
GB	W Y	YT	W YT	YT	Y	W YT			W							
GBL	W YT	W YT	W YT	W YT	W Y	W YT	W		W YT		YT		W			
GBO	W YT	W Y	W Y	W YT	W Y	W YT			W YT		Y					T
GMD	W YT	W YT	W YT	W YT	Y	W YT	W T		W YT		W		Y			
GN	W YT	W YT	W YT	W YT	W YT	W YT	T		W YT		W		Y			

GTM	W YT	W TY	W Y	W	Y	W Y			Y							T

*Colors of samples by cave in parentheses.

While not significantly different between cave and surface or among mat colors, *Acidobacteria* are a major phylum across all samples. Jones *et al.* [58] studied *Acidobacteria* across 87 soils and found them to be both ubiquitous and abundant. Their abundance relative to other taxa was variable, but correlated strongly with pH ($R= -0.80$, $p<0.001$). They suggest that pH is an effective habitat filter for *Acidobacteria*, with the highest abundance below pH 5.5. pH in lava caves seldom drop to this level, but may on a microhabitat scale. In Jones *et al.* [58] the proportion of the community comprised of *Acidobacteria* dropped to about 20% between pH 5.5 and 8.5, leading to progressively more narrowly defined lineages as pH deviates from neutrality.

Ortiz *et al.* [24] studied microbial communities swabbed from ten cave surfaces in one room of Kartchner Cavern, Arizona, which is a karst cave located in a semi-arid environment similar to ours in LABE. Previous studies of this oligotrophic cave had shown unexpected microbial diversity associated with speleothems [59, 60]. They identified 21 phyla and 21 candidate phyla that grouped into three distinct community profiles; one dominated by *Actinobacteria*, one by *Proteobacteria*, and a third by *Acidobacteria*. Overall the community dominated by *Actinobacteria* had the lowest diversity and the *Proteobacteria* dominated had the more diverse community. The breakdown of phyla in the study by Ortiz *et al.* [24] of carbonate speleothems showed 46% of the bacteria were unclassified (in contrast to 5% unclassified surface and 3% unclassified cave at the phylum level in our study). The difference in unclassified bacteria has to do largely with better bioinformatics in the last few years. They concluded that speleothem communities are very sensitive to subtle variation in nutrient inputs and environmental factors. The taxonomic profile from this study [24] differed from that reported for nine other speleothems [59], supporting high variability among samples.

In a second study, Ortiz et al [25] collected surface soils from three locations above Kartchner Cavern. Analysis of their three soil samples was limited to qPCR for comparison of domain distributions within their cave samples. They found bacterial abundance in the cave was comparable to their soil samples, but *Archaea* were significantly higher in the cave, and fungi were below the detection limit in cave samples. Metagenomic analysis suggested that the speleothems they tested supported a unique chemoautotrophic community based on nitrogen with potentially novel nutrient cycling pathways. Comparison of cave samples with one of the soil samples from above the cave showed an overlap of 16% between cave and surface [24], which is comparable to our study of seven cave and surface soil samples, with an overlap of 11.12% at the OTU level. Our study and the Ortiz *et al.* [24,25] studies support the hypothesis that cave bacterial communities are significantly different from surface soil bacterial communities.

Another study that also examined surface soils was Wu *et al.* [26]. They found carbonate cave wall communities dominated by *Gammaproteobacteria* and *Actinobacteria* and identified related groups that use atmospheric carbon and inorganic nitrogen. Sinkhole soil was comparable to surface soil and more closely resembled aquatic sediment communities than cave wall communities.

The soil clearly can be a source of bacteria for colonization of the underlying caves; however, the actual overlap in OTU is only 11.2%. Microbes in caves enter from the surface through drip waters, air currents, gravity, floodwater, or human or animal activity, but quickly adapt under the selection pressure of oligotrophic and mineral conditions of lava caves as evidenced by the low level of overlap between cave and surface OTUs. It appears that over time, these communities become very different as the bacteria adapt to the cave and those that can't adapt die off. As an example of possible cave-adaptation, Snider *et al.* [61] compared UV sensitivity in bacteria isolated from Carlsbad Cavern, NM, compared to isolates from surface

soil and rocks. Most of the cave isolates were more sensitive to the effects of UV than surface bacteria, but many of the cave microbes retained their ability to repair UV-induced DNA mutations.

Actinobacteria are more abundant in cave samples

A major difference between surface and cave bacterial communities in our study, and in comparison to the Ortiz *et al.* [24] study is the abundance of *Actinobacteria*. While *Actinobacteria* occurred in moderate numbers in surface soils in our study (21%), they occurred in much higher numbers in cave samples (39%). Surface soils were also dominated by *Gammaproteobacteria* (18%) and *Alphaproteobacteria* (17%). *Actinobacteria* are also probably the dominant forms seen in the scanning electron micrographs (Fig 10).

The scanning electron microscopy (SEM) studies of white and yellow-pigmented samples from Caves S-L280, L-V460, and Hopkins Chocolate, showed many commonalities with SEM studies in other lava and carbonate cave studies around the world. Notably, the beads-on-a-string morphology has been found in Lechuguilla and Spider Caves in Carlsbad Caverns National Park [62] lava caves [57,64], Hair-like extensions, which may be pili/fimbrae, have been commonly found in other lava caves in the Azores [57,64] and in Hawai`i [21]. The filaments that are covered with hair-like extensions with spheroids on the tips are a novel morphology in our lava cave studies (Fig 10A,B). As with our other lava cave microbial community studies, LABE microbial mats display a range of interesting morphologies that overlap with these other studies.

Actinobacteria include organisms that give caves their musty odor due to the chemical geosmim [65]. Barka *et al.* [66] recently reviewed *Actinobacteria* taxonomy, physiology, and secondary metabolite production. *Actinobacteria* comprise the largest phylum among the Bacteria and are now subdivided into six classes, but about 80% are members of the class *Actinobacteria*. They are Gram positive with high G+C ratios and have a wide ecological

distribution in aquatic and terrestrial environments, and survive periods of nutrient depravation by production of exospores. Most *Actinobacteria* are heterotrophic, feeding on organic carbon, and some are known to fix nitrogen both as symbionts and free-living. New studies have also established that they can be chemolithoautotrophic, such as in the case of the uncultured T3 subdivision of *Actinobacteria* that have been shown to exhibit nitrate-dependent iron oxidation [67].

Actinobacteria are key members of the microbial community in caves (e.g. [68]). Snider *et al.* [69] showed that *Actinobacteria* within two lava caves with roots penetrating the ceiling tended to be where more moisture is available, and their numbers fall off substantially in drier areas of the caves.

Some evidence exists that the coloration of the microbial mats may be produced by the bacteria present, in particular *Actinobacteria*. Production of melanoid pigments varies by strain, nutrients, and age [66]. Pigment colors range from red, yellow, orange, blue, green, and black [66]. Porca *et al.* [68] also reported that several *Pseudonocardiaceae* (relatives of their OTU group III) were shown to produce yellow pigments [70,71] which could result in some of the coloration observed. Lee [72] identified two new members of the *Peudonocardiaceae* from soil and dry bat guano from a cave in the Republic of Korea that produced yellow and grey-white colored colonies. One note of caution is that universal bacterial primers have been shown to be unable to amplify the 16S rRNA gene in many *Actinobacteria* despite 100% homology to the primers [73]. Thus, it is entirely likely that both our study and others are missing a portion of the *Actinobacteria* present in the environments sampled.

Within the *Actinobacteria* recovered from our cave samples, the dominant organisms belonged to the *Pseudonocardiacea.* Barton *et al.*, [74,75] hypothesized that the *Pseudonocardiaceae*-related phylotypes found at one of their sites are degraders of plant

matter coming into the caves. The *Pseudonocardiacea* are also known to produce a variety of secondary metabolites [76].

A comparison [24] of *Actinobacterial* diversity in volcanic caves across four locations (HI, NM, and two islands in the Azores) showed dominance and exceptional diversity of *Actinobacteria*, with 62% of OTU having less than 97% similarity to known sequences. Of the *Actinobacteria*, 71% were singletons. Five of the OTUs (3.05%) represented 74.1% of sequences, with the most predominant family the *Pseudonocardiaceae* from all four locations.

Spilde et al. [62] sampled microbial communities in Hawaiian lava caves across a range of rainfall (47-401 cm per year). They sampled white, yellow, and unique pink-orange mats, along with white mats floating on water, and organic ooze. Of the microbial mats from the walls, white mats had the greatest diversity, while the yellow and pink-orange mats were dominated by *Actinobacteria*. Yellow and pink-orange mats were the most similar. The major diversity agrees with the patterns reviewed in Table 3.

Results of these studies and our LABE study suggest a common core of phyla in caves from around the world, reflecting a subset of phyla from the overlying soils.

Other bacteria of interest in lava cave microbial mats

In addition to the *Actinobacteria*, the *Gammaproteobacteria*, in particular the orders *Xanthomonadales* and unclassified *Gammaproteobacteria*, were slightly elevated in cave samples (20%) in comparison to surface soil communities (18%). The order *Xanthomonadales* shows up in other cave studies on NCBI (i.e accession numbers: DQ066611, FJ347998, HM592533). A newer and not well described taxon, *0319-6A21*, shows up with much greater abundance in the lava tube caves then in the surface soils. *0319-6A21* was first isolated in 2004 from Australian desert soil [77]. *0319-6A21* is classified

as belong to the class *Nitrospirales* and is likely to provide clues into nitrogen cycling in caves. *Chitinophagaceae*, from the phylum *Bacteroidetes* are elevated in soils where insect chitin is higher than in the cave, a defining characteristic of this family [49], although new isolates from oligotrophic lake waters lacked this ability [50].

These results, together with the elevated *Pseudonocardiacea*, parallel the findings of Porca *et al.* [68] who found these groups to be major constituents of microbial communities in their studies of yellow colonies in carbonate caves in Spain, Slovenia, and the Czech Republic. Their study revealed three major core groups that included the *Pseudonocardiacea*, the *Chromatiales* and the *Xanthomonadales*, which Porca *et al.* [68] suggest may be "true cave dwellers." One of the intriguing things in the study by Porca *et al.* is that their clone sequences were sometimes 98-99% similar to clone sequences from another of our lava cave studies [21].

One of the few lava cave microbial mat papers that extends the discussion of bacterial diversity to the level of genus is the Hawaiian study from 16 caves by Spilde *et al.* [62] The samples included white and yellow microbial mats along with pink-orange mats. At the genus level they reported an abundance of *Bacillus*, which were not abundant in our LABE *Nitrospirae*, and two new genera of *Actinobacteria*; *Crossiella* and *Euzebya*.

One bacterial community is clearly different

Valentine 13 (Fig 5) is clearly not typical of either cave or surface samples. *Betaproteobacteria* (36.92%) dominate Valentine sample 13, while *Actinobacteria* make up only 8.56% of the total phyla. At the L6 level, only 1.44% of sequences are *Pseudonocardia*. Several variables may account for the differences. The site is located at a junction where the passage splits into two tubes. Very near the sample site is a large pile of breakdown, the only occurrence of breakdown of this size in the entire cave. The breakdown includes packed, wet sediment that has fallen through cracks in the ceiling above the pile. LABE personnel have

observed millipedes and springtails around the edges of the breakdown indicating a small connection with the surface. The site is wetter than other locations in the cave. We speculate that the ceiling connection may be a source of microbes or nutrients unique to this site. The shape of the passage, with a higher ceiling at the collection site, may also direct more visitors to pass by the sample site, possibly also affecting the community structure or nutrient inputs. The floor also changes from pahoehoe to a'a lava at this point, perhaps indicating a change in rock chemistry. Regardless, we kept the sample in for analysis, and point out the need for multiple samples to mitigate such variations.

Differences in bacterial diversity among differently colored mats

Mat color composition did not appear substantially different among the colors. However, some minor interesting differences were observed. Members of the *bacteria* dominated lava cave microbial mats of all colors. The *Actinobacteria* vary somewhat by mat color (Fig 5), with 37% in tan mats, 39% in white mats, and 44% in yellow mats. *Gammaproteobacteria* varied the most by color with 19% in tan mats, 37% in white mats, and 21% in yellow mats.

Northup *et al.* [14] compared white and yellow microbial mats and selected secondary minerals based on color from four lava caves in each of three different locations; tropical and semi-arid lava caves in Hawai`i, temperate lava caves in the Azores, and semi-arid lava caves in New Mexico. Mats are more extensive in areas with greater rainfall. They found 13 Phyla across all white and yellow mats from all three locations. All mats had *Actinobacteria*; *Alpha-, Beta-, Delta-,* and *Gammaproteobacteria*; *Acidobacteria*, and all but one had *Nitrospirae*. The number of phyla per cave ranged from 5-11, with slightly greater diversity at the phylum level in yellow over white mats, but not in all phyla.

The study by Hathaway *et al.* [21] was the first in-depth comparison of white and yellow microbial mat communities from lava caves on different archipelagos, and concluded that geographic location is important in determining the composition of microbial communities,

with Hawai`i showing greater diversity than Terceira. These differences may be partially due to the greater range of habitats in Hawai`i, ranging from semi-arid to tropical rainforest conditions. Novel bacteria were found in all sites showing the need for conservation of caves as sources of novel bacteria and as simplified natural ecosystems for study of larger ecological questions.

Principle Coordinate Analysis (PCoA) by island showed that only 13.7% of the variability was explained by geographic location and levels of nitrogen, organic carbon, and copper. Rainfall, especially in Hawai`i, accounted for another 10.8% of the variability. PCoA by cave showed a strong influence of geography. *Actinobacteria* (16%) dominated the clones recovered from Hawaiian samples, while on Terceira *Acidobacteria* dominated with 21% of the clones recovered. *Alphaproteobacteria* made up 13% of the diversity in Hawaii and 15% in Terceira. Bacterial sequences recovered with no known phyla were 14% of the total in Hawai`i and 12% in Terceira. A nearest neighbor comparison among caves in Hawai`i and Terceira with other caves in the ARB database showed only thee OTUs (0.22%) overlap. This high level of alpha diversity means that bacterial communities found in each cave are different. These differences may be partially due to the greater range of habitats in Hawai`i, ranging from semi-arid to tropical rainforest conditions.

Riquelme *et al.* [57] studied white, yellow, and tan mats from 14 caves on two islands in the Azores using 16S rRNA clone libraries. Environment and chemistry showed no relation to OTU diversity and composition of the microbial mats. Similar to other studies including ours, there was a dominance of cosmopolitan OTUs. There was a greatest influence on β diversity (composition) between islands and caves than α diversity (community). The absence of clear differences across mat colors they suggested could be due to insufficient geologic time on these islands for microbial communities to diversity, and/or covergent evolution due to the selective pressure of extreme environments in caves.

We predict that a comparison of LABE microbial mats with those of the Azores, New Mexico, and Hawai`i, as next gen sequencing becomes available for these sites, would show lower diversity in LABE than in Hawai`i in particular. Color of the mat was not a predictive factor in diversity, much to their surprise, but is consistent with our findings in LABE.

A study of Icelandic lava caves by Northup *et al.* [78] compared surface soil microbial communities with microbial communities of different types (mats, slimes, snottites, organic oozes, and soil) from four lava caves. Surface soils were the most diverse at two sites. As in our study the mats were similar at the phylum level with *Actinobacteria* dominant followed by *Acidobacteria*, and *Alpha-, Beta-,* and *Gamma- Proteobacteria*. Unlike our study there was a lack of *Nitrospirae* in the Icelandic caves—which may be due to cold They found sample type to be the most important factor in bacterial diversity.

Porca *et al.* [68] compared yellow microbial communities on the walls of karst caves and found a common core of microorganisms that they compared to the Azorean and Hawaiian yellow microbial mats reported in Northup *et al.* [14]. They studied three geographically distinct caves from Spain, the Czech Republic, and Slovenia. Sixty percent of the OTUs formed three cores common to all three caves made up of *Actinobacteria* and two within the *Gammaproteobacteria* sequences. Seven percent were common to two of the sites, and the remaining 35% were site-specific. Several estimates of diversity showed the greatest diversity from Slovenia, then Czech Republic, then Spain. There was no leveling off of diversity below the phylum level. They concluded that the overall similarities of these and results from other studies reflects the similarity of limestone caves in terms of environmental conditions, geochemistry, and availability of organic matter to support microbial growth and may represent a group of true troglobiont microorganisms.

Abiotic variables impact bacterial diversity differences

We selected our study caves to include as broad a range of abiotic variables possible among

LABE caves. Richness by environmental variable (S1 Fig) shows an effect only with Distance from the nearest entrance and Temperature, but the two factors are related. As you go deeper into a long enough cave the temperature reaches the stable temperature around the man annual surface temperature, as modified by many factors, particularly cave geometry. The remaining environmental factors of age of lava flow, elevation a.s.l., length of the cave, branch complexity (nodes), and pool water pH, have no significant effect on cave microbial diversity.

Cave geomicrobiological studies show that caves are nutrient-limited with redox interfaces in microniches where we see the interaction of microbial activities and minerals [79,80]. Interactions with minerals may be the dominant force driving microbial diversity in lava caves. An experimental study by Jones and Bennett [81], using a range of mineral substrate types with two different cave microbial inocula, found that under nutrient-limited conditions different microbial communities can develop at the level of individual mineral grains. The nature of mineral composition selects for the microorganisms that can grow on a given rock. Rocks and minerals are not homogeneous and can influence microbial diversity at the microniche level. Components of the substrate may be toxic or beneficial to microbial growth by providing mineral nutrients, pH buffering, or other advantageous conditions. Given the long evolutionary history of microbes in geologic time, Jones and Bennett [81] hypothesize that each mineral surface is specifically altered by the best-adapted and most comprehensive microbial community that can use the mineral surface to the greatest advantage. While the surface may be a source for immigration of microbes, most are unable to adapt to the extreme cave environment. Isolation from the surface and from other caves allows for adaptation and the evolution of novel taxa. Further study of lava geochemistry at LABE is needed to test this hypothesis in the caves, but it seems likely that mineralogical factors do influence the within cave diversity differences.

Levels of PO_4^-, SO_4^{2}, NO_3^- in LABE lava caves were compared to those reported in lava caves

from Terceira Island, Azores [82]. Phosphate levels in LABE samples are low, but much higher than in Terceira (ranges LABE 0.4697-0.6715 ppm vs. Terceira 0.003-0.0696 ppm), much lower in sulfate (ranges LABE 0.3251-1.1231 ppm vs. Terceira 1.42-37.29 ppm), and higher in nitrate (ranges LABE 0.5483-8.0036 ppm vs. Terceira 0.08-6.92 ppm). The nitrate results are unexpected given the large number of cattle roaming the surface above Terceira lava caves. Pools in Lechuguilla Cave [83], a carbonate cave in NM, varied depending on isolation from environmental factors. Levels of all measured minerals did not vary much over time in isolated Lake Louse, but did in Lake Lechuguilla, which is nearer the entrance. In 2006 in Lake Louise, levels of Cl^- were 4.16 ppm, SO_4^{2-} 34.7 ppm, $N\text{-}NO_3^-$ 1.49 ppm, and $N\text{-}NH_3$ 3.05 ppm compared to Lake Lechuguilla with Cl^- 3.65 ppm, SO_4^{2-} 34.7 ppm, $N\text{-}NO_3^-$ 1.65 ppm, and $N\text{-}NH_3$ <0.03. Levels in Lake Lechuguilla spiked as high as Cl^- up to about 45 ppm, SO_4^{2-} up to about 240 ppm, and $N\text{-}NO_3^-$ up to about 40 ppm.

Dissolved organic carbon (DOC) in standing waters in LABE is compared to DOC in water samples from four lava caves in the Azores and in Hawai`i, and carbonate caves within Carlsbad Cavern National Park (Fig 4). Results across the caves are comparable, showing low levels of DOC from all of the caves sampled, with the lowest overall levels of DOC from Lechuguilla Cave which are always <1-1.7 ppm [83]. LABE samples ranged from 3.7-17.3 ppm DOC and showed a greater range than observed in the other sites. A study of ecosystem dynamics in a karst cave in the Ozarks [84] showed DOC levels ranging from 0.77-3.4 ppm over the course of a year, and a relationship (r^2= 0.85) between DOC and peak microbial biomass. DOC inputs in drip water into Kartchner Cavern ranged from 1.5 to 9.5 ppm [24] and 0.5 to 2.7 ppm over a year [25]. We correlated LABE DOC with species richness and found no correlation, but our water sources are transient pools which are often no assocated with microbial mats.

Thurman [85] reports the range of DOC in groundwater from 0.2-15 ppm with a median value of 0.7 ppm and most less than 2 ppm. Barton and Jurado [75] defined cave waters

below 2 ppm as oligotrophic. By these standards, our LABE water samples ranged from oligotrophic to mesotrophic. In addition to drip water with dissolved organic carbon, organic carbon enters the LABE caves as occasional debris, like sticks, grass stems, and human-associated debris.

Cave biologists are recognizing the importance of DOC as the base of the food chain, supporting the growth of microbes, rather than the traditional inputs of organic debris [23]. Chemoautotrophic contributions were once thought to be negligible in most caves have been shown to be major contributors in some ecosystems [86], and likely contribute to the food base in many caves.

Nitrogen cycling bacteria in LABE caves

Nitrogen is often a limiting nutrient in oligotrophic environments. Our cave sequences document the presence of several key organisms in the nitrogen cycle, some of which occur in elevated numbers in comparison to surface soil samples. The *Nitrospirae* contain nitrite oxidizers, the *Alphaproteobacteria* include nitrogen fixers, and *Betaproteobacteria* contain ammonia oxidizers, all key players in the nitrogen cycle. A new genus of *Nitrospira* has been described by Daims *et al.* [87] that can complete the entire nitrification cycle, taking ammonia to nitrate. We will investigate whether this species is present in our samples once primers become available. Differences from cave to cave could be further investigated with a study of nitrogen available in the basaltic matrix and from infiltrating waters. Nitrite levels in our limited sample (Table 2) were largely low, but three caves had slightly elevated nitrate levels. S-L280 at 8.0036 ppm, L-V460 at 2.7485 ppm, and Hopkins Chocolate at 1.5608 ppm were above the limit for drinking water of 1.0 ppm, and may indicate either surface inputs with nitrate or *in situ* production in these caves.

We found the *Nitrospirae*, which contain nitrite oxidizers and a *Nitrospira* sp. that can carry out complete nitrification, to be higher in cave samples (7%) in comparison to surface soil

samples (3%). *Nitrospirae* OTUs varied from 9 to 1064 across different caves and colored mats, with the highest number of OTUs occurring in tan mats from Golden Dome and GE-L350 Caves. Other nitrogen cycle bacteria of interest in our samples are the *Alphaproteobacteria*, which contain nitrogen fixers, and the *Betaproteobacteria,* which contain ammonia oxidizers, all key organisms in the nitrogen cycle. Previous studies have suggested that nitrogen is low in cave environments (i.e. [14,88]) and the ability of microorganisms to cycle nitrogen is key to supplying community needs in an oligotrophic environment. The fact that these phyla vary from cave to cave is of interest and suggests that a study of the nitrogen available in the basaltic substrate and additional studies of infiltrating water would be useful.

Other studies have suggested the importance of nitrogen-based systems in caves. Hathaway *et al.* [82] investigated the diversity of ammonia oxidation (*amoA*) and nitrogen fixation (*nifH*) genes in lava caves of Terceira, Azores, Portugal. They found that *Nitrosospirae* related sequences dominated the ammonia-oxidizing bacteria and that a key nitrogen fixation gene, *nifH,* was found among *Klebsiella pneumoniae*-like sequences (*Gammaproteobacteria*). Tetu *et al.* [88] found evidence that microbial slime curtain communities in the submerged Weebubbie Cave under the Nullarbor Plain in Australia had primary productivity based on the combined activity of ammonia-oxidizing *Archaea* and bacterial nitrite oxidizers, especially *Nitrospirae*. These studies support the importance of groups, such as the *Nitrospirae* and *Proteobacteria* in nitrogen cycling in caves.

Actinobacterial diversity in cave samples and the search for bioactive compounds

Caves are an extreme habitat and the cave microbiome has great potential as a novel resource for drug discovery [64,66,90,91]. Antimicrobials are one example of secondary metabolites produced by microorganisms, but Zheng [93] broadened our understanding of the role of these products as toxins, ionophores, bioregulators, and signal molecules produced by "metabolically talented" microbes. Actinobacteria produce 2/3 of antibacterial agents in use,

but are also important in biotechnology, medicine, and agriculture. A review of their extensive secondary metabolites by Barka *et al.* [66] include antibacterials, anifungals, antivirals, antihelminths, antitumor, immunosuppressive and immunostimulatory agents, biopesticides, herbicides, and plant growth promoters. Natural products are the most promising source of novel antibiotics and cave environments have great potential for the development of new bioactive microbial metabolites especially from *Actinobacteria*, which are dominant in lava caves [64].

The opposite of antibiotic production is antibiotic resistance; both are natural processes. A study of cultivatable oligotrophic isolates from Lechuguilla Cave, New Mexico, showed high levels of antibiotic resistance in an environment isolated from anthropogenic surface inputs for four million years [93]. Of 93 isolated strains from an oligotrophic environment, most were multiply drug-resistant to the 14 tested commercially available antibiotics. Resistance was more common against natural antibiotics, but not to all. There was little resistance shown to synthetic antibiotics. *Streptomyces* isolates overall showed the highest levels of resistance. The authors concluded that antibiotic resistance is natural and ancient, and "hard-wired" in the microbial genome. If bacteria produce antibiotics to reduce competition for scarce resources, then oligotrophic cave environments are good places to look for production of novel bioactive metabolites.

Impact of human visitors on bacterial diversity

We divided our LABE study caves into high visitation which are visited by about 30,000 people a year, and low visitation which have controlled access and are rarely visited by researchers and park personnel, perhaps receiving up to 10 visitors some years. High and low visitation (Table 1A), are comparable in terms of alpha diversity and show no significant differences in microbial community structure. When you make the jump to Carlsbad Cavern with over 400,000 visitors per year, Griffin *et al.* [94] reported significant human impacts of visitors on the microbiota. Using culture techniques they reported that *Staphylococcus* spp.

were the dominant bacteria in the air along tourist trails compared to *Knoellia* spp. off trail. *Knoellia* is a new genus of *Actinobacteria* first isolated from a cave in China [95]. Fungal spores of *Penicillium* and *Aspergillus* showed a general decrease with distance from the entrance, but with a peak in the lunch room where visitors rest, eat, and wait for the elevator to return to the surface. A second study in Griffin *et al.* [94] using molecular techniques showed *Enterobacteriaceae* dominating along the descent rail and in the lunchroom. They concluded that humans were important sources of non-indigenous microorganisms into Carlsbad Cavern, and recommended mitigation steps. There was only one *Staphylococcaeae* identified, and only 25 OTUs of *Enterbacteriacae* identified to the level of family detected at any LABE cave or surface sample. What we may be seeing is a threshold of visitors before we see human impacts. Right now, that threshold may be somewhere between 30,000 and 500,000 visitors per year, and merits further study.

Conclusions

Overlap in OTUs between surface and cave sampled communities at LABE is only 11.12%, revealing that the subsurface cave bacterial communities are not a subset of the surface soil communities as previously assumed. Of particular significance are the differences in *Actinobacteria*, *Alphaproteobacteria*, *Nitrospirae*, and *Gammaproteobacteria* composition between surface and cave samples. Surface soil diversity is higher than that observed in cave samples and considerable novel diversity exists in both surface soil and cave mat samples. However, because many of the earlier studies are based on clone sequences that are limited in number, this conclusion may change as more next generation sequencing is applied to these sites. Communities in different mat colors do not appear to differ substantially in composition, which is similar to the findings of Hathaway *et al.*[21]. The only environmental factors which did influence microbial diversity in our caves were distance of the sample site from the entrance and temperature, which correlate.

LABE microbial cave community diversity at the phylum and *Proteobacteria* class level is

comparable to diversity found in other major lava cave areas in New Mexico [8,10], Hawai`i [21,62], and the Azores [21,57,64,82]. Communities in different mat colors show significant overlap but also many unique members that may contribute to the color of the mats in our study. Comparison with other lave cave mat studies support a common core microbiome of *Actinobacteria*, *Acidobacteria*, *Nitrospirae*, and *Alpha-*, *Beta-*, *Delta-*, *Gamma-Proteobacteria*.

So what does account for the differences in mat colors in lava caves? Caves are not homogeneous environments. There are zones related to distance from the entrance; seasonal variations; three-dimensional geometry; differential cooling and deposition of minerals in lava caves; and microhabitats which may vary at the level of the individual grain. We think the microhabitat differences and the species composition at the microhabitat scale account for the color differences seen in the microbial mats.

Our study is the most extensive bacterial diversity study of lava caves to date, comparing the bacterial diversity in three colors of microbial mats across seven caves, and with surface soil from each cave. Variability inherent in sampling supports the need for replicated study of microbial community structure. Further studies should examine diversity in other lava cave areas around the world using newer sequencing technologies. There are preliminary indications of world-wide microbial biogeography and we should work to fill in the gaps.

Supporting Information

S1 Fig. Linear models of all environmental parameters plotted against bacterial richness of lava cave microbial mats. Grey area is the 95% confidence interval.

S2 Fig. Alpha diversity indices box plots of surface soils and cave microbial mats by color.

S3 Fig. *Actinobacteria* tree of samples from surface soils and cave microbial mats. The outer

bars are the proportion of each sequence found in either the cave or surface. Approximate maximum likelihood tree.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

Conceived and designed the experiments: DEN. Performed sample collection: DEN. Performed the experiments: KJH, EH, MNS. Analyzed the data: ASW, KHL, DEN. Wrote the paper: KHL, DEN, ASW.

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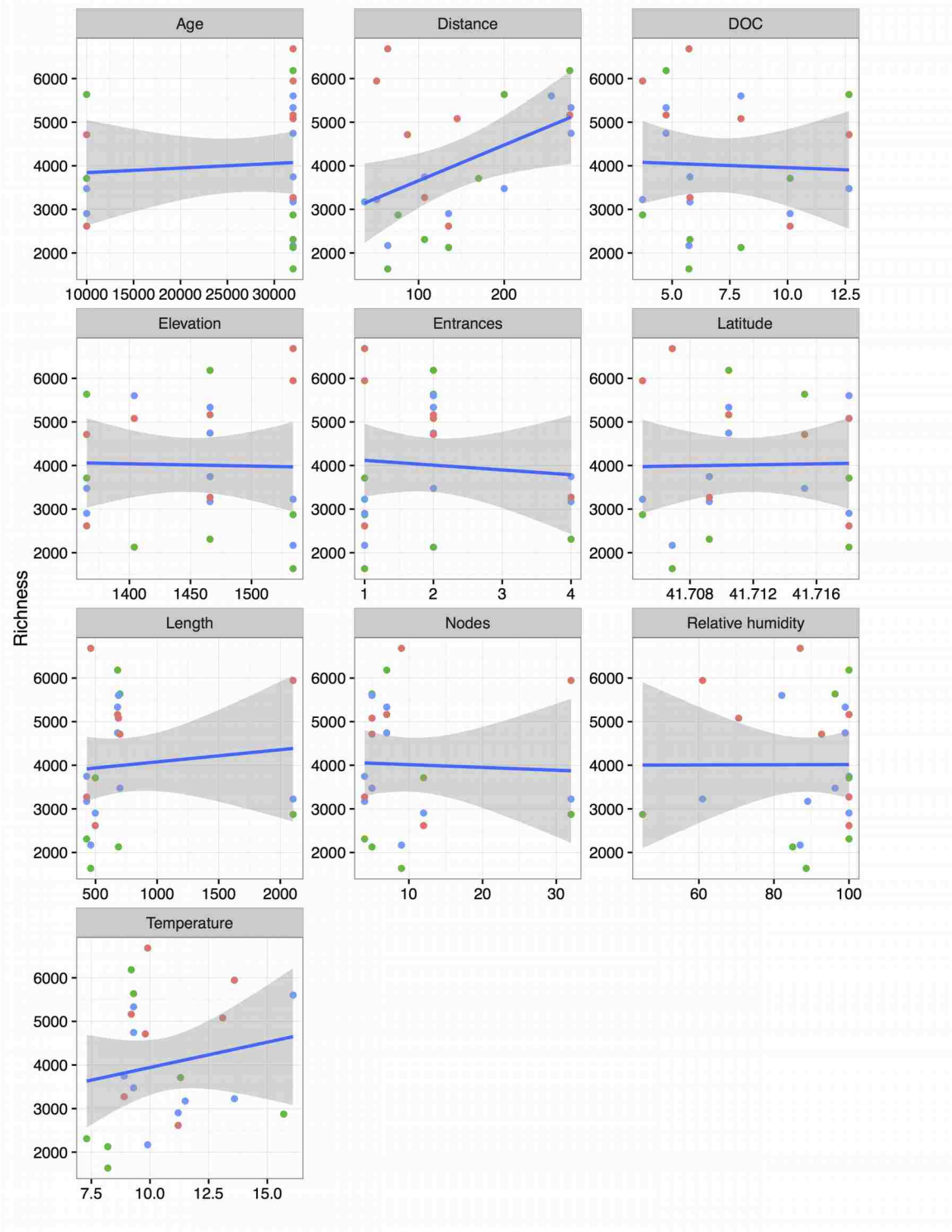
S1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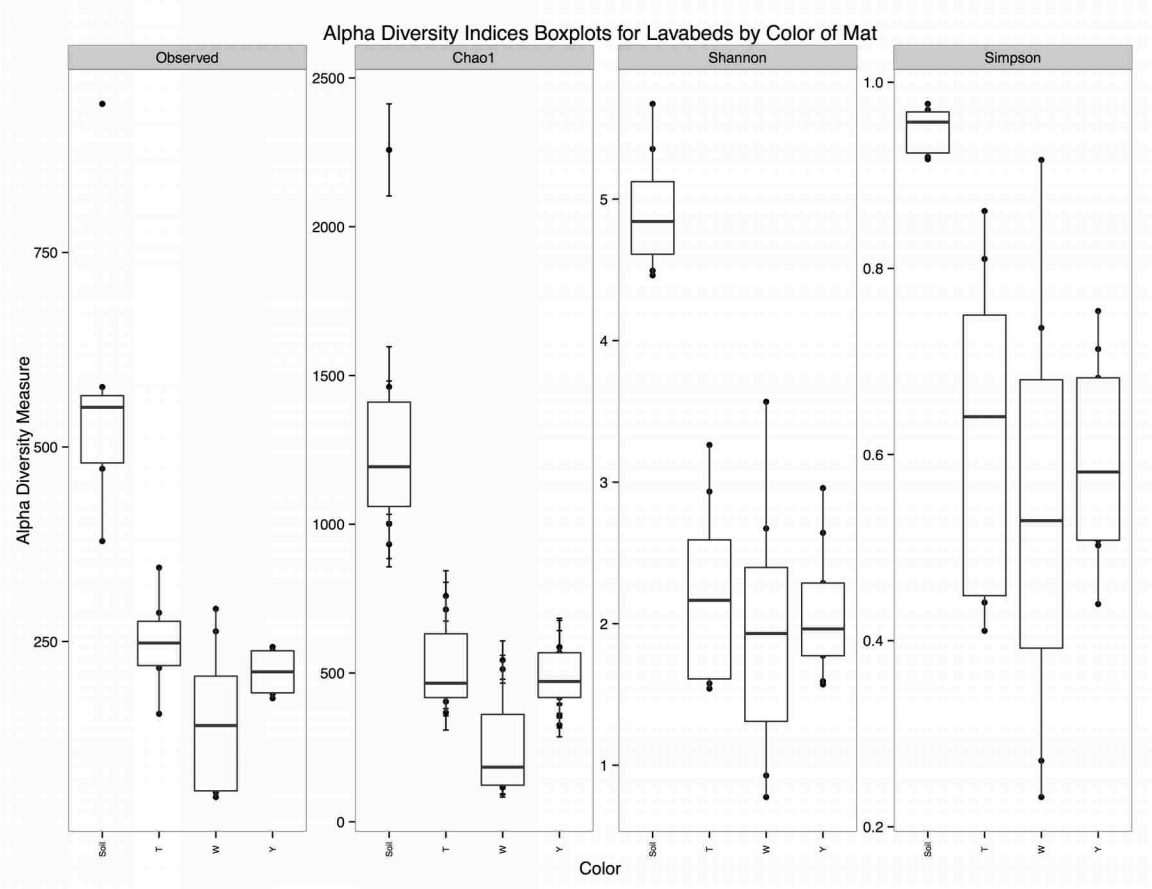
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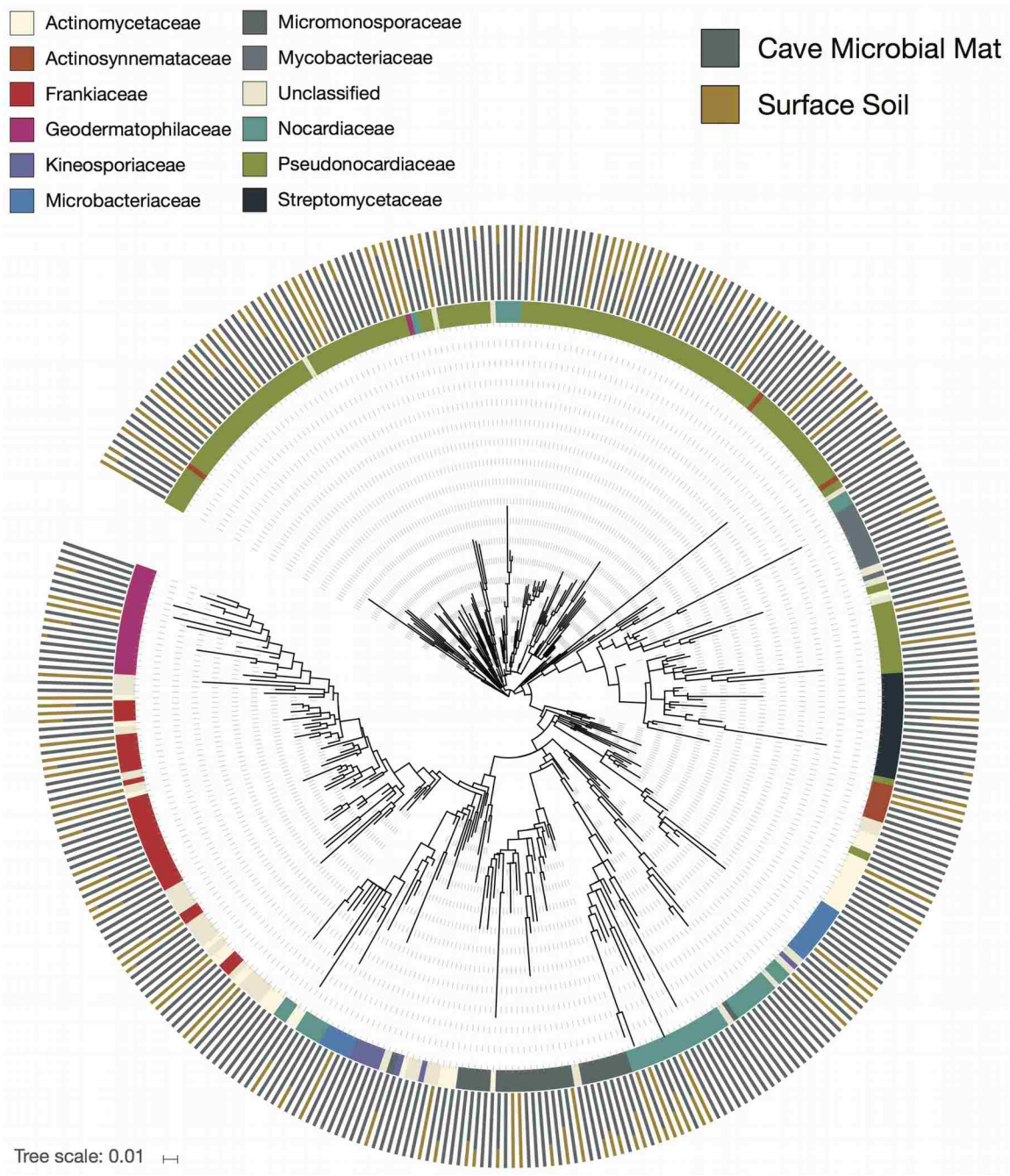
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Cave Microbial Mat
Surface Soil
Tree scale: 0.01

Local and Landscape Factors Affecting the External Bacterial Diversity on Bats in the Southwestern United States

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Abstract

Recently microbiomes of humpback whales, dolphins, shrimp, and external body parts on humans have captured the imagination of scientists and provide a new framework for microbial ecology, drug discovery, conservation management, and important links to macroecology. However, we have little to no understanding of the external bacteria microbiome on bats or factors that influence the structure of these communities. White-nose syndrome (WNS) is a newly emergent disease that results in moderate to very high mortality in affected bats. Our results provide a first insight into the distribution of external bat

bacteria in a pre-WNS environment. For this study we used 16S rRNA gene 454 pyrosequencing on 186 bats from 14 species sampled across southeastern New Mexico to northwestern Arizona. The microbial communities on bats in the region were highly variable with representatives from Actinobacteria, Firmicutes, Nitrospira, and Cyanobacteria. The patterns could be partly explained by environmental and local factors, with cave-caught bats sharing more similar external microbial communities based on Bray-Curtis dissimilarity. Bats caught in caves had a distinct microbial community by compared to those that were netted on the surface. Our results also suggest that bats caught in the cave have a more homogenized external microbiome.

Introduction

There are approximately 45 species of bats that occur throughout the continental United States, with over half of these species found in the Southwest (Humphrey, 1975; Hall, 1981) (Figure 1). The high diversity of bats in the Southwest, particularly in southern

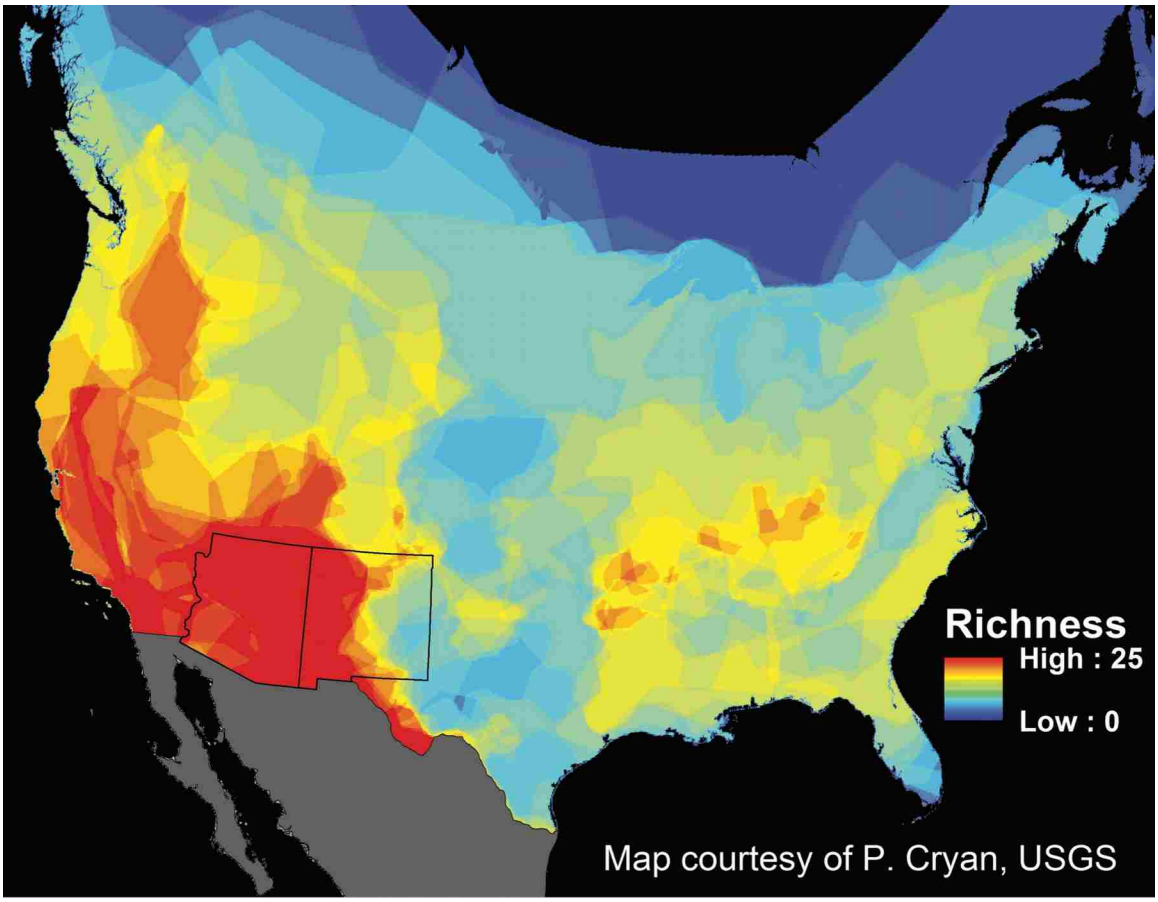


Figure 1. Map of bat species richness in the United States (US) and Canada. Total number of bat species occurring in each area calculated by counting the number of overlapping species distributions, as represented by the US National Atlas Bat Ranges geospatial data set (available at https://catalog.data.gov/dataset/north-american-bat-ranges-direct-download). Warmer colors represent areas with higher species richness and cooler colors represent areas with lower species richness. Map courtesy of P. Cryan, US Geological Survey.

Arizona and New Mexico, is attributed to the presence of some species occurring at the northern limits of their range from Mexico (Findley, 1975; Hoffmeister, 1986; Frey, 2004). Ecology and topography of the Southwest (e.g., Colorado Plateau and Sky Islands) also likely contribute to suitable habitat that is used by many bat species for roosts. For example, several species such as the spotted bat (*Euderma maculatum*) often use crevices in high, sandstone

cliff faces for day roosts (O'Shea et al., 2011).

White-nose syndrome (WNS) was introduced into the eastern region of the United States 10 years ago (Frick et al., 2010). To date this disease, caused by a cold-loving fungus (*Pseudogymnoascus destructans*), has killed millions of hibernating bats in the East and is spreading westward. Given the high diversity of bat species in the western and southwestern United States, the potential threat to bat diversity at a regional scale is very high. *P. destructans* is a novel species for cave ecosystems in North America and it is likely affecting the natural microbiota of bats and caves. It is likely that some of the naturally occurring microbiota found on bats have undergone various interactions with other novel microbiota over time (Phillips, et al., 2012), with the present faunal composition representing the more resilient or even beneficial species to the ecosystem or organism where they reside. However, most current microbiome studies on bats focus on the gut or fecal microbiome (Carrillo-Araujo, et al., 2015; Borda, et al., 2014), and knowledge on a regional scale of the external bat microbiome in a WNS-free area is lacking. The influence of local factors including abiotic and biotic variables in geographic patterns of the bat external microbiome at the local and regional scale still needs to be determined.

In this study we used 186 bats collected from southeastern New Mexico to northwestern Arizona (Figure 2) to gain insights into regional scale patterns of external bat

Figure 2. Map showing the general locations of the sampling sites in the southwestern United States. PARA (Grand Canyon Parashant National Monument), ELMA (El Malpais National Monument), FS (Fort Stanton-Snowy River Cave National Conservation Area), HGL (High Grasslands), CCNP (Carlsbad Caverns National Park). Elevation base map by Stamen, CC-BY OpenStreetMap Terrain.

bacteria and the factors that drive these patterns. Specifically, we address two questions: First, to what extent are the changes in distributions of bat bacteria a function of geographic location, ecoregion (Omernik and Griffith, 2008), and climatic variables. Second, we examined whether being cave-caught (6-8 hours in the cave before capture), in contrast to surface-netted, had a significant effect on the external bacteria community on bats. This may be significant given that bats are susceptible to WNS while hibernating in caves and differential exposure to microbes might explain differing levels of susceptibility.

Methods

Sampling. We sampled 186 bats belonging to 14 species using 16S rRNA gene analysis for external microbiome identification. These samples came from a total of five study locations in the Southwest: Grand Canyon-Parashant National Monument (PARA), in Arizona, and Carlsbad Caverns National Park (CCNP), Fort Stanton-Snowy River Cave National Conservation Area (FS), El Malpais National Monument (ELMA), and Bureau of Land Management high grasslands (HGL) caves near Roswell, in New Mexico. Bat sample collection was allowed under the following permits: 2014 Arizona and New Mexico Game and Fish Department Scientific Collecting Permit (SP670210, SCI#3423, SCI#3350), National Park Service Scientific Collecting Permit (CAVE-2014-SCI-0012, ELMA-2013-SCI-0005, ELMA-2014-SCI-0001, PARA-2012-SCI-0003), Fort Collins Science Center Standard Operating Procedure (SOP) SOP#: 2013-01, and an Institutional Animal Care and Use Committee (IACUC) Permit from the University of New Mexico (Protocol #15-101307-MC) and from the National Park Service (Protocol #IMR_ELMA.PARA.CAVE.SEAZ_Northup_Bats_2015.A2).

Samples were collected from 2012 through 2014. Cave-caught bats were plucked from the walls of the caves in ELMA, FS, and HGL and netted in Carlsbad Cavern in CCNP in a location along their flight path out of the cave. Cave-caught bats were typically sampled 6-10 hours after returning to the cave in the early morning. Surface-caught bats were netted using sterilized nets near water sources in CCNP, ELMA, FS, and PARA. Captured bats were handled with clean gloves and swabbed for DNA before other measurements were taken to limit contamination by human-associated microbiota. The skin (i.e., ears, wings and uropatagia) and furred surfaces of each bat were swabbed with sterile swabs soaked in sterile Ringer's solution (Hille, 1984). Each swab was placed in a sterile 1.7ml snap-cap microcentrifuge tube containing 100 ul of RNAlater, and immediately frozen in a liquid nitrogen dry shipper or placed on dry ice. Samples were transported to the University of New Mexico and stored in a -80°C freezer. We used MR DNA Molecular Research LP, Shallowater, Texas (http://www.mrdnalab.com/) for genomic DNA extraction and 454 sequencing diversity assays of bacterial 16S rRNA (27F universal bacterial primer). The 186 samples were sequenced in nine runs. Barcoded amplicon sequencing processes were preformed by MR DNA® under the trademark service (bTEFAP®). The 16S rRNA gene 27F PCR primers were used in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycle used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Sequencing was performed at MR DNA on an Ion Torrent PGM following the manufacturer's guidelines.

454 Processing. All 454 reads were processed in QIIME (Caporaso, et al., 2010). Bacterial sequences shorter than 200 bp or longer than 500 bp were exclude and bases with a quality score lower than 30. The quality control and trimming was carried out using the split_libraries command. Bacterial samples were denoised and clustered (at the 97% level) with pick_denovo_otus.py pipeline using the sumaclust option (Mercier, et al., 2013).

Chimera checking was done using usearch (Edgar, 2010) to detect artifacts created during sequencing. Taxonomy was assigned using the Greengenes 13_8 core data set (McDonald, et al., 2012) with uclust. This yielded a total of 186 bacterial 16S rRNA gene samples.

Statistical analysis. Alpha diversity indices were carried out in QIIME using alpha_diversity.py command. NMDS analysis was carried out using the phyloseq package (McDonald, et al., 2012 McMurdie and Holmes, 2013) and ggplot2 (Wickham, 2009) in R (R development core team, 2012). Beta diversity was analyzed using non-metric dimensional scaling (NMDS) with the Brays-Curtis distance. The Brays-Curtis distance was picked because it is invariant to changes in units and unaffected by additions and removals of species, and NMDS was chosen because it entails fewer assumptions about the data. Random forest models were run in QIIME (supervised_learning.py) using 10-fold cross-validation with 1,000 trees. Random forest models, a type of supervised classification, was used to test the predictive power of the ecological variables for the NMDS. The goal of random forest model is to classify unlabeled communities based on a set of labeled training communities. This will generate a ratio of estimated generalization error and baseline error. A reasonable ratio of the estimated generalization error compared to the baseline error should be two or greater, i.e. the random forests classifier does at least twice as well as random guessing for an unlabeled community. Mantel tests were carried out using the vegan (Oksanen, et al., 2007) package in R with 999 permutations. Multiple regression on distance matrices (MRM) was done in the ecodist (Goslee and Urban, 2007) package in R with 1000 permutations. Bayesian t-test was carried out in the BayesianFirstAid (Bååth, 2013) package for R using bayes.t.test. The paired geographic distance matrix for these analyses was calculated from the latitude and longitude using an R function written by Peter Rosenmai, last accessed at: http://eurekastatistics.com/calculating-a-distance-matrix-for-geographic-points-using-r March, 4th, 2016. Sorting of the distance matrix was done using the dendextend (Galilee, 2015) package. Retrieval of the paired scores (distance and similarity) was done using an R function from http://stackoverflow.com/questions/21180464/distance-matrix-to-data-frame-pairs-in-r, last accessed March, 4th, 2016. Environmentally associated taxa were taken from

Barberán et al, 2015 with the exception of the freshwater taxa (Newton, et al., 2011) and cave taxa (S5 table).

Biome files, QIIME mapping files, workflow, and R scripts are available at https://github.com/bioinfonm/microBat/tree/batmicrobiom and are archived at https://zenodo.org/record/17577#. All raw sequence data with the quality files and mapping files are available at: [sequence storage link]. Full metadata table is available in the supplemental data (S1 table). Cave names and location are encoded to protect park and BLM resources. The full cave names and sampling locations are protected by law by their respective agencies.

Results and discussion

Microbial diversity on bats. Our study stands apart from culture-based studies and other next generation sequencing studies by focusing on the diversity of the external bacteria from 186 bats (S2 Table). The number of reads after quality control range from 843 to 20515. Sample coverage was measured by calculating the Good's coverage. Good's coverage values ranged from 81% to 99% with an average of 95.3%. The average bat sampled had ~6,000 taxa on its external surfaces out of a total of 36,042 bacterial taxa identified across all bat swabs. OTUs of the 36,042 taxa were assigned to 47 phyla, 157 classes, 340 orders, 576 families, and 1,143 genera of Bacteria. Twenty-seven classes, 113 orders, 292 families and 621 genera had no representatives in the sequence database. The number of different bacterial phylotypes (richness) did not vary in a consistent manner across geographic distance [Surface-netted samples R_m= 0.06, P= 0.0046 ; cave-caught samples R_m= 0.09, P= 0.0039; Mantel test].

Bats sampled from cave-caught bats were dominated by the phylum Actinobacteria, while surface-netted bats were dominated by Cyanobacteria, Actinobacteria, and Alphaproteobacteria. Most of the phylotypes were restricted to relatively few samples with very few shared taxa. Eighty percent of surface-netted bats shared 15 phylotypes. In cave-caught bats, 80% of the samples shared only 8 phylotypes. Across 80% of all bats sampled

only 6 phylotypes were shared and they belonged to the classes Actinobacteria, Flavobacteria, and Gammaproteobacteria. These bacterial classes are widely distributed across a range of environments. Bats are likely exposed to bacteria common in air, soil, and chloroplasts. Given the ability of bacteria to disperse over long ranges one might expect surface-netted bat bacterial communities to be more homogenous than caves but this is not the case. In addition, there were a large number of Chloroplasts hits across all samples.

Common bacteria found in the air above guano piles included: Chryseomonas, Klebsiella, Micrococcus, Salmonella, Staphylococcus, and Streptococcus in a culture dependent study (Borda, et al., 2014) . Bacillus, Enterobacter, Enterococcus, Escherichia, Klebsiella, Pantoea, Pseudomonas and Serratia were found in the gut of the short-nosed fruit bat (Daniel, et al., 2013). On the ocular surfaces (Leigue, et al., 2014) of 36 bats the most common isolated bacteria were Staphylococci, Bacillus, Corynebacterium, Shigella, Hafnia, Morganella, and Flavobacterium. In our study many of the same bacterial genera were present on the external surfaces of bats. The exception was the lack of Chryseomonas, Klebsiella, Samonella, Pantoea, Serratia (found in six samples in low amounts), Shigella, and Hafnia in our samples.

Structuring of Community Similarity Patterns. The bacterial community composition was highly variable within a sampling sites and across geographic regions. Community similarity patterns suggest that factors such as location (e.g. cave-caught vs. surface-netted) and ecoregion help to structure the bacterial communities on bats (Figure 3 and 4). Cave-caught

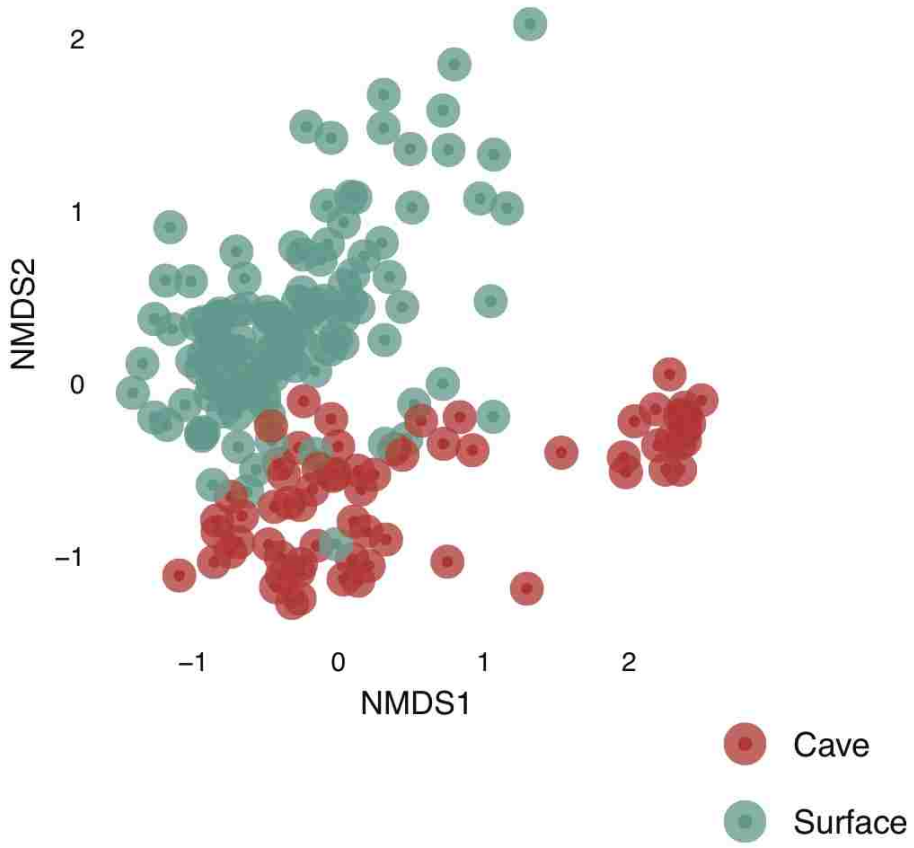


Figure 3. Similarity in the composition of the bacterial communities was quantified using NMDS (stress = 0.084) with the Brays-Curtis distance metric. Symbols are colored by location of capture. Samples closer together represent samples with more similar bacterial communities. The samples tend to cluster by cave-caught or surface-netted.

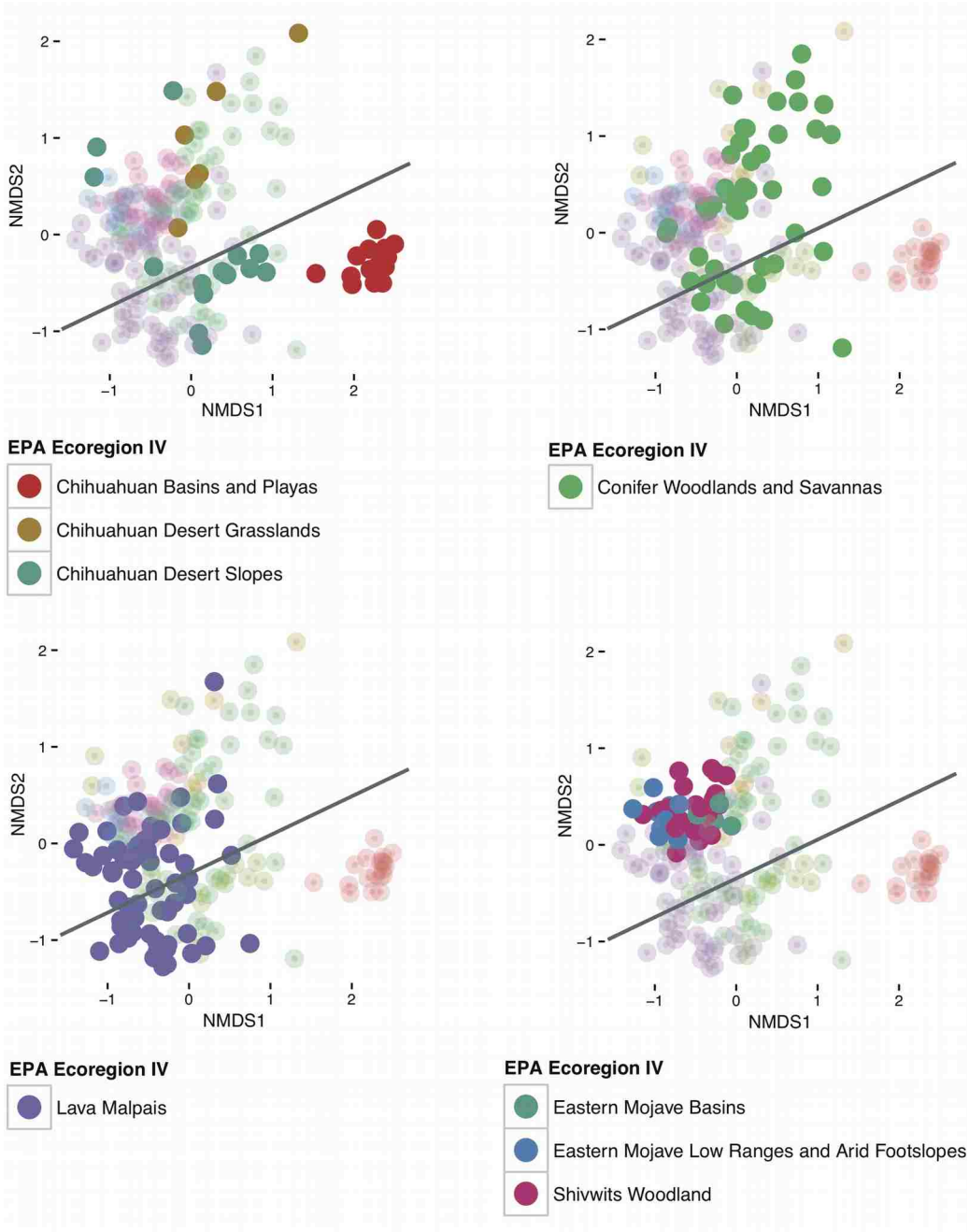
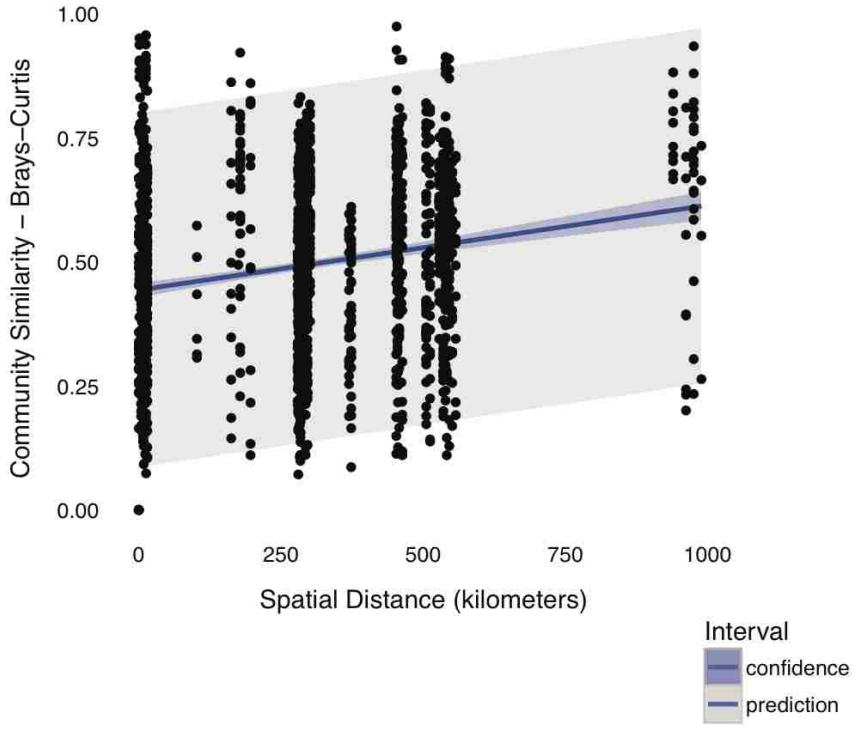


Figure 4. Similarity in the composition of the bacterial communities was quantified using NMDS (stress = 0.084) with the Brays-Curtis distance metric. Symbols are colored by EPA Ecoregion IV. Samples closer together represent samples with more similar bacterial communities. The grey line represents the split between surface-netted and cave-caught bats.

or surface-netted was the strongest driver of the NMDS.

Community similarity pattern variables were tested by using a random forest model. Random forest models were minimally successful for determining sampling site (2.76), bat species (2.28), and seasonality (2.61) associated with each sample. The models were successful for determining cave-caught or surface-netted with a ratio of 8.43 and ecoregion with a ratio of 3.20. Bacterial community similarity was related to geographic distance (Figure 5). Communities that were geographically closer shared more similar communities,



as indicated by a significant Mantel test [R_m=0.09, *P*=0.003], but the regression coefficient was weak. A similarly, significant but weak effect has been seen in a continental scale study of dust-borne bacteria (Barberán, et al., 2015).

Other microbiome projects from household dust, to whales and shrimp noted that factors such as net primary productivity (NPP), rainfall, temperature, and seasonality were correlated with patterns of bacterial richness and diversity (Apprill, et al., 2014; Larsen, et al., 2015). Using multiple regression on distance matrices (MRM) on our target variables, we

determined other factors correlated with richness, similarity, and phylogenetic diversity (as measured by Faith's PD) (Table S3). For all bat samples, richness was best correlated with soil pH and bat surface area [overall correlation: MRM R^2=0.026 (P = 0.003)]. Similarity, as represented by the MDS1 (axis 1), was correlated with mean annual precipitation, mean annual temperature, soil organic carbon, soil pH, and log of NPP [overall correlation: MRM R^2=0.24 (P = 0.001)]. For phylogenetic diversity, surface soil pH and bat surface area mattered most [overall correlation: MRM R^2=0.034 (P = 0.001)]. However, it is likely that climatic, soil, and bat variables influence cave-caught or surface-netted bats differently (Table S4). Climate, NPP, and soil makeup are highly linked, so it is difficult to assign which factors are directly responsible for structuring the bacterial communities on bats.

Effects of cave and surface. Basic information on how roosting in a cave or flying on the surface affect a bat's external microbiome is lacking. This question is important in light of WNS. Since bats contract WNS while hibernating in caves, it is possible that the external microbiome may offer natural defenses against WNS. Thus, the overall distribution of bacteria among phyla shifted between cave-caught or surface-netted bats (Fig 6) after a period of 6-8 hours is important for bats vulnerable to WNS. Cave-caught bats have proportionally more Actinobacteria and Nitrospira while surface-netted bats had proportionally more Cyanobacteria, Firmicutes, and Synergistetes. Similar shifts in community structure between surface soil and cave samples were seen in a carbonate cave in Arizona (Ortiz, et al., 2014) and the photic and aphotic zone in two caves in the Antarctic (Tebo, et al., 2015).

In addition to seeing the effects of roosting in the cave on the external microbiome, we expected the source of microbes to vary between cave-caught and surface netted bats. To test this hypothesis, we identified specific bacterial taxa typically associated with environmental sources. We visualized source associated taxa using violin plots (Fig 7). Violin plots are similar to box plots but also show the probability density at a given value. Environmentally associated taxa included sources from: plants (Chloroplasts), soil, insect, freshwater, and caves. We would expect that for bats netted on the surface there would be

proportionally more surface-associated taxa than for cave-caught bats. For example, we would expect more plant or freshwater associated bacteria in bats netted on the surface. We did detect differences, using a Bayesian t-test, in the mean proportions for plant associated taxa (BEST mean difference for cave -0.11, 95% CI -0.15 - -0.083) and weak evidence for cave associated taxa (BEST mean difference for cave 0.058 , 95% CI 0.017-0.061). There was no evidence for differences between insect, soil, and freshwater associated taxa proportions (BEST mean difference for cave -0.00047, -0.0077, and -0.0026, respectively). We hypothesize that the few samples with high freshwater associated taxa are likely bats who were netted shortly after dipping into local water sources. In addition there were several samples (~20) with high numbers of insect-associated taxa; these likely belong to bats that had recently fed on insects before being netted or had a high parasite load. Future bat microbiome studies will test these hypotheses.

Our results show that the external microbial community on bats follows similar local and regional scale patterns to household dust and internal bat microbiome studies. We might expect some of the microbial patterns to be driven by differences in rates of bacterial dispersion. Unmeasured variables can contribute to both local and regional patterns. For example: average plant height and composition at a sampling site, local bat foraging and roosting behavior might influence the communities on a smaller scale. We can show the importance of sourcing associated taxa with bats, i.e foraging habits. Our data show that surface-caught bats carry proportionally more plant taxa (Chloroplasts), whereas cave roosting bats carry more cave taxa (Nitrospira and Actinobacteria). The bacteria found on bats caught in the cave tend to be more homogeneous (Fig 6). Bats in caves are likely exposed to cave bacteria and a reduced number of surface taxa. The bats caught in caves trended towards having more Actinobacteria while other taxa (except Nitrospira) were reduced in porpotions of OTUs when compared with surface caught bats.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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Author Contributions A.S.K contributed to the bacterial data analysis, methods, results, discussion and fieldwork. J.C.K contributed to the writing, results, and discussion. J.M.Y. contributed to the data analysis, methods, results, discussion, and fieldwork. E.W.V. contributed to funding acquisition, data collection, writing, and interpretation of data in relation to bat ecology. A. P-A contributed to writing, editing, and workflow. J.J.H. contributed to the writing, editing, and data analysis. D.E.N. contributed to study design, funding acquisition, data collection, editing, and interpretation of habitat characteristics and bacterial sequencing results. D.C.B. contributed to study design, funding acquisition, data collection and discussions regarding bat ecology.

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Figure 5. Relationship between paired community similarity and distance scores. The slope is $1.710e^{-07}$ with a corrected R^2 of 0.04. Blue is the 95% confidence interval and grey is the

predicted from the linear model.

Figure 6. Proportion of phyla (with Proteobacteria by class) of all bats that were cave-caught or surface-netted. Cave bat photo by Debbie Buecher. Surface-netted bat photo by Kenneth Ingham.

Figure 7. Square root proportion of bacterial sequences identified as indicator taxa of cave-caught or surface-netted bats. Scale is the proportion of the total number of OTUs in a sample.

Metabolic asymmetry drives the distribution of marine predators

John M. Grady, Ara Winter, Brian Maitner, James H. Brown, Kristin Kaschner, Derek Tittensor, Anthony Dell, Felisa Smith

Endothermic mammalian and avian lineages have independently invaded the sea over a dozen times during the Cenozoic and are ecologically significant predators in many habitats. Remarkably, the radiation of endotherms has occurred primarily in cold, thermally stressful waters, and counter to general biogeographic patterns of diversity. Here we link metabolism to biogeography by showing that the energetic constraints on foraging lead to metabolic and foraging asymmetries that favor endotherms in cold waters. We compile a comprehensive database of over one thousand species of large predatory fish, sharks, reptiles, mammals and birds to assess global patterns of distribution and consumption at sea, and derive theory to link foraging success to metabolism. After controlling for food availability and other factors, thermal drivers of consumption lead to ~20fold increase in mammal consumption and abundance from the equator to the poles. This corresponds to an increase in taxa with slower and solitary feeding styles among endotherms as prey become easier to capture. An increase in abundance and foraging breadth can account for the striking patterns of richness in marine endotherms.

Endothermic mammals and birds, such as cetaceans, pinnipeds, and penguins, are top predators in the ocean, structuring trophic interactions, community organization, and ecosystem fluxes of energy and matter. Endotherms have independently invaded the sea over a dozen times[1,2] despite numerous hurdles to entry, including the high rates of heat loss associated with water (~25x greater than air), lack of available oxygen and substantial energy costs associated with surfacing to breathe, incumbent predators and competitors, and for many species, energetic and geographic restrictions imposed by terrestrial birth. Remarkably, marine endotherms have largely diversified in cold temperate waters, despite the thermal stresses and counter to nearly all biogeographic trends of diversity in major taxa. They dominate predatory richness at large body sizes (Fig. 1) and the energy flux through upper trophic levels in cold seas[3,4].

To account for this biogeographic puzzle, and shed light on the ecological advantages of endothermy, we first illustrate the empirical patterns of distribution among endothermic, ectothermic and mesothermic marine predators and highlight their covariation between richness and thermoregulation. We build on qualitative theory[5] to derive foraging principles that link ecological scales of individual metabolism to ecosystem trophic fluxes, with implications for patterns of global diversity.

Empirical Patterns

Ecologists have long noted that biodiversity on land tends to peak in the tropics, particularly within the productive and structurally complex tropical rainforests [6,7]. This pattern hold for virtually all major taxa, includes terrestrial mammals, birds, reptiles, amphibians, plants, insects and fungi [8]. In the ocean, similar patterns are observed, with peak richness for fish, sharks, coral, seagrasses, and mangroves occurring in the coastal tropics, often within the

structurally complex and productive coral reefs, particularly in the Indo-Pacific[9]. Large predatory ectotherms, including sharks (*Selachimorpha*), groupers (*Epinephalinae*), barracuda (*Sphyraena*), large jacks (*Caranx* and *Seriola* in *Carangidae*), sea snakes (*Hydrophiini* and *Latidicauda*) all fit this general pattern (Fig. 2). In contrast, most cetacean families forage primarily in cold temperate seas. Pinnipeds are virtually absent from the tropics, and all major clades of swimming birds that pursue prey via swimming (penguins, auks, cormorants, grebes, loons), rather than aerial diving, are predominantly temperate (Fig. 2, S1). Not a single species of penguin, auk or pinniped frequents the tropical central Indo-Pacific, the center of marine biodiversity. Mesothermic tuna (*Thunnini*, billfish (*Istiophoridae* and *Xiphiidae*), mackerel sharks (*Lamnidae*), and thresher sharks (*Alopius vulpinus* and *A. superciliosus*), which metabolically elevate their body temperature but do not defend a thermal set point[10,11], all have intermediate, cosmopolitan ranges and lack a strong latitudinal signal.

The rare exceptions to these pattern are instructive, possessing novel feeding styles and elevated hunting speeds. Dolphins (*Delphinidae*) are among the fastest[12] and most agile cetaceans and use their social intelligence to cooperatively herd and capture prey[13]. Sperm and beaked whales forage in depths that are cold at all latitudes, and herbivorous manatees are predominantly tropical (Fig. S2). Among birds, only aerial hunters are common in the tropics (Fig. S3). Many of these species rely on the element of surprise and great speeds attained by plummeting through the air; plunge-diving gannets, for instance, have been recorded to enter the water at 24 m/s to capture fish[14]. Only Delphinids and aerial hunting birds are common hunters in warm tropical waters, as well as cooler seas. Their shared reliance on high speed foraging is unlikely to be a coincidence.

Endothermic mammals and birds have high metabolic rates, which require high consumption rates. Some have posited that endotherm restriction to cold temperate waters is related to productivity in the temperate seas[15]. While high productivity may be necessary for

many endothermic species, it is hardly sufficient. Analysis of annual NPP at global scales reveals a very weak latitudinal signal (Fig S4, S8). NPP for phytoplankton peaks along parts of the Indo-Pacific, tropical western Africa and South America just as much as it does in the North Atlantic, North Pacific and the southernmost coastlines of South America, Africa and Australia. Indeed, the correlation between latitude and NPP, or sea surface temperature and NPP is low, and counter to this argument, there is a modest increase towards the tropics (Fig. S4). Similarly, fishery catch rates, a measure of fish productivity, also bear, little relationship to latitude or sea surface temperature[16]. Further, records of benthic productivity in tropical coral reefs are among the highest annual rates of benthic marine productivity recorded[17], rivaling temperate kelp dominated coastal systems[18]. Finally, the diversity of oceanic dolphins and aerial foraging seabirds thriving in the tropics attests to the availability of sufficient food to support endotherm populations (Fig S2, S3), if it can be procured.

Instead we argue that critical, thermally mediated aspects foraging are more important. A closer look at the energetics of endotherm/ectotherm foraging will illuminate the underlying causes of this unique and general biogeographical pattern.

A Mechanistic, Metabolic Model of Foraging

To consume food, animals must search, encounter, capture and handle prey items. We first consider the elements of foraging, link their rates to temperature, and finally bridge foraging with ecosystem rates to address the mechanisms underlying the patterns of richness.

The first component of foraging is a search rate, which will reflect the detection radius of the

predator, and the dimensionality of search space (3D pelagic vs 2D benthic or surfacing foraging[19]). These features will not, however, directly vary with water temperature. The encounter rate δ reflects the rate at which consumer (predator) and prey's paths intersect. Path intersection can be modeled as a function of predator-prey relative velocity v_r, or the root mean square of the velocities of consumer v_c and prey v_p across a landscape[19]:

$$1.\ v_r \propto \sqrt{{v_c}^2 + {v_p}^2}$$

Controlling for body size and shape, velocity in ectotherms will generally reflect the thermal effects of metabolism20,21 on muscle contraction rates:

$$2.\ v_{ecto} \propto B \propto e^{-E/kT}$$

where E is an activation constant (~0.65 eV), k is Boltzmann's constant and T is temperature (Kelvin). While velocity $\propto e^{-E/kT}$ for ectothermic predators and prey, it is constant for endotherms. For marine endotherms feeding on fish and squid, the temperature dependence of encounter rates δ can be modeled as

$$3.\ \delta \propto v_r \propto \sqrt{C^2 + e^{-2E/kT}}$$

where C is constant representing routine swimming speed for endotherms, ~1.5 m/s 22,23, while for prey fish, ~20 cm, they are ~0.2 m/s at 10 ºC24, increasing by a factor of ~2.5 for every 10 ºC change, following Eq. 3. Mean annual temperature varies from approximately 30 ºC in the shallow tropics to 0 ºC in the polar seas. From 0 to 30 ºC, v_r will increase ~40% (Fig. S5).

Encounter rate ϵ is a function of v_r and as well as prey density X_p (per area or volume), where $\epsilon = \delta X_p$. Data for global prey density is scarce, and overfishing has reduced historical quantities, but chlorophyll density X_{chlor} can be used as an index of historical prey density X_p,

where $X_p \propto X_{chlor}$. Similarly, Net Primary Production (NPP) can be used as an index of prey production rates.

Consumption rate C is the product of encounter rate ϵ, the capture rate per encounter c/ϵ, and handling time h (including digestion and satiation): $C = \epsilon(c/\epsilon)h$. The capture rate per encounter c/ε reflect on differences between consumer and prey speeds Δv (v_c - v_p), favoring the predator when the differential is high and the prey when the differential is low or negative. The difference in velocity (or acceleration) between ectothermic consumers (e.g., sharks) and ectothermic prey (e.g., small teleosts) will generally not vary with water temperature, as their response are generally symmetric with respect to thermal gradients. In contrast, endothermic consumer and ectothermic prey speeds are asymmetric, with ectotherms rates falling in cold waters but endotherms staying constant. As a result, differences in locomotory rates will favor endotherms in cold temperatures, where Δv increases:

$$4. \quad \Delta v = C - v_0 e^{-1/kT}$$

Just how Δv affects capture rates has not been measured, though a positive relationship is to be expected. In perhaps the simplest scenario, $c/\epsilon \propto \Delta v$. Prey for most marine mammals range from 10 – 40 cm. Utilizing Eq. 1, and using empirical data[25] for burst speed in a typically sized prey (~35 cm), we expect Δv to increase ~fourfold from 30 to 0 ºC for an endotherm predator. However, capture rates per encounter may involve multiplicative metabolic processes (e.g. detection and locomotion rates) that are exceed Δv :

$$5. \quad c/\varepsilon \geq v_d$$

If capture involves multiplicative processes, c/ϵ will exceed this rate. Thus, Eq. 7, represents a minimum bound on consumption rates. Handling time is frequently treated as a Monod

function (type II functional response)[26], leading to an asymptote in consumption rates. Handling time and satiation impose limits on the rate an endotherm can consume food, no matter how successful it is at capturing prey. Excess prey, however, represents resources that can potentially be converted into offspring. If this occurs, total consumption C_{endo} at ecosystems scales rates will reflect changes in c/ϵ and prey density X_p.

$$6. C_{endo} \propto X_p V_r \left(\frac{c}{\varepsilon}\right)$$

Ultimately, prey production rates impose limits on collective endotherm consumption. If endotherms approach one hundred percent capture of all food production (i.e., no competition), then consumption rates will take on a sigmoidal shape:

$$7. C_{endo} \propto \left(\frac{N_p}{1+e^{Y_0 X_p v_r \left(\frac{c}{\varepsilon}\right)}}\right)$$

where Y_0 is a normalization constant. In practice, this is likely rare in all but the most favorable circumstances.

Wherever ectotherms are important consumers and endotherm consumption does not approach prey production, a more analytically tractable form should be observed in Eq. 6. Using NPP and chlorophyll as proxies for prey production and density, we can isolate the thermal effects on consumption. Considering the effects of temperature on predator and prey velocity, as well as thermal effects on capture rates per encounter, we expect a minimum of ~fourfold increase in collective endotherm consumption from 30 to 0 ºC, after controlling for food availability. Consumption rates exceeding a fourfold increase in cold waters will reflect the multiplicative metabolic processes at work, e.g., detection and pursuit rates. An increase in collective consumption rates will be reflected in a corresponding increase in

population abundance if body size distributions across thermal gradients are similar.

Testing Model Predictions

We compiled rates on cellular, locomotory and visual processing rates for fish and endotherms from the literature (see Methods). Muscle contraction rates, acceleration, and routine swimming speeds increase in an approximately exponential fashion with temperature (Fig. S6), and close to theoretically expected[27] energy rate of –0.65 eV. Visual rates in fish, including flicker fusion frequency and saccadic eye movement, also increase at rates equaling or exceeding theoretical values (Fig. S7). The ecological significance of these rates are underscored by the unique mesothermic physiology of billfish, which metabolically elevate temperatures in their eyes and brain but no other organ[28].

The thermal sensitivity to ambient water in ectotherms contrasts with endotherm insensitvity; taken together they generate the velocity differential parameter (Δv) that informs our model (Eq. 4; Fig 4). Comparisons between endotherm Δv values taxa may be instructive in assessing their foraging niche. Dolphins have burst speeds at nearly twice the rate of pinnipeds and penguins, expanding thermal range in which they can be effective foragers. In contrast, prey speed can exceed pinniped and penguin speeds at ~15 ºC (Fig. 4). It is probably not a coincidence that the richness of these taxa declines precipitously above 15 ºC (Fig. 2, S8).

To connect individual components of foraging to ecosystem patterns we assessed patterns of collective consumption in mammals, focusing our attention on pinnipeds and small odontocetes, which predominantly feed in shallow waters where sea surface temperatures offer an accurate reflection of thermal foraging conditions. Calculations of marine endotherm consumption have been determined from abundance records, scaling of consumption with body size, range estimates, and expert knowledge of their habitat preferences within their range[29,30] (see Methods). Current estimations lack resolution on

covariation of consumer abundance with food availability, constraining tests of model predictions with food. The relationship between temperature and abundance, however, has been independently modeled for marine mammals, and also corresponds closely with latitudinal restrictions on species distributions. Therefore, our most novel and important predictions of relating thermal drivers of consumption can be assessed.

We performed both ordinary least squares linear models (LM) and spatial autocorrelation correction methods to assess the significance of temperature on food consumption. Our spatial autocorrelation correction method, known as Integrated Nested Laplace Approximation or INLA, employs a computationally efficient, Bayesian Hierarchical approach to removing autocorrelation and assessing predictors[31,32]. As there is some disagreement in the literature as to which provides better, unbiased estimates of coefficients[33]; we show both here, and employ sensitivity analyses to quantify error associated with model selection choices (SI). We also considered the environmental predictors of ocean depth, NPP, distance from land, and coastline length (Fig. S8). The lowest AICc value was for the full model (Table I).

Our results exceed minimal estimates of endotherm consumption and illustrate the importance of temperature in modulating endotherm foraging success and abundance. GLM coefficients indicate an 18fold increase in consumption from 30 to 0 C° across the globe, with temperature accounting for 69% of the variation; INLA calculation provide a similar but somewhat lower value of 13fold increase. These results are robust; variation in scale size, analytical techniques and NPP estimation do not qualitatively alter our results (Table S2). These results are significantly greater than our minimal estimate of fourfold increase in consumption and suggest multiplicative metabolic processes at work.

Linking Abundance to Richness

Theoretical and empirical predictors of richness are varied and no simple cause is likely to be sufficient to fully explain patterns of global diversity. Nonetheless, it is clear that species cannot exist in areas where there is too little food to support minimum population sizes. Specialist species, in particular, should be vulnerable to low food availability. The more individuals hypothesis (MIH) links higher densities of individuals to higher community alpha richness[34,35], and has received empirical and theoretical support in terrestrial systems36-40. In the marine realm, where ecosystems are more stable on annual and geological time scales, and dispersal barriers are less imposing, food limitations on abundance and richness should be more apparent, particularly for upper trophic predators with relatively low population sizes.

The overall greater consumption rates observed by marine endotherms permits higher abundance in cold waters. If size structure is relatively stable across thermal gradients, then abundance should track global consumption patterns. Indeed, this is what we observe; relying on empirical compilations of global mammal abundance, we observed a ~20fold increase from from 30 to 0 ºC (Table 1, Table S1, Fig. 5, 6). In colder waters mammals consume a higher fraction of ecosystem production, leading to corresponding increases in the number of individuals (Fig. 6).

Foraging difficulties for endotherms imposed by warm waters may lead to threshold effects that bar entry for organisms with certain body plans and foraging styles. In particular, solitary and slower moving taxa should be disadvantaged in the shallow tropics. Body plans and foraging styles tend to be similar at the family level in marine endotherms. Consequently, relatively slow and solitary foraging alcids, penguins, pinnipeds, loons, grebes and ducks largely disappear from the tropics. This can be observed in plots of familial richness in swimming birds and mammals (Fig. S9, 2). This suggests that two fundamental

forces are at work in constraining marine swimming mammalian and avian richness in the tropics: lower abundances due to difficulties in capturing food, and restrictions on foraging strategies and body plans that impose significant phylogenetic barriers to entry.

This disparate pattern of biogeography among thermoregulatory guilds can be visually summarized by plotting the ratio of endotherm to ectotherm diversity (Fig. 7). Marine mammals and birds dominate apex predator richness in coastal habitats at ~45 º latitude, or ~15 ºC sea surface temperature. Somewhat surprisingly, they comprise the majority of predator richness in pelagic habitats at all temperatures and latitudes. Pelagic tropical habitats are home to a diversity of fast–moving cooperative dolphins, as well as mesothermic tuna, billfish and sharks (e.g., mako and thresher sharks). While our compilation of ectothermic species is not exhaustive, it is also clear that large ectothermic teleosts are comparatively rare, as are pelagic sharks (Fig. 2). Clear, pelagic tropical seas offer little in the way of refuge to smaller juveniles, and it may be in these environments a premium is placed on elevated locomotory and sensory rates.

The success of marine endotherms in cold waters reflects foraging advantages afforded by thermal kinetics, but it also hints at something of equal significance: competition. The effects of temperature are symmetric for ectothermic predators and their prey; both increase their speeds in warm waters and decline in cold waters. Thus, from a foraging perspective, cold water offers equally appealing habitat for sharks as does the tropics. Further, ectothermic sharks and predatory fish, with their low metabolic demands, can survive on fewer fish than endotherms, and could potentially reduce prey populations to densities too low to support metabolically demanding endotherms[41]. Yet, clearly endotherms are thriving in cold coastal oceans (Fig. 2, 5). One reason may be that the foraging advantages afforded to endotherms (Fig. 3) allow them access to prey that may be otherwise too difficult for ectotherm predators to signficantly reduce. Faster sensory and locomotory rates may allow endotherms to exploit transient patches of food more quickly and at the expense of ectotherms. Further, the higher

metabolic rates can benefit endotherms in the case of more aggressive forms of competition, including interference competition and predation on competitors' offspring (i.e., intraguild predation). In these instances, faster locomotory and sensory rates in endotherms should prove advantageous, with the magnitude of their advantage increasing as water temperatures decline.

Despite the advantages of higher metabolic power observed in endotherms, large predatory sharks are nonetheless cosmopolitan and important consumers in most marine ecosystems. This pattern is in stark contrast with ectothermic predators on land, where large reptiles are absent or inconspicuous in many terrestrial habitats. We suggest the reason may reflect the unique advantages that gills offer to sharks and fish, which lack a counterpart to terrestrial ectotherms. Without gills, mammals and birds face considerable challenges in deep sea foraging and employment of basic feeding strategies, such as sit-and-wait hunting. In addition, heat loss stress associated with water constrains mammals and birds to relatively large sizes, reducing the competitive and predatory burden on smaller fish and sharks. On land, however, reptiles are more vulnerable to mammalian predators and competitors. It is notable that the largest lizard, the Komodo dragon, occupies islands free of mammalian carnivores, and large crocodiles are confined to aquatic habitats where food is generally not abundant enough to support comparably sized mammals. Without any special anatomical advantages, low-power reptiles tend to persist in body sizes where their small stature offers a measure of protection from warm-blooded hunters by concealing them from a predatory gaze. In the ocean, the dangers posed by endotherms are comparatively reduced, permitting a diversity of thermoregulatory lifestyles to flourish.

Methods

Range distributions of large predatory ectotherms, mesotherm and endotherms were collected for our analysis. Range data for birds was acquired from BirdLife International (www.birdlife.org) and all mammal data from the IUCN (www.iucnredlist.org). Teleost

clades where most members are capable of reaching 1 meter in length and contain at least five species were considered. All fish and shark data was acquired from the IUCN. In addition missing species of barracuda and jacks were supplemented from aquamaps (www.aquamaps.org), which utilizes observation data stored in OBIS (www.iobis.org). OBIS is an open access database of ecological and environmental information that serves as repository for fish ranges and environmental correlates.

Data on locomotory and metabolic rates were compiled from the literature. See citations in the supplemental captions. Contraction time t and body length L can be used to calculate maximum speed S, where $S = 0.7L/2t$.[25] This formula was used to generate the maximum swimming speeds of fish shown Figure 4, based on muscular contraction rates reported from Wardle[25]. See also Figure S5. Rates for pinnipeds42-47, penguins[48-51], and dolphins52,53 were compiled from the literature.

Consumption data determined from empirical compilation of abundance records and simple niche modeling to assess their spatial distribution[29,30]. Abiotic niche variables of distance to land, distance from ice, and water temperature preferences were determined from the literature and used to construct range maps for marine mammals. Ranges were validated by comparison to independently constructed ranges based on presence/absence observation.

All spatial analysis were performed in R 3.2.4[54] and QGIS[55]. All variables were log transformed except for temperature. Spatial autocorrelation techniques include INLA, or Integrated Nested Laplace Approximation, R package INLABMA[56]. INLABMA utilizes Bayesian hierarchical modeling approach to reduce spatial autocorrelation.

Figures

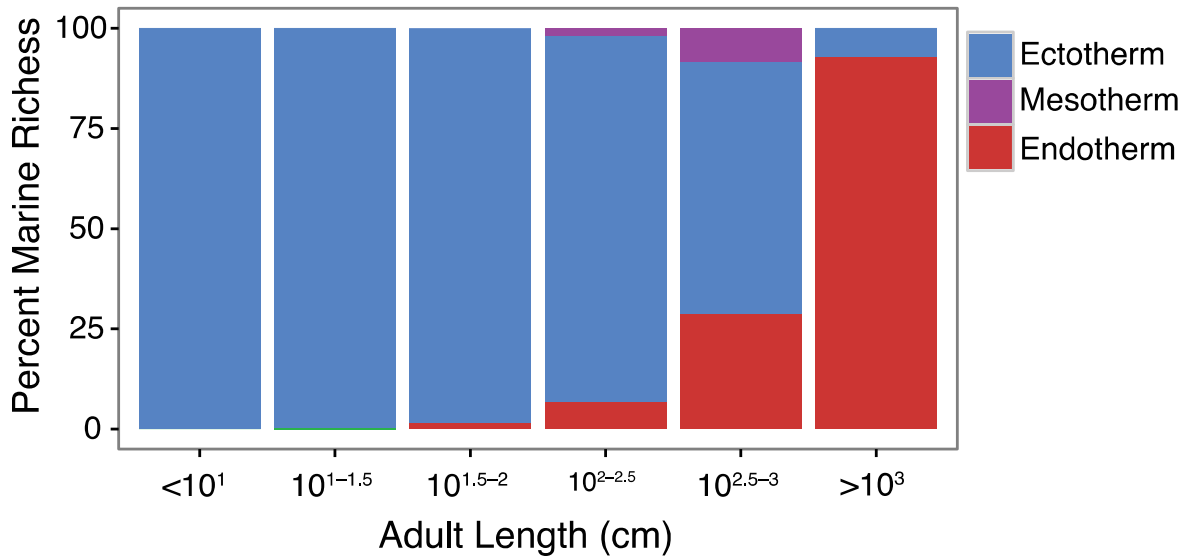

Figure 1. Global marine diversity for thermoregulatory guilds across body sizes. The total diversity of marine fish, sharks, reptiles, mammals and birds are considered, and percentages of marine diversity were calculated for each length bin. For fish, body length data for some species were not available. The percentage of species without length data was determined, and a correction was applied by adding a value corresponding to the missing percentage to each bin. Each bin represents one half an order of magnitude of length, where $10^{0.5}$ cm equals 3.16 cm, $10^{1.5}$ cm = 31.6 cm, etc.

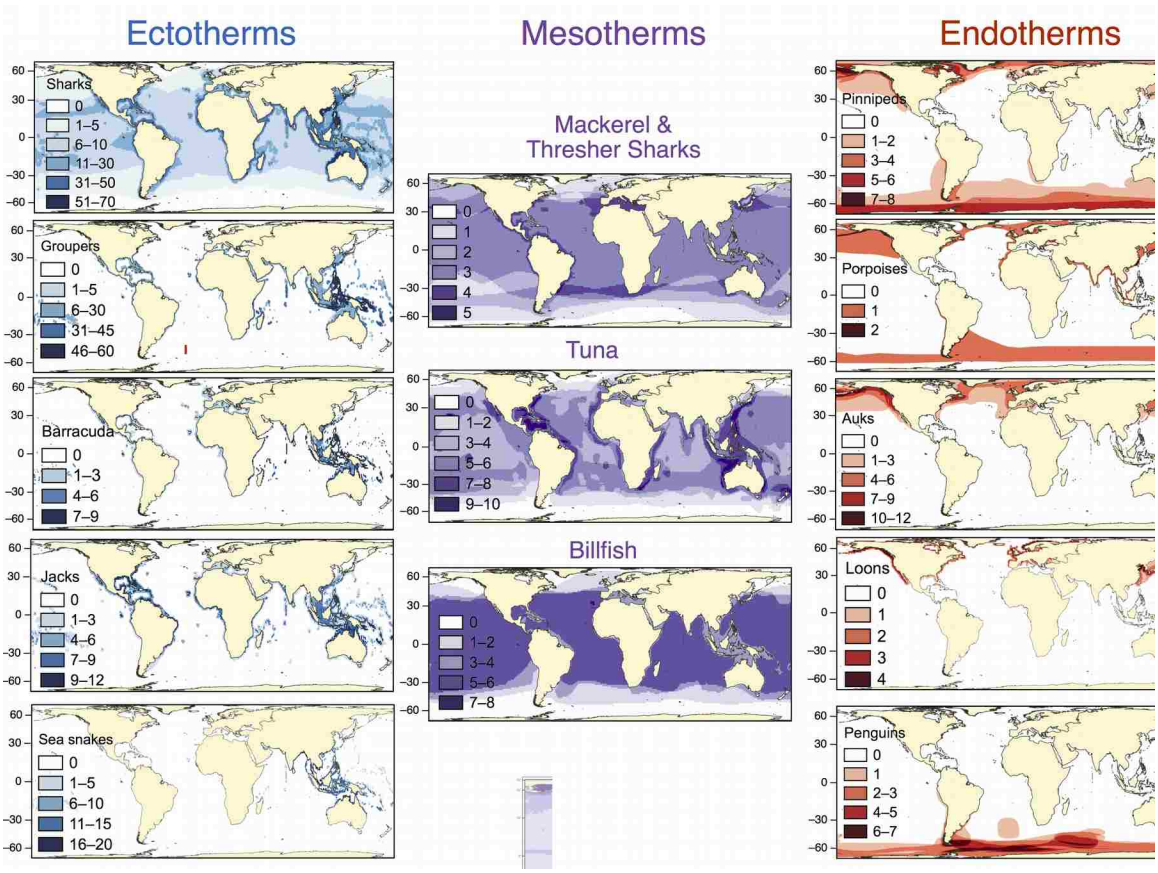

Figure 2. Global patterns of richness in large marine predators. Ectothermic apex predators are most diverse in tropical and warm-temperate coastal habitats, particularly in the Indo-Pacific region. Mesothermic predators (see text) are equally diverse in the tropics and mid temperate latitudes, only declining above 45°. Endothermic marine mammals and swimming birds are generally absent or low diversity in the tropics, with diversity peaking above 30–45°.

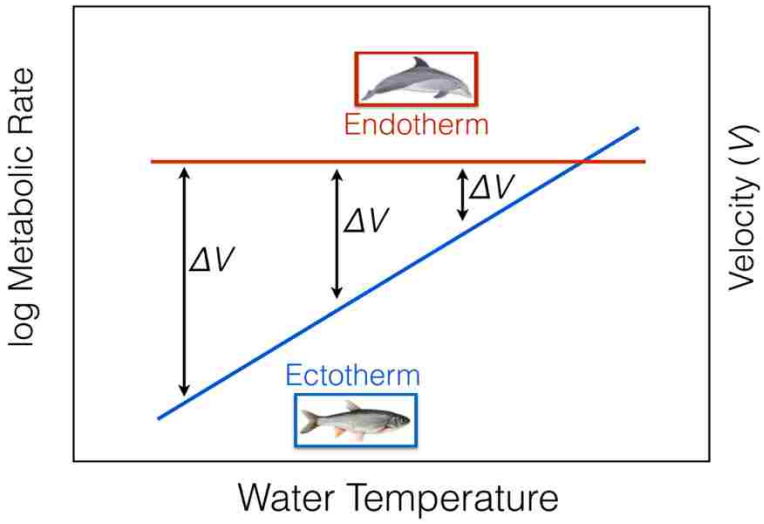

Figure 3. A schematic of the metabolic and performance asymmetry existing between endothermic predators and ectothermic prey. Endotherm metabolic and performance rates are generally insensitive to water temperature, while ectotherm rates generally respond in an exponential fashion. Note that, for endotherms, the most favorable difference in velocity (ΔV) or other performance rate will be at the coldest temperature, when ectothermic prey metabolism and performance rates are lowest. The same relationship also applies to ectothermic predators of endotherms. Endotherms will have the best chance of escape from sharks in cold temperatures, where shark speed and response rates are lowest.

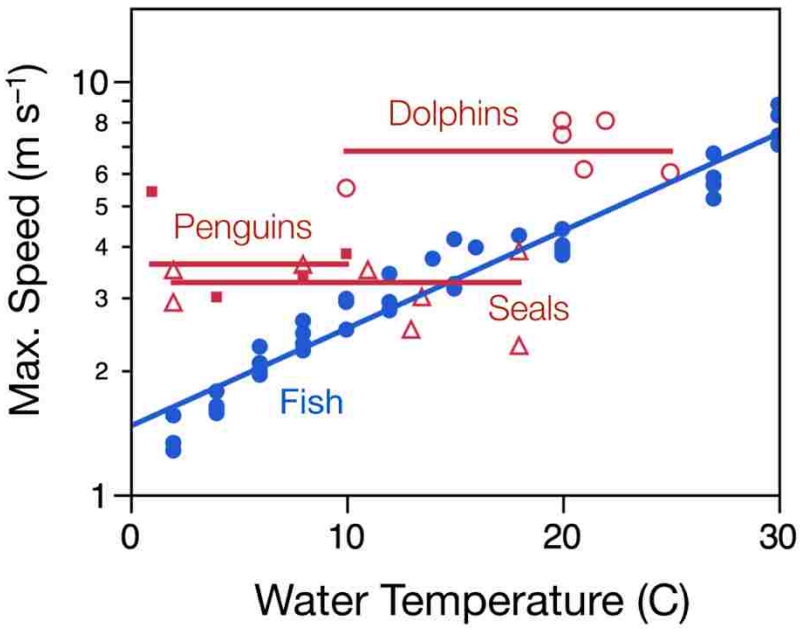

Figure 4. Metabolic asymmetry in marine endotherms and ectotherms. Data on fish and endotherm speed supports our schematic (Fig. 3). While endotherm maximum speeds varying with body plan, they are effectively insensitive to water temperature. Conversely, ectothermic fish show an exponential increase in speed as water temperatures increase. The mean speed for dolphins was 6.83 m/s (s.d. = 1.10); penguins 3.90 m/s (s.d. = 1.05); pinnipeds 3.15 m/s (s.d. = 0.566). For fish, $\log(y) = 0.068x$, $r^2 = 0.98$).

Parameters	LM Coefficients (C.I.)	INLA Coefficients (C.I.)
Consumption		
Annual SST (°C)	-0.0421 (-0.0428 – -0.413	-0.0370 (- 0.0361 – 0.0378)
SST Shift, 30 – 0 °C	18.3 (17.3–19.2)	12.9 (12.1–13.6)
1/kT	0.692 (0.679–0.704)	
Ocean Depth (m)	-0.109 (-0.117– - 0.101)	0.212 (0.206–0.218
Distance to Land (km)	-0.0803 (-0.0885 – - 0.101)	-0.227 (0.240 – -0.214)
NPP (mgC m^{-2} d^{-1})	0.181 (0.152–0.209)	0.0571 (0.0517 – 0.0624)
Abundance		
Annual SST (°C)	-0.0431 (-0.0471 – -0.0420)	
SST Shift, 0 – 30 °C	19.8 (18.2 – 25.9)	
1/kT	0.584 (0.575 – 0.594)	
Ocean Depth (m)	0.0778 (0.0623 – 0.0920)	
Distance to Land (km)	-0.0381 (-0.4713 – -0.0291)	
NPP (mgC m^{-2} d^{-1})	0.181 (0.152 – 0.209)	

Table 1. Spatial modeling of endotherm consumption and abundance. The coefficient outputs for the linear model (LM) and spatial autocorrelation correction model INLA (Integrated Nested Laplace Approximation) are shown above. The term 1/kT, where k is Boltzmann's constant and T is temperature (kelvin), is an alternative temperature variable to Celsius. The results are strikingly close to the inverse prediction from metabolic theory of -0.65 eV for individual rates. All terms but temperature are log transformed. The variables shown represent the model with the lowest AICc value (695.3 for consumption; 5333.9 for abundance).

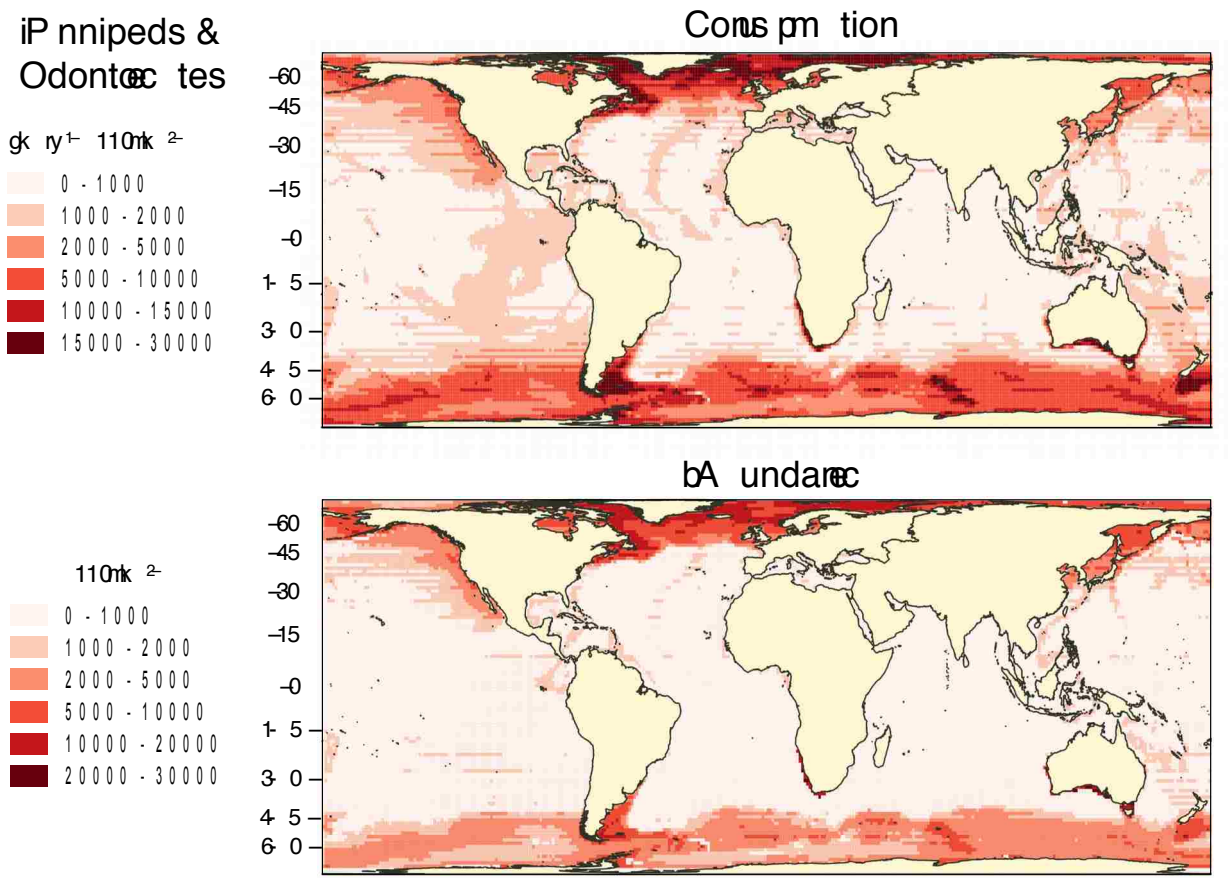

Figure 5. Global abundance and consumption in marine mammals. Pinnipeds and small toothed whales show increasing rates of collective consumption and abundance in colder coastal waters.

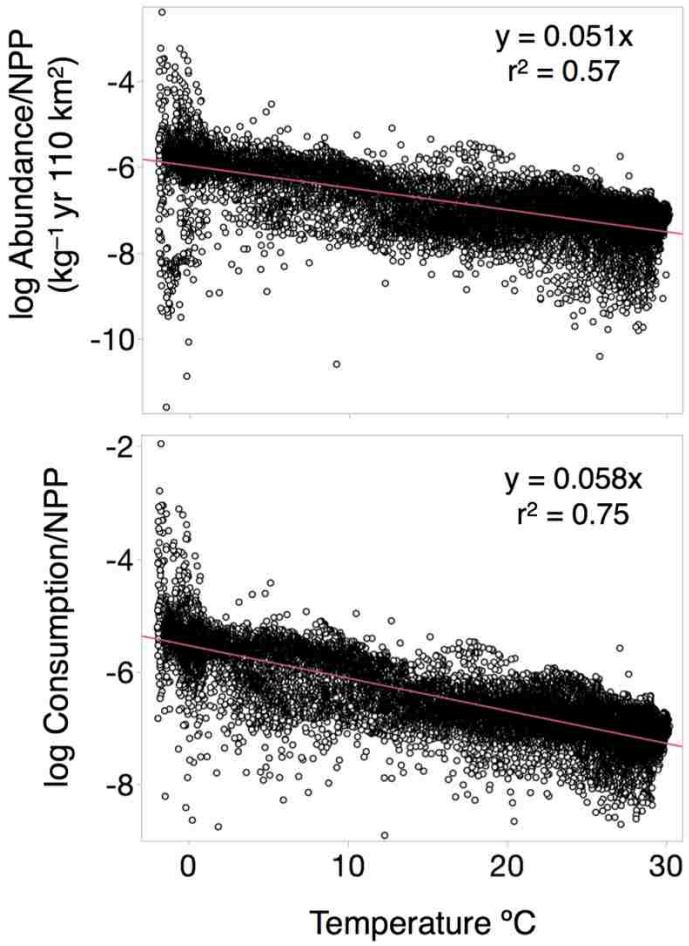

Figure 6. Relative consumption and abundance in marine mammals. As sea surface temperatures decline, pinnipeds and small odontocetes increase in abundance and collectively consume more of the available production. The slopes are similar, reflecting the mechanistic linkages between these two currencies. Each point represents data for a 110 km^2 cell. For the bottom figure, the units are the same, generating a unitless ratio. Pinnipeds and odontocetes typically have a trophic level of ~4, and marine trophic transfer efficiencies are ~10%; therefore, the maximum consumption/NPP ratio (bottom graph) is a log value of -3.

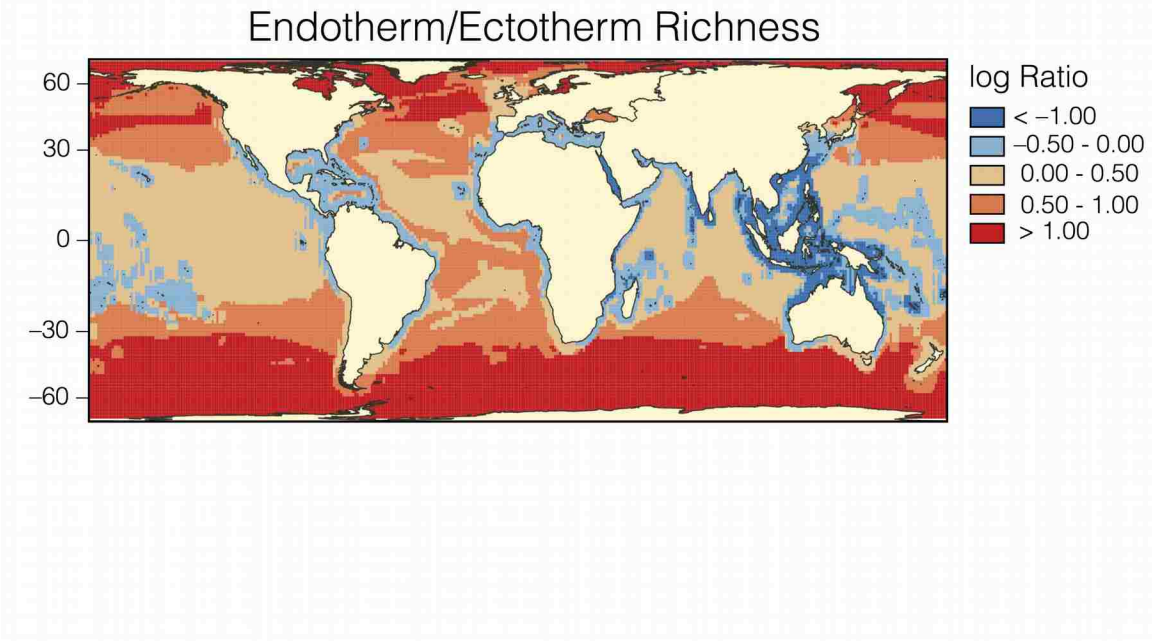

Figure 7. Relative richness of major predatory taxa. Large ectothermic predators contribute the highest fraction of community richness in tropical and warm temperate coastal waters, while endothermic swimming birds and mammals dominate cold waters and open oceans. In areas where no large predatory ectotherms were recorded, e.g., near coastal Antarctica, the deepest red color was assigned.

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Supplemental Materials and Methods

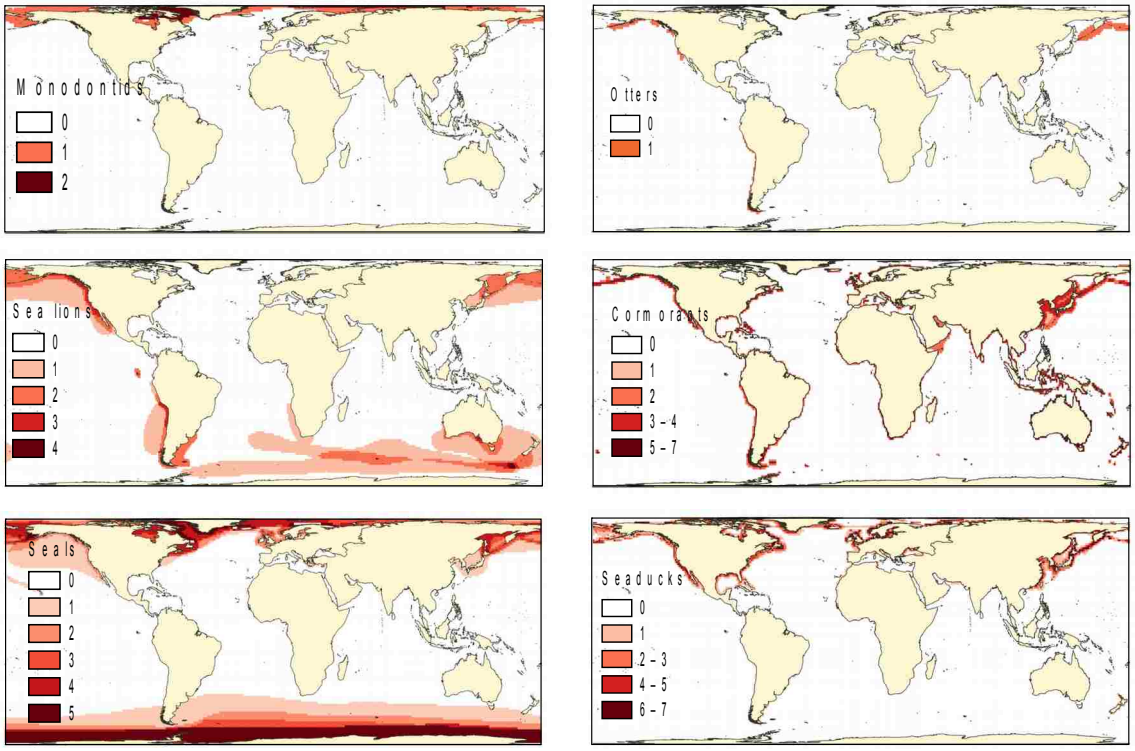

Figure S1. Additional richness patterns in marine mammals and swimming birds. Monodontids are composed of narwhals and belugas. Sea lions are Otarriidae, seals are Phocidae, cormorants are Phalacrocoracidae, and seaducks are Merginae, within Anatidae.

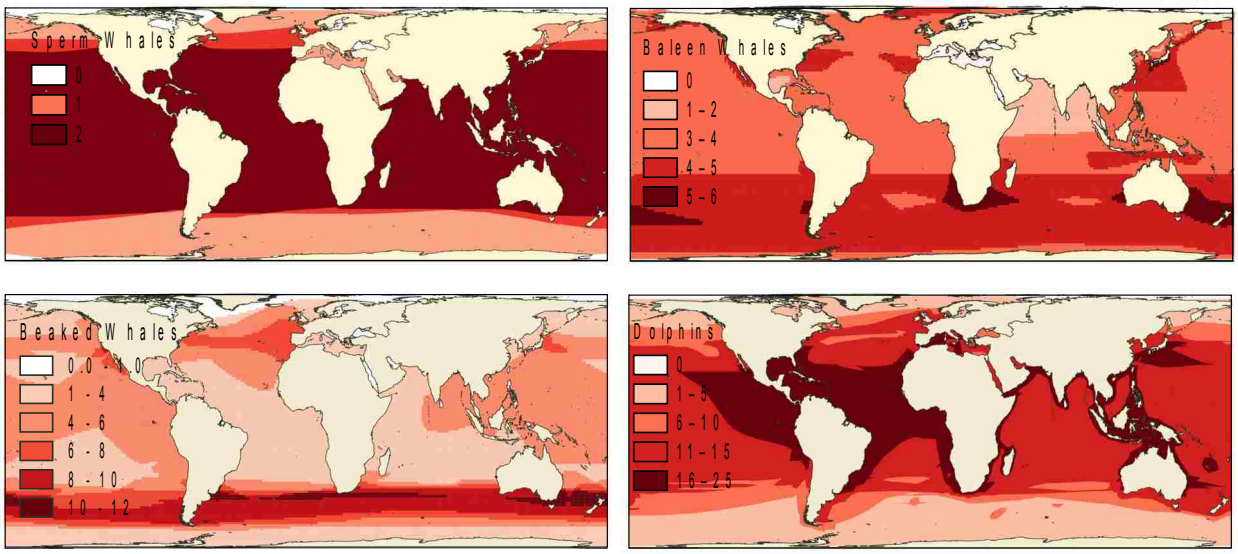

Figure S2. Exceptions that prove the rule. Marine mammals that forage at depth (in cold waters) or are large planktonic feeders are not expected to show a systematic bias towards temperate waters. Sperm whales (Physeteridae & Kogiidae) are cosmopolitan, whale beaked whales (Ziphiidae) show peak diversity in low southern latitudes. Baleen whales (Mysticeti) largely feed in temperate latitudes where swarms of zooplankton can be found. Dolphins (Delphinidae) are exceptionally fast and cooperative, and are able to exploit prey items in tropical as well as temperate seas.

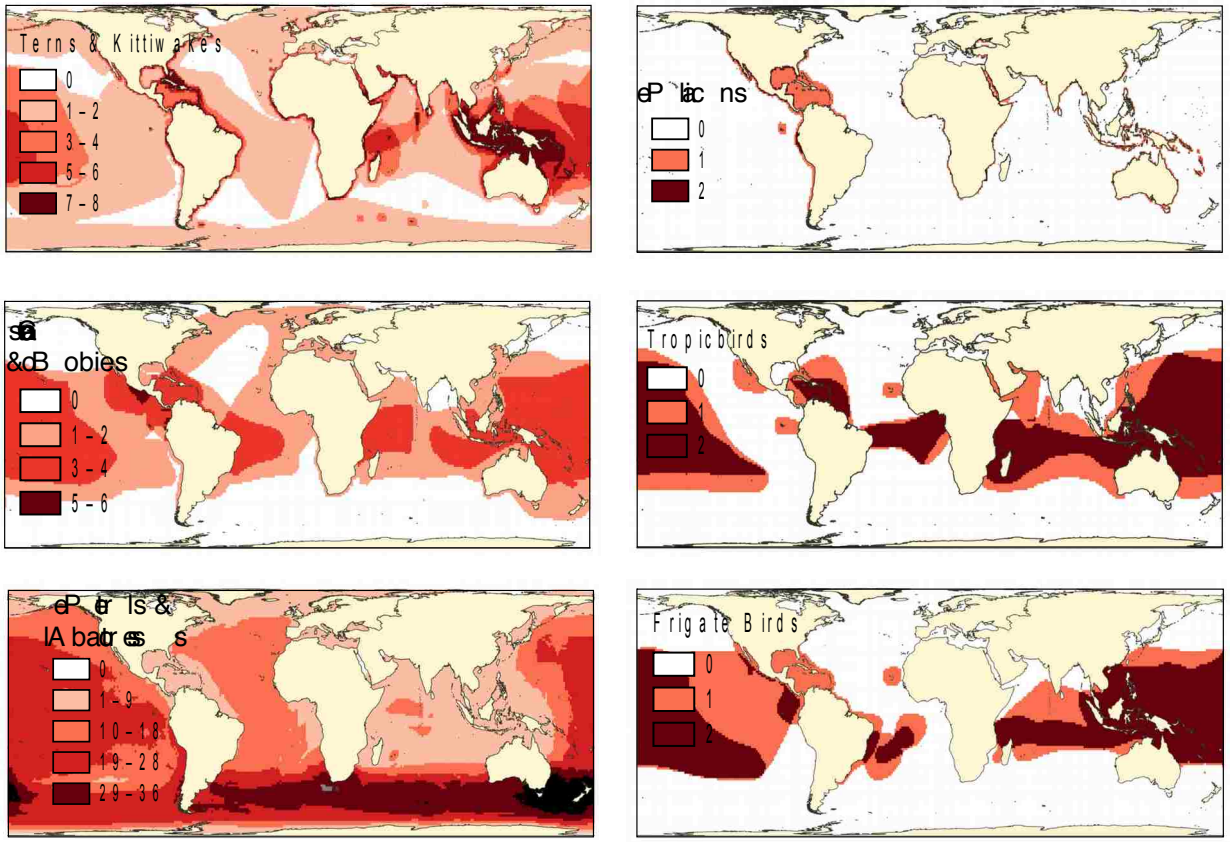

Figure S3. Avian exceptions that prove the rule. Aerial feeding birds can opportunistically feed on surface foods or capture fish with fast plunging dives. This strategy permits species to live throughout the globe, and show diverse distributions. Most families are predominantly tropical, but the members of the most diverse families, found within Procellariiformes (petrels and albatrosses), show peak diversity in the cool southern seas. Terns and kittiwakes belong to Laridae, gannets and boobies comprise Sulidae, pelicans are Pelecanidae, Tropicbirds are Phaethontidae, and frigate birds are Fregatidae.

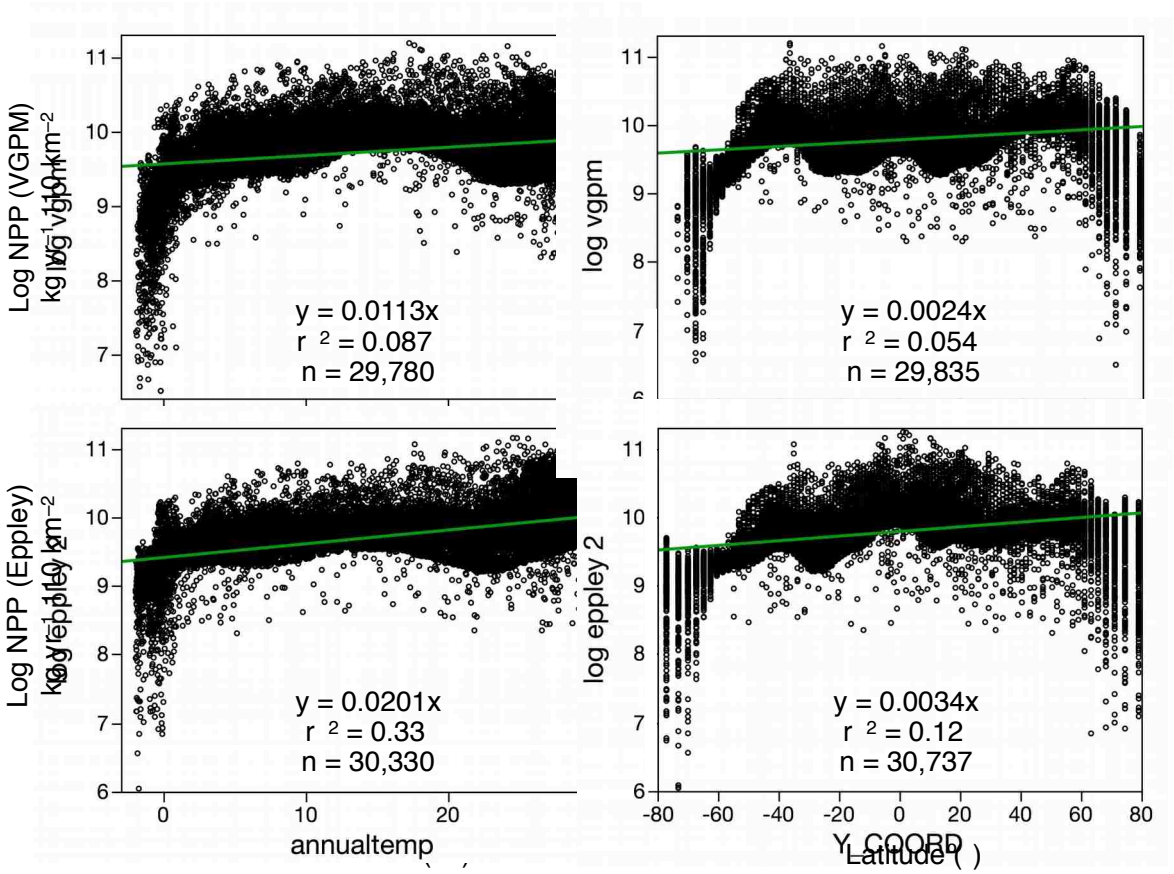

Figure S4. Plots of Net Primary Production (NPP) with sea surface temperature (SST) and latitude. Shown her are two of the more common forms of NPP calculations, the vertically generated production model (VGPM), and Eppley model57-59. SST and latitude are only weak predictors of production for both models, and in the wrong direction to explain elevated endothermic consumption and richness in cold waters. In polar seas, subzero water and seasonal ice cover significantly reduces NPP.

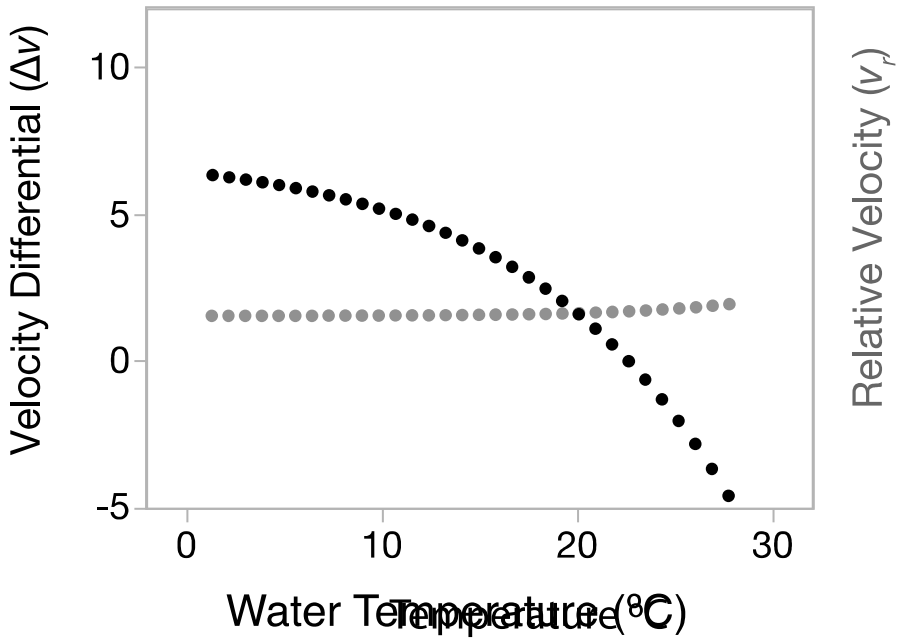

Figure S5. Predicted changes in differential velocity and relatively velocity across thermal gradients. Gray dotted lines represent the relative velocity v_r of endothermic predator and ectothermic prey, which will increase modestly as water temperatures warm. A greater shift will be observed in the difference in velocities Δv between predator and prey across thermal gradients (black dotted line). Endotherms will be much faster than their prey in cold water, where ectotherm metabolism and locomotory rates are low, but will be disadvantaged in warmer waters. If realistic values for Δv are used (Fig. 4) a straight line is fitted through Δv, a ~fourfold shift in Δv is observed from 0 to 30 ° (Δv = 0.146°C).

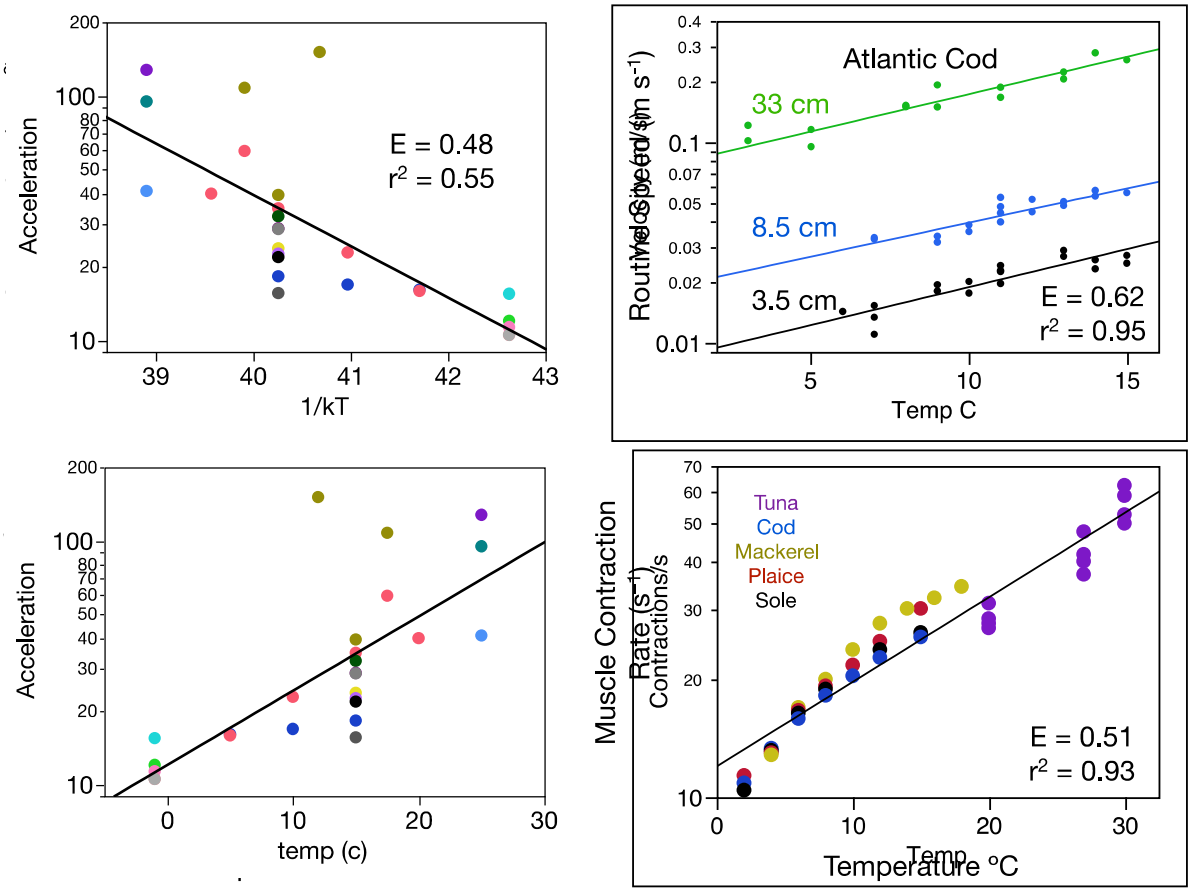

Figure S6. Thermal dependence of metabolic rates. Metabolic rates generally increase in an exponential fashion with temperature. Metabolic theory suggests the rate of increase corresponds to an 'activation energy' of 0.65 eV. This value can be determined by plotting against $1/kT$ (inverse temperature), where k is Boltzmann's constant and T is temperature in Kelvins (upper left panel). For ease of understanding, all calculations where performed using inverse temperature plots but are shown in the conventional manner with temperature on the x axis. Note that the upper left and lower left panels are equivalent. Acceleration (left panels) is considered to be a mass-independent rate in fish[60], so standardization of body size is not necessary, but for velocity (upper right panel) it is important to control for size. Date for acceleration is from Domenici[60]; contraction rates from Wardle[25], and swimming speed from Peck[61].

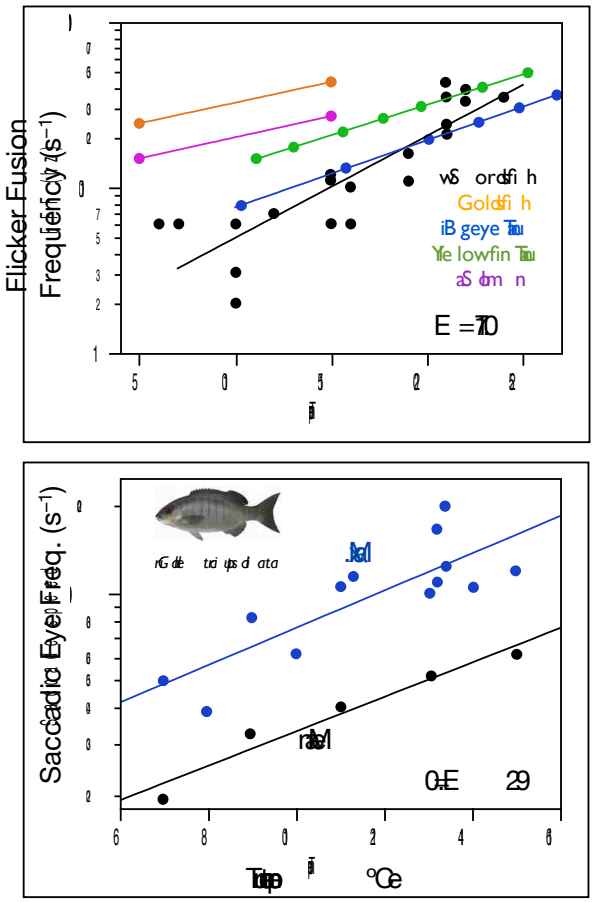

Figure S7. Thermal dependence of visual detection and processing speeds. In the lower panel, saccadic eye movements, important for receiving visual information, showed an exponential dependence on temperature. In the upper panel, the flicker fusion frequency – i.e., the rate at which flickering light pulses can be registered as discrete – increase markedly with temperature, consistent with theory. Data from saccadic eye movement from Montgomery[62] and flicker fusion frequencies from Fritsches et al.[63]

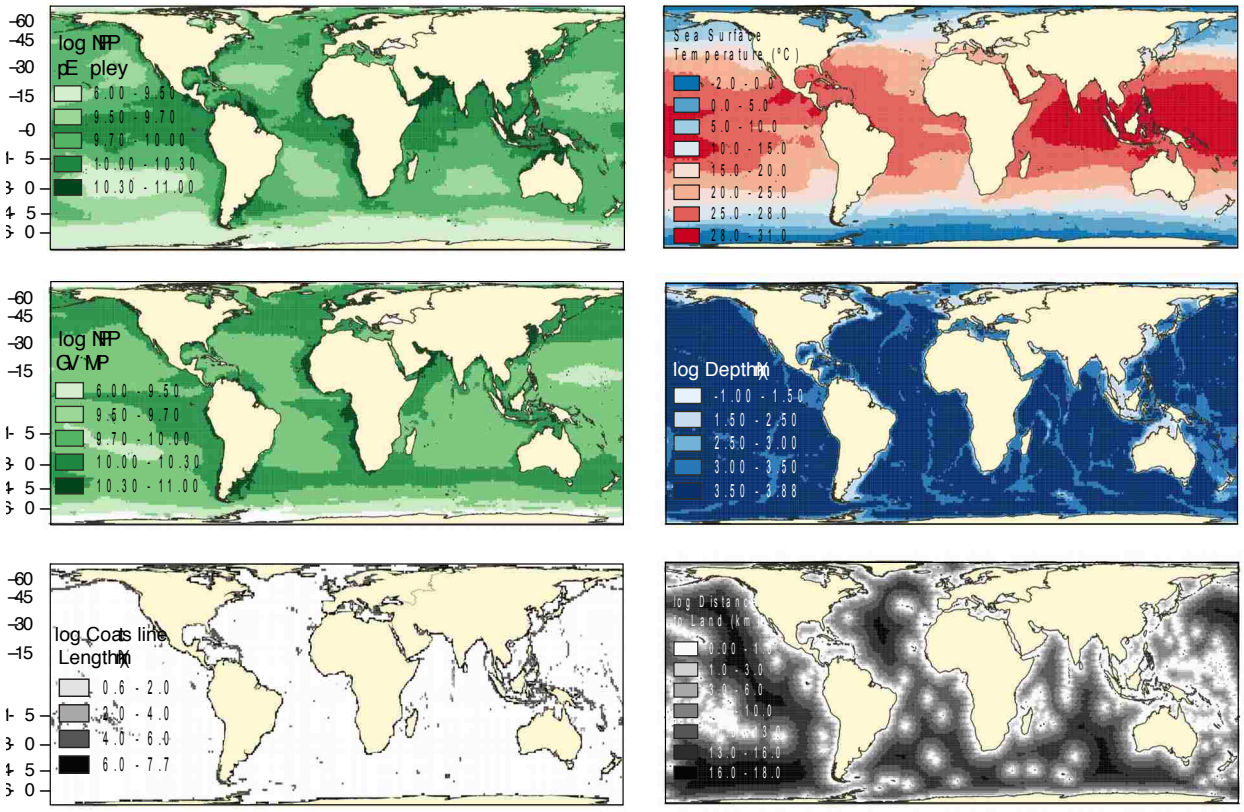

Figure S8. Environmental predictor variables of endotherm consumption and richness.

Parameters	LM Coefficients (C.I.)	INLA Coefficients (C.I.)
Consumption		
Annual SST (°C)	-0.0494 (-0.0543 – -0.0448)	-0.0433 (- 0.0517 – 0.0345)
SST Shift, 30 – 0 °C	30.3 (22.1–43.3)	19.9 (10.8 –35.6)
1/kT	0.737 (0.683–0.790)	
Ocean Depth (m)	-0.305 (-0.381– - 0.279)	0.322 (0.288–0.356)
Distance to Land (km)	-0.904 (-1.21 – - 0.719)	-0.642 (0.826 – -0.456)
NPP (mgC m^{-2} d^{-1})	0.170 (0.152–0.209)	0.338 (0.265 – 0.411)

Table S1. Model parameters at 880 km² grid cells. Both linear model and INLA methods were performed to test environmental predictors of mammal consumption at 880 km^2. The results were not qualitatively different from calculation using 110 km2 grid cells (Table 1).

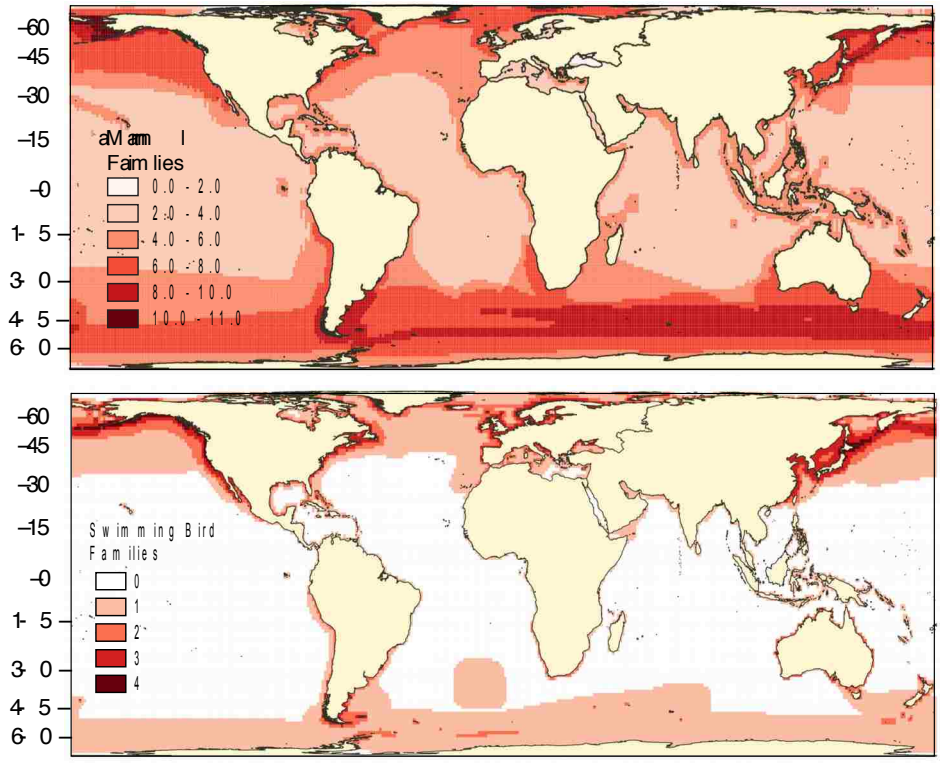

Fig S9. Familial level richness in marine mammals and swimming birds. Marine mammal families are composed of pinnipeds, otters and cetaceans; bird families are penguins, auks, ducks, grebes, loons and cormorants.

Conclusions of patterns of richness from bacteria to apex predators

Conclusions

These studies involved large groups of people that formed an interdisciplinary group to address questions of what drives the patterns of richness and diversity we see from cave bacteria to apex predators in the ocean. The projects ranged in both geographic and body size scales from lava cave microbial mat communities to sperm whales in the deep oceans. This require a range of techniques borrowed from microbiology, macroecology, and geographic information systems. There were three fundamental philosophies that helped to guide these works:

Hanson, et al., 2012 argued that we need to move beyond just showing patterns (in particular bacterial biogeography) to identifying drivers and mechanisms that give rise to these patterns.

In talking about modeling building Levin, 1966 "Truth is at the intersection of independent lies." In other words having two different approaches to your models to help verify what you are seeing.

From Gelman et al.,2013. "Bayesian inference is the process of fitting a probability model to a set of data and summarizing the result by a probability distribution on the parameters of the model and on unobserved quantities such as predictions for new observations."

With these guiding philosophies in mind the research present here followed most closely to Hanson and Gelman. In studying microbial mats and bacteria on bats we showed biogeographic patterns but tested these patterns against local and regional environmental and physical predictors. In each case with our models we fitted the model to the data and tested if the model was corrected. Studying marine predators captured that whole approach from visualizing global patterns, to discovering what drives these patterns, and finally to a metabolic theory to describe the patters. Levin's statement about two lies was harder to

apply. In the case of the microbial mats in lava tubes we were able to tell the same story with two different sets of data. One was OTU counts which told a story of cave microbial mats being different from the overhead soils. While the phylogenetic story showed a more nuanced evolutionary history.

Importance of patterns and predictor variables

Across the range of distances and body plans one driver stood out, temperature. Temperature was the strongest driver of similarity between basalt surface soils and the microbial mats in caves; within lava caves temperature was also a driver of similarity; however it is was a weak predictor of bacterial richness. In bacteria on bats temperature was a predictor of similarity in cave-caught bats but not for richness or phylogenetic diversity. For surface-caught bats temperature was predictive of richness, similarity, and phylogenetic diversity. Many of the predictors for bacteria richness on bats also predicted bat species richness across Arizona and New Mexico. For top ocean predators temperature is a major component that predicts not only species richness but also foraging and consumption habits. However for endotherms their peak richness was anti-tropical and for ectotherms there peak richness was tropical but in structural complex (reefs, mangroves, islands, and oceanic shelves) areas like the Indian Ocean.

However, for each of these studies there were numerous other factors that also helped to explain the patterns in richness and diversity. In the microbial mats in the lava caves relative humidity help to structure community similarity and distance from the entrance predicted richness. Bat bacteria had a range of other predictors from local (bat richness, precipitation, NPP) to larger landscape features like ecoregion classification and landscape complexity. In the apex ocean predators we saw effects from proximity to land, bathymetry, structural complexity of the environment, and evolutionary history. The findings across all three studies showed a complex interaction of predictor variables that also varied by geographic scale.

Future studies

While macroecology is a robust and mature field, the areas of microbial biogeography and applying macro principles to micro-problems is fairly young (~10 years, Fierer et al.). With the advent of new sequencing technologies and exponential expanding microbial gene databases microbial biogeography is catching up. Identifying the mechanisms that drive bacteria patterns in the first two studies would be key to establishing links between micro- and macro-. Testing the classic mechanisms such dispersal, drift, mutation, and selection in cave environments should be done due to caves having a more restrictive environment then the surface. On the marcoecology side testing our predictors across timescales is an important next step. The pinniped peak richness showed anti-tropical patterns. This is due to a combination of foraging strategies and evolutionary history. Ultimately making the link between microbial process and macroecologic patterns is required to further both fields.

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