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**Invited Review: Microbiome Evolution Along Divergent Branches of the Vertebrate Tree  
of Life: What's Known and Unknown**

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**Abstract:**

Vertebrates harbor microbes both internally and externally, and collectively these microorganisms (the “microbiome”) contain genes that outnumber the host’s genetic information ten-fold. The majority of the microorganisms associated with vertebrates are found within the gut; where they influence host physiology, immunity, and development. The development of next generation sequencing has led to a surge in effort to characterize the microbiomes of various vertebrate hosts, a necessary first step to determine the functional role these communities play in host evolution or ecology. This shift away from a culture-based microbiological approach, limited in taxonomic breadth, has resulted in the emergence of patterns suggesting a core

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vertebrate microbiome dominated by members of the bacterial phyla Bacteroidetes, Proteobacteria and Firmicutes. Still, there is substantial variation in the methodology used to characterize the microbiome, from differences in sample type to issues of sampling captive or wild hosts; and the majority (>90%) of studies have characterized the microbiome of mammals, which represent just 8% of described vertebrate species. Here, we review the state of microbiome studies of non-mammalian vertebrates and provide a synthesis of emerging patterns in the microbiome of those organisms. We highlight the importance of collection methods, and the need for greater taxonomic sampling of natural rather than captive hosts; a shift in approach that is needed to draw ecologically and evolutionarily relevant inferences. Finally, we recommend future directions for vertebrate microbiome research, so that attempts can be made to determine the role that microbial communities play in vertebrate biology and evolution.

## **Introduction**

Microorganisms, primarily bacteria, can be found living both on and in all animals. It has generally been thought that the number of bacterial cells associated with an animal exceeds the number of the host animal's cells at least tenfold (Savage 1977; Berg 1996), although newer estimates suggest that this ratio may be more in the range of 1:1 (Rosner 2014; Sender *et al.* 2016). Regardless of the total number of microbial cells, collectively, the genomes of these microorganisms may contain 10-100 times as many genes as the host's genome (Berg 1996; Savage 1977; but see Rosner 2014; Sender *et al.* 2016). These microbes aid in the host's nutrient acquisition and immune response, and can influence host behavior, development, reproduction and overall health (Fraune & Bosch 2010; Colombo *et al.* 2015). The influence of the host on their microbiome is still being determined, but both host diet and phylogeny have been shown to

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be important predictors of endogenous (gut) microbial community composition (Ley *et al.* 2009; Sanders *et al.* 2013; Clements *et al.* 2014; Mikaelyan *et al.* 2015). Much of this information is derived from culture-independent (i.e. molecular) studies, which have primarily sequenced fragments of the bacterial 16S rRNA gene. The development of next-generation sequencing (NGS) technologies over the last decade has greatly facilitated such studies, allowing both rapid and affordable sequencing at the depth needed to sufficiently characterize diverse bacterial communities (Turnbaugh *et al.* 2007; Gloor *et al.* 2010; Arumugam *et al.* 2011a; Bartram *et al.* 2011)

The apparent relationship between host phylogeny (or genotype) and microbial community composition has led to much discussion of co-evolution of microbial communities and their multicellular hosts (Ochman *et al.* 2010; Anderson *et al.* 2012; Phillips *et al.* 2012b; Moeller & Ochman 2014). However the vast majority of work on gut microbial communities has focused on mammals, particularly humans (Ley *et al.* 2006, 2008, 2009; Arumugam *et al.* 2011b; Yatsunenko *et al.* 2012). Furthermore, the majority of non-human mammalian microbiome studies have tended to characterize fecal microbiomes from captive animals, often from laboratories or zoos (Ley *et al.* 2009a; b & refs within). Given that we know that human gut microbiomes are largely developed at an early age and are related to both the diet and environmental conditions of the individual host (Koenig *et al.* 2011; Lozupone *et al.* 2012), it is questionable whether work on captive animals can be used to predict the gut microbiomes of animals in the wild. This problem has been suggested before (Amato 2013), yet there is still a substantial lack of studies that have attempted to characterize enteric microbial communities in hosts within a natural environment (Table 1, Table S1 Supplementary Information). This lack of knowledge becomes even more pronounced when we extend the focus beyond mammals to other

vertebrates, with the gut microbial communities of major branches of the vertebrate tree of life such as amphibians, reptiles, birds, and fish being very poorly described (Figure 1).

The microbiome has been linked to changes in host growth rate and metabolism (De Winter *et al.* 2015), host phylogeny (Anderson *et al.* 2012; Phillips *et al.* 2012a; Colman *et al.* 2012), host ecology and life history (Wong & Rawls 2012; Coon *et al.* 2014; Dill-McFarland *et al.* 2014) and geography (Hird *et al.* 2014). These emerging patterns have led to the hypothesis that endogenous microbiomes reflect the evolutionary signatures of their hosts, and that ecological and evolutionary forces act on both the host and its resident microbiome. Microbes may in turn affect the evolution of the host, and microbes may have influenced vertebrate host evolution for millions of years, potentially contributing to the evolutionary trajectories of entire vertebrate communities (Zilber-Rosenberg & Rosenberg 2008; Ley *et al.* 2009; Fraune & Bosch 2010). Ecological studies stand to gain much by incorporating knowledge of the host organism's microbiome, and microbiome research can fundamentally change how we approach questions in evolutionary biology. As we assess the existing knowledge of non-mammalian vertebrate microbiomes, with particular attention to the endogenous (gut) microbiome in the context of better-known mammalian (i.e. human and primate) taxa, we highlight questions that remain unaddressed in these systems and make recommendations for future avenues of research in light of rapidly advancing sequencing technologies.

### **Patterns in the microbiome along the vertebrate tree of life:**

#### ***Fishes***

Fishes are the most diverse group of vertebrates with nearly 34, 000 described species as of early 2016, and ray-finned fish encompass half of all known vertebrate species (fishbase.org).

Fish are anamniotic ectotherms that require aquatic habitat for survival. One of the most successful vertebrate groups, fish occupy marine and freshwater habitats across the globe, and have adapted to live in some of the most extreme environments of any vertebrate (e.g. some species tolerate hydrogen sulfide streams). Fish have a variety of reproductive strategies and in terms of diet may be herbivorous, omnivorous or carnivorous (Nelson 2006), suggesting that the microbiomes of fish could be highly variable, depending upon both host phylogeny and environmental conditions.

The microbiomes of fish are among the better characterized of non-mammalian vertebrates, likely because of the greater importance of fish as a food resource or for recreational activity. As with studies of the microbiota of other host taxa, much of the work on the autochthonous microbial communities of fish has been largely culture-based, with culture-independent methods being used only recently. Increased ease of analysis from advancing technologies such as NGS, coupled with the importance of fish in aquaculture and their breadth of ecologies, has meant that the microbiomes of fish have received much attention in recent years, and patterns in fish microbiome structure has previously been reviewed (Clements *et al.* 2014).

#### *Exogenous microbiomes of fishes*

The mucosal and skin microbiomes of fishes has been less studied than that of amphibians, but more so than that of reptiles including birds. Mucosal microbiota appear to be important in host physiology, and help with the development of adaptive immunity in mammals, particularly humans (Human Microbiome Project Consortium 2012), although this relationship is less understood in other vertebrates. The transition from aquatic to terrestrial life over the course of vertebrate evolution also provided opportunity for adaptive shifts in mucosal microbiomes

(Lowrey *et al.* 2015), so that examining the mucosal or cutaneous microbiomes of fish could be critically important in assessing these shifts. NGS and targeted bacterial 16S rRNA gene scans were used to assess the bacterial diversity of five mucosal surfaces of captivity-raised rainbow trout (*Oncorhynchus mykiss*), revealing that the Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Tenericutes were the dominant bacterial phyla, and that the skin had the most diverse bacterial communities of any surface investigated (Lowrey *et al.* 2015). *Flectobacillus* and *Flavobacterium* were the dominant bacterial genera found both on the skin and gills, but the proportions of these genera were variable and they comprised 3.5-35% of the total community in different samples. External mucosal surfaces shared the most similar microbial communities, but all five mucosal surfaces examined (both internal and external) were shown to have distinct “core” microbial communities (Lowrey *et al.* 2015). Mucosa were also screened for the presence of known fungal pathogens to amphibians and fish as well as for the presence of anti-fungal properties in the microbiome; 28% of the identifiable bacterial operational taxonomic units (OTUs; a surrogate for bacterial species based on sequence similarity) matched with cultivable, fungal-resistant bacteria known from amphibian skin (Lowrey *et al.* 2015). While this study had limited sample size (just six individuals), and used captive-reared rather than wild hosts, it provides the first thorough picture of a teleost fish microbiome, and suggests that there may be some overlap between the cutaneous microbiomes of fish and amphibians.

While some studies have suggested that the skin and mucosal microbiomes of freshwater fish show lower bacterial diversity than gut communities (Boutin *et al.* 2014; Leonard *et al.* 2014), high bacterial diversity has been found on the skin of killifish (*Fundulus grandis*) (Larsen *et al.* 2015), as well as in the afore-mentioned study of rainbow trout (Lowrey *et al.* 2015). For aquatic organisms, constantly exposed to bacteria in the medium that they inhabit, this finding

would not be surprising as the microbiome of the aquatic environment itself is generally more diverse than the skin surfaces of other aquatic organisms (Apprill *et al.* 2014; Bik *et al.* 2016). Thus, the cutaneous microbiome of fish may share some properties with that of larval amphibians (Kueneman *et al.* 2014), in that it may be influenced by both the host and the bacterial composition of the surrounding water. It would be interesting to compare the skin microbiomes of different organisms inhabiting the same aquatic environment (e.g. a shared pond) to see if there is overlap in the microbiomes of coexisting aquatic vertebrates, potentially a result of each being influenced by the same microbial community in the water column or sediment.

Comparisons between the skin and gut microbiomes of fish are more limited, although culture-dependent approaches have suggested that the skin microbiome of captive reared catfish (*Clarias gariepinus*) has similar levels of culturable diversity to that of the gut microbiome (Olojo *et al.* 2012). Temporal variability in the microbiome may also be important, and methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) analysis of 16S rRNA genes have suggested seasonal shifts in skin microbiome structure observed in both wild (Wilson *et al.* 2008) and captive fishes (Le Nguyen *et al.* 2008). In an effort to characterize the role of host specificity in the skin microbiome, over 100 individuals representing six fish species from four families were sampled in the Gulf of Mexico and their skin microbiomes analyzed by ribosomal intergenic spacer analysis (RISA) and 16S rRNA gene NGS (Larsen *et al.* 2013). Sequencing revealed that Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were the dominant bacterial phyla, with each fish species containing species-specific skin bacterial communities (Larsen *et al.* 2013). RISA results were less clear, however, and suggested a statistically significant effect of sampling date and

individual, as well as host species. Overall, the data suggested a pattern of association in microbiome composition with sample date and locality, but also a strong influence of host species specificity. This supports the hypothesis that skin microbiomes of aquatic vertebrates are comprised of bacteria present in the surrounding environment (Wilson *et al.* 2008), but that those that are able to actually colonize and establish on a host show a phylogenetic component (Bik *et al.* 2016).

#### *Endogenous microbiomes of fishes*

Host physiology (McDonald *et al.* 2012), phylogeny, and ecology (Wong & Rawls 2012) have all been implicated in structuring the gut microbiota in fishes (reviewed in Clements *et al.* 2014). Time of day, seasonality and active digestion has also been shown to affect fish gut microbiota (Kohl *et al.* 2014; Fortes-Silva *et al.* 2015) suggesting a complex community impacted by multiple environmental variables. Clements *et al.* (2014) highlight the need to accurately classify host ecology (i.e. diet) in these analyses, in order to truly determine the functional roles of fish gut microbial communities, and some studies may have misclassified diet, drawing inaccurate conclusions on the role of the microbiome (Sullam *et al.* 2012). However, both culture-dependent studies and those based on DNA sequencing have generally confirmed the importance of members of the Proteobacteria and Tenericutes (Clements *et al.* 2014; Givens *et al.* 2015). Since 2014, >30 studies have been published that examined the gut microbiomes of fishes using NGS techniques (Figure 2), and these data require a re-evaluation of what we know about the endogenous microbial community of fish.

Surprisingly, given the potential for fish to acquire microbial populations from the surrounding water, it has yet to become standard practice to compare the microbial composition of the water that fish inhabit to that of their gut microbiome. Marine mammals harbor gut



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microbial communities consisting of species found in seawater but forming distinct assemblages specific to the host species (Bik *et al.* 2016). It would be expected that gut microbial populations of fishes are also acquired from the environment (particularly for omnivorous or herbivorous fish species). Indeed, the gut bacterial communities of omnivorous fish have been shown to cluster with free-living aquatic bacterial communities rather than with the gut communities of herbivorous mammals, whereas the gut bacterial communities of carnivorous fishes cluster with those of carnivorous mammals (Sullam *et al.* 2012). Similarly, when fishes of different species, reflecting highly divergent phylogenetic positions and ecologies, are raised together in experimental ponds, they tend to have highly similar gut bacterial communities (Larsen *et al.* 2014), suggesting a strong influence of the local environment on the gut microbiome. That study found that the gut communities of three commercial fish species (channel catfish *Ictalurus punctatus*, largemouth bass *Micropterus salmoides* and bluegill *Lepomis macrochirus*) were dominated by Fusobacteria, and that just 11 bacterial genera, shared between the three species, made up approximately 98% of the bacterial sequences recovered from all samples. The gut microbiome of each of the three host species showed similar levels of bacterial species richness, but evenness was significantly lower in largemouth bass, potentially a reflection of this species' trophic status as a top predator (Larsen *et al.* 2014).

Commercially important fish species dominate studies on the fish gut microbiome. These studies have revealed interesting data that indicate fish gut microbiomes are not only structured according to dietary type and environmental conditions, but are significantly influenced by first feeding (Ingerslev *et al.* 2014), metabolic activity (Ni *et al.* 2014) and starvation (Xia *et al.* 2014). Interestingly, the gut microbiomes of a commercial trout species (*Oncorhynchus mykiss*) were found to be dominated by Firmicutes rather than Proteobacteria when juveniles were first

fed plant-based rather than marine-based food; and this effect was still seen after individuals were switched to marine-based food only, indicating the importance of first colonization effects in structuring the gut microbiome into adulthood (Ingerslev *et al.* 2014).

The Trinidadian guppy (*Poecilia reticulata*), a species that exists as two predation dependent ecotypes that differ in diet, morphology, life history and physiology, has been used as a model system to investigate the role of host life history on gut microbial community composition (Sullam *et al.* 2015). Despite differences in gut physiology and diet between the two ecotypes, gut microbial composition did not seem to be affected by ecotype in the wild guppies, which showed significant structuring in their gut microbial communities based on their stream of origin, as well as a temporal effect (Sullam *et al.* 2015). In contrast, captive guppies showed distinct gut microbial communities based on fish ecotype regardless of diet over the course of the experiment. Both wild and captive guppies had distinct core microbial communities that were different from one another, as well as from environmental water and sediment samples (Sullam *et al.* 2015). This suggests that the ecotype of wild guppies does not present a strong enough selection pressure to override the effects of locality or host genetics on the gut microbiome, but that the same is not true for guppies raised in captivity, an interesting finding as it highlights the care that needs to be taken when making inferences about the microbiome from captive animals. However, the dominant bacterial phyla in the guts of both wild and captive guppies, regardless of ecotype, were Tenericutes, Spirochaetes, Proteobacteria and Fusobacteria (Sullam *et al.* 2015). At a finer resolution, the host populations with the most dissimilar gut microbial communities were those that were most distant genetically, suggesting the influence of host evolutionary history (regardless of ecology) in the gut microbiome (Sullam *et al.* 2015).

Members of the Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria were detected in the gut microbiomes of 15 different fish species (12 bony fish, 3 sharks), and although the proportions of each phylum varied by both fish species and individual, Proteobacteria tended to dominate (Givens *et al.* 2015). Despite a high amount of variation at the individual level (likely a result of limited sample size), each of the fishes had a distinct microbial community and the three shark species contained gut microbiomes that were highly similar (69-97% shared OTUs; Givens *et al.* 2015). There were also significant differences in the gut microbial communities of wild vs cultured mummichogs (*Fundulus heteroclitus*) and between life stages (juvenile, intermediate and adult) of pinfish (*Lagodon rhomboides*), suggesting influences of diet, age, and the environment on the fish gut microbiome. Interestingly, no single OTU was shared across all 15 host species, but several core OTUs were present in multiple host species (i.e. with the three shark species; Givens *et al.* 2015).

The influence of growth rate on the fish gut microbiome has been investigated in killifish (*Kryptolebias marmoratus*) and Atlantic cod (*Gadus morhua*; Forberg *et al.* 2016). DGGE and 16S rRNA gene sequencing revealed that genetically identical killifish of the same age but of different size (i.e. therefore at different growth rates) had significant differences in the richness of their gut bacterial community, and clustering analyses separated the communities of fish with a large body size from those of fish with a small body size, albeit not significantly so (Forberg *et al.* 2016). The gut communities of small and large Atlantic cod (again, of the same age, but in this case genetically heterogeneous) were also significantly different in bacterial richness, and host size (growth rate) was significantly correlated with microbial composition. That the two species differed in their genetic diversity suggested the strong influence of host genetics, which was at least partially influencing growth rate, on the initial establishment of the fish gut

microbial community (Forberg *et al.* 2016). Laboratory studies on the model organism zebrafish (*Danio rerio*) have shown that both the composition and population size of the initial bacterial colonizers can affect subsequent colonization ability in the fish gut (Stephens *et al.* 2015), so that host genetic variability could have important implications for the microbiome in later life. The influence of genetic variation at the individual level also highlights the need for increased numbers of individuals to be sampled during microbiome studies, as much of our knowledge of fish microbiomes, and vertebrate microbiomes in general, is often based on just a few individuals that may not necessarily be representative of the genetic diversity within wild populations.

An example of a study that did sample extensively at the individual level is that of Llewellyn *et al.* (2015), who sampled the gut bacterial communities of 96 wild Atlantic salmon (*Salmo salar*) across their range in both freshwater and marine life stages, in order to examine biogeographic patterns. NGS of bacterial 16S rRNA genes revealed that individual salmon in their freshwater stage have similar gut microbial communities, regardless of locality, whereas dramatic differences in gut microbiome composition were detected between salmon in marine and freshwater stages, with adults retaining much of their marine microbiome when they reenter freshwater to spawn (Llewellyn *et al.* 2015). Proteobacteria and Tenericutes (particularly *Mycoplasma* sp.) were the dominant bacterial phyla in marine adults, whose gut microbiomes were generally characterized by low bacterial diversity and high inter-individual variability compared with those of juveniles; possibly indicative of both seasonality and dietary complexity (Llewellyn *et al.* 2015). Clearly migratory fishes can undergo drastic changes to their gut microbial community composition during ontogeny, but those changes do not appear to be a reflection of purely environmental factors. This supports the hypothesis that while the gut microbiomes of fish are more similar to the microbiome of their environments than those of

mammals, both phylogenetic factors and host ecology interact to structure the fish endogenous microbial community (Ghanbari *et al.* 2015).

### ***Amphibians***

Amphibians (frogs, salamanders & caecilians) are ectothermic, anamniotic vertebrates which occupy terrestrial, arboreal, fossorial and freshwater aquatic habitats in both temperate and tropical regions (Vitt & Caldwell 2013). Amphibians typically undergo a complex lifecycle which includes a larval aquatic stage and undergo drastic changes in physiology during metamorphosis. As of early 2016 there are 7, 517 described species of amphibians (amphibiaweb.org) with new species still being discovered at a rate of >120/year over the last ten years. All amphibian species are carnivorous or omnivorous as adults, although some juvenile stages are herbivorous/detritivorous or forego feeding altogether. Thus, while amphibians represent a diverse range of taxa found in a variety of environments, they show fundamental similarities (primarily carnivorous/omnivorous, larval aquatic stage, and subject to environmental fluctuations in temperature) that could influence their associated microbial communities.

Little is known of the microbiomes of amphibians, and the majority of studies on microbial communities associated with amphibians have focused on the cutaneous (skin) microbiome (Table S1, Supplementary Information). Even within those studies, many have utilized culture-dependent techniques to test for the presence of bacteria with anti-fungal properties or antimicrobial peptides (Culp *et al.* 2007; Brucker *et al.* 2008; Harris *et al.* 2009; Hacıoglu & Tosunoglu 2014). These studies have largely been motivated to investigate factors associated with amphibian chytrid fungus, an emergent pathogen caused by *Batrachochytrium dendrobatidis* that has been linked to global declines in amphibian populations and widespread

species' extinction (Briggs *et al.* 2010; Olson *et al.* 2013; Jani & Briggs 2014). Few studies have utilized culture-independent techniques to characterize the microbial communities found on amphibian skin (or any other part of the host) in a natural setting (Table 1, Table S1 Supplementary Information). This lack of culture-independent studies is alarming, as it has long been recognized that the majority of bacteria cannot be readily cultured using standard techniques (Amann *et al.* 1995; Pace 1997) and only 3-7% of bacterial species identified by 16S rRNA gene analyses of cutaneous communities were likely cultivable (Walke *et al.* 2014).

#### *Exogenous microbiomes of amphibians*

The few studies that have investigated natural amphibian populations suggest that the microbial communities living on adult amphibian skin are likely to be host-species specific rather than simply being bacteria acquired from the environment. (McKenzie *et al.* 2012; Kueneman *et al.* 2014). The opposite may, however, be true for larval amphibians, whose skin communities, like those of fish, have been found to be at least partially comprised of bacterial species found in the surrounding environment (Kueneman *et al.* 2014). In a study of the skin microbiome of terrestrial red-backed salamanders (*Plethodon cinereus*), much of the bacterial diversity was derived from that found in the soil where the host lived (Loudon *et al.* 2014). That said, 90% of the OTUs identified were shared across 65 individuals, suggesting that there was some core bacterial community on the salamanders skin. The same study also showed that the composition of the amphibian skin microbiome changed during captivity, regardless of the environment (natural or artificial) in which the host was raised (Loudon *et al.* 2014). Again, this raises the question of the usefulness of microbiome studies on captive animals.

### *Endogenous microbiomes of amphibians*

Even less is known about the gut microbiome of amphibians, with the gut community of just a single amphibian species (the leopard frog, *Lithobates pipiens*) being investigated in two studies (Kohl *et al.* 2013, 2015). The first study found that leopard frog gut bacterial communities undergo significant changes throughout metamorphosis, presumably related to physiological and environmental changes to the host. Gut communities of larval *L. pipiens* were largely comprised of bacterial species found in the water column in which they reside, while the gut bacterial communities of adults were unique and composed of species significantly different from that of the environment (Kohl *et al.* 2013). Larval amphibians from two other species (*Bufo terrestris*, *Pseudacris crucifer*) experienced an increase in the abundance of Gram-negative bacteria during metamorphosis, a shift that could have occurred as a result of depressed immune system function during metamorphosis that might allow for increased colonization of certain bacteria in the gut (Fedewa 2006). Elevated bacterial diversity in the gut of the leopard frog occurred following exposure to the pollutant polychlorinated biphenyl (PCB), which also could be a response to a weakened host immune system (Kohl *et al.* 2015). The immune system of adult amphibians fundamentally resembles that of mammals (Colombo *et al.* 2015) with the gut immune components being the largest immune system compartment (Weiner *et al.* 2011). Amphibians might therefore be expected to be excellent models for investigations on relationships between the gut microbiome and immune system function, which makes the lack of studies on amphibians even more surprising.

Diet has been shown to strongly influence gut bacterial community composition in other vertebrates (Ley *et al.* 2009; Sullam *et al.* 2012; Mikaelyan *et al.* 2015), but the impact of diet on the microbiomes of amphibians has not been addressed. Metamorphosis from larvae to adults

typically includes dietary changes, but broader developmental changes during that process likely have a greater impact than diet alone. Bacterial isolates obtained from the skin of frogs fed a carotenoid rich diet differed significantly from those of wild populations of the same host species and consisted of more bacterial species, a finding that the authors suggested might be beneficial (Antwis *et al.* 2014). Dietary and developmental changes as well as exposure to environmental contaminants certainly have the potential to influence the amphibian microbiome (both cutaneous and gut), but no broad conclusions can be drawn from such a limited amount of research. Studies have also generally been limited to the analysis of just one region of the amphibian host (i.e. either the cutaneous microbiome or the gut microbiome, but rarely both). An exception is a study by Montel Mendoza *et al.* (2012) that investigated culturable bacteria in multiple regions of an amphibian host, examining both the skin and the cloacae of captive bullfrogs (*Lithobates catesbeianus*). The cloaca (the opening for both digestive and reproductive tracts in amphibians) harbored strains of lactic acid bacteria not found on the skin (Montel Mendoza *et al.* 2012). As with many studies, however, it was limited to culture-dependent analyses of captive animals, although the finding that amphibians would have different bacterial communities in different parts of the body is not surprising given that clear differences are seen in different regions of the human microbiome (The Human Microbiome Project Consortium 2012; Cho & Blaser 2012).

### ***Reptiles***

Extant, non-avian reptiles (amphisbaenians, lizards, snakes, crocodylians, turtles and the Tuatara) are amniotic ectothermic vertebrates which occupy every continent except Antarctica, and nearly all biomes including terrestrial, freshwater and marine habitats (Vitt & Caldwell 2013). As of early 2016 there are 10, 272 described species of living reptiles making them nearly



twice as diverse as mammals (reptile-database.org) and the second most diverse clade of amniotic vertebrates behind birds (Pincheira-Donoso *et al.* 2013). Reptiles utilize a wide range of life history and reproductive strategies including sexual and asexual reproduction and in some cases have the ability to shift parity mode, a fairly plastic trait in this group. Reptiles have a variety of feeding strategies, including herbivory and omnivory, but the vast majority of species are carnivorous.

In contrast to amphibians, reptile associated microbiome studies have focused on the gastrointestinal tract and, to our knowledge, there are no published studies to date on the cutaneous microbiomes of non-avian reptiles. This is surprising, as snake fungal disease, an emergent pathogen caused by *Ophidiomyces* fungi, is rapidly spreading in the eastern United States (nine states to date since 2009) and has been identified as a potential global threat to snake populations and of high conservation concern (Rajeev *et al.* 2009; Sleeman 2013; Sutherland *et al.* 2014). Studies of the skin microbiome could provide insights into both the spread and potential susceptibility of the reptile host to this disease, in much the same way that studies of the amphibian skin microbiome may provide insights into chytrid fungus disease. Rather, studies of the microbiome of reptiles have focused on the endogenous microbial community so that, much more than amphibians, some patterns in the gut microbiome of reptiles are becoming apparent.

#### *Endogenous microbiomes of reptiles*

Culture-independent examination of the gut microbiota of two crotaline snake species by DGGE of amplified 16S rRNA gene fragments and subsequent sequencing found that while the species differed in community composition, the dominant bacterial phyla in both snakes were Bacteroidetes and Firmicutes (Hill III *et al.* 2008), the same phyla that dominate the gut microbiome of terrestrial mammals. A cross-taxonomic survey examining the presence of

members of the Bifidobacteria in various animals also used DGGE, in combination with quantitative PCR, to assess the gut microbial community of single individuals of eight lizard species and four turtle species housed in the Prague Zoo (Kopečný *et al.* 2010). Bifidobacteria comprised up to 22% of the bacteria found in the digestive tracts of the studied reptiles, and that bacterial group was also found to be abundant in the digestive tracts of wasps, cockroaches, and bumblebees (Kopečný *et al.* 2010). While the insects sampled were collected from wild populations, insects are a major food item for captive reptiles so these findings may be correlative (i.e. the gut microbiome of prey could contribute to the gut microbiome of a predator). However, Bifidobacteria are also often the dominant bacteria in the gut of human infants (Sela *et al.* 2008; Yatsunenکو *et al.* 2012) and their presence in captive reptiles could reflect a limitation in diet breadth as well as specific dietary composition.

DGGE has substantial limitations compared to next-generation sequencing and provides only a cursory overview of the diversity of a microbial community. The first use of NGS to examine the gut microbiome of reptiles investigated how the gut microbiome of Burmese pythons (*Python bivittatus*) changed during the digestion of prey items (Costello *et al.* 2010). As is the case for almost all of these studies, the animals were housed in captivity, although to some extent the study confirmed the work of Hill III *et al.* (2008), and found that the python gut microbiome was dominated by members of the bacterial phyla Bacteroidetes and Firmicutes. However, the relative abundances of these phyla and overall bacterial species diversity changed significantly during digestion, with an overall increase in abundance and diversity of Firmicutes during the digestive process (Costello *et al.* 2010). The increase in diversity could not be attributed to bacteria found in or on the prey item, so the observed changes in bacterial community composition likely represent shifts in bacterial populations indigenous to the host

rather than an accumulation of those associated with the meal (Costello *et al.* 2010). At a finer scale, the study also investigated bacterial community composition in different regions of the GI tract, sampling both the small and large intestines which were found to have similar phylum level bacterial composition (Costello *et al.* 2010). Members of the Bacteroidetes only dominated the large intestine during fasting periods, with Firmicutes (largely members of *Clostridium*, *Lactobacillus* and the Peptostreptococcaceae) gradually outnumbering the Bacteroidetes during periods of active digestion. This pattern was also observed in the small intestine, with Bacteroidetes dominating in fasting individuals, although the authors lacked sufficient sampling in the small intestine to test this statistically (Costello *et al.* 2010).

A more detailed examination of the differences in microbiome composition in different regions of the gut was performed on a wild crotaline snake, the cottonmouth (*Agkistrodon piscivorus*; Colston *et al.* 2015). Next generation sequencing was used to examine multiple samples taken from the small intestine, large intestine, and cloaca. As found by Costello *et al.* (2010), members of the phylum Bacteroidetes were the dominant bacteria of the large intestine; however, the composition of the small intestine differed from previous findings in that the Firmicutes were not the dominant bacterial phylum present (Colston *et al.* 2015). Rather, bacteria belonging to the phylum Proteobacteria dominated samples taken from the small intestine and cloaca (the single opening for both excretory and reproductive organs in reptiles). The increased prevalence of Proteobacteria suggests a gut microbiome more similar to that of birds (Hird *et al.* 2014), and also suggests that, again, the enteric microbiomes of wild individuals may be substantially different from those of animals raised in captivity (Colston *et al.* 2015). This question is intriguing both from an evolutionary standpoint and from that of experimental design. The earlier, limited studies that suggest that reptiles share gut microbiota similar to terrestrial

mammals should probably be re-investigated using samples collected from wild rather than captive hosts, and across a broader taxonomic range.

Dietary shifts are known to drive speciation in animals and shifts from carnivory or omnivory to herbivory lead to shifts in the gut microbiome of mammals (Ley *et al.* 2008). While the majority of extant reptiles are carnivorous, a small number (roughly 2% of known species) are herbivorous (Stevens & Hume 2004). Examination of the feces of herbivorous marine iguanas (*Amblyrynchus cristatus*) and land iguanas (*Conolophus subscristatus* and *C. pallidus*) of the Galapagos Islands suggested gut microbiota dominated by members of the Firmicutes (Hong *et al.* 2011). Fecal microbial composition was dependent on host species and land and marine iguanas, as well as terrestrial tortoises occurring on the same islands, each harbored specific bacterial communities. The land iguanas also showed some similarities in fecal microbial composition to terrestrial iguanas from a different species and locality (Hong *et al.* 2011). These results suggest potential similarities between the gut bacterial communities of herbivorous reptiles and those of herbivorous mammals. Fecal microbial communities of the iguanas also varied according to geographic location of the host, and while the primary differences in bacterial community structure were related to host species and ecotype (marine or terrestrial), within each species or ecotype, bacterial communities were structured by geographic location, with more proximal hosts having a more similar fecal microbial community (Lankau *et al.* 2012). This suggests either localized environmental impacts on the gut microbiome or the potential exchange of gut populations between hosts that are spatially closer. The latter could indicate some degree of microbiome heredity if spatially closer individuals are genetically related, or the exchange of gut bacteria through mechanisms such as shared feeding or coprophagy. While either concept seems plausible, similarity in gut microbiota between individuals that are

geographically close together has not always been found for other reptiles. For example, while herbivorous gopher tortoises (*Gopherus polyphemus*) appear to have similar gut microbiota to other herbivorous reptiles, their microbial communities showed no relationship to geographic locality of the host or to local plant variation (Gaillard 2014).

Gopher tortoises may represent an interesting variation in reptile microbiome composition, as while members of the Firmicutes and Bacteroidetes dominate their fecal microbiota, these taxa have been found in near equal proportions (Yuan *et al.* 2015). This is unusual as other herbivorous reptiles tend to have fecal communities that are overwhelmingly dominated by Firmicutes (Hong *et al.* 2011). However, turtles and tortoises have the highest proportion of herbivorous species among major reptile lineages (Vitt & Caldwell 2013), so this trend of a more taxonomically balanced gut microbiome may be more widespread in herbivorous “grazing” reptile species than is typically assumed. Bacterial species richness was found to be higher in samples from adult gopher tortoises compared to juveniles (Yuan *et al.* 2015), a pattern that has also been noted in studies of the human gut microbiome (Koenig *et al.* 2011; Yatsunenکو *et al.* 2012). While gut bacterial community composition in gopher tortoises was not strongly related to geographic distance between hosts, a weak relationship existed between gut microbiomes and conservation management practices (prescribed fire treatments) of the environment (Yuan *et al.* 2015). The lack of broader geographic patterns in gopher tortoise gut bacterial community structure may be a function of the ecology of this species, which can traverse great distances and often have large home ranges. The same study also found a weak association between microbiome structure and kinship, with parent-offspring and full siblings showing similar microbiome structure. These relationships could have arisen from direct parental

transmission during egg development, sibling association in the nest, or coprophagy biased towards close kin (Yuan *et al.* 2015).

Whether comparing genetically related individuals or not, little work has been done on understanding how reptiles acquire their associated microbial community. For mammals, acquisition of the gut microbiome begins during the birth process, and the infant microbiome develops further following nursing and other maternal contact. Broader environmental acquisition continues to occur so that infants begin to develop a microbiome that resembles that of cohabiting individuals, not just that of the mother (Koenig *et al.* 2011). The gut microbiome of juvenile reptiles likely develops from environmental exposure, for example, hatchling iguanas eat soil as they exit the nest and also acquire kin-associated microbes through coprophagy (Troyer 1984). Coprophagy also occurs within several turtle and tortoise species, although whether this is the primary mode of acquisition of gut microbiota remains untested (Yuan *et al.* 2015). This generalization remains underexplored in other reptile lineages where other mechanisms for microbiome acquisition may be present. For example, coprophagy is unlikely to be present in strictly carnivorous species, and the acquisition of bacterial populations from prey items may be more important.

Of the 25 species of extant crocodylians (Vitt & Caldwell 2013) microbiome analyses have only been performed in the American alligator (*Alligator mississippiensis*; Keenan *et al.* 2013; Keenan & Elsey 2015). However, the microbiome of this species has been quite well studied, with investigation of the bacterial community along the entire GI tract from mouth to cloaca in both wild and captive individuals, as well as during winter and spring months which represent periods of fasting or active feeding, respectively (Keenan *et al.* 2013). The oral, upper GI tract and lower GI tract harbored distinct bacterial communities, with the mouth containing

the richest bacterial community, presumably because of its exposure to the aquatic environment (Keenan *et al.* 2013). The microbiome of the oral cavity and upper GI tract were dominated by members of the Proteobacteria, while the lower GI tract was more variable (Keenan *et al.* 2013). In feeding alligators, the lower GI tract microbiome was dominated by members of the Firmicutes and Fusobacteria in both wild and captive individuals, although a dramatic increase of Firmicutes in wild individuals once feeding began in spring was not observed in captive individuals, whose proportion of Firmicutes remained relatively constant. The unexpected prevalence of Fusobacteria rather than Proteobacteria or Bacteroidetes in the gut of wild alligators may reflect the hosts ecology, as wild alligators frequently feed on carrion and Fusobacteria have previously been found to comprise the majority of the endogenous microbiome in vultures, another carrion feeder (Keenan *et al.* 2013; Roggenbuck *et al.* 2014). During periods of fasting, the lower GI tracts of both wild and captive alligators were dominated by members of the Proteobacteria and Bacteroidetes (Keenan *et al.* 2013). The much reduced shifts in microbiome structure of captive alligators vs. wild individuals during fasting or feeding months again calls into question the usefulness of microbiome data acquired from captive animals. Furthermore, when fecal samples were considered alone, they were overwhelmingly dominated by Bacteroidetes, although Bacteroidetes represented less than 10% of the composite microbiome when all other GI regions were included. This could lead to a false impression of the “gut microbiome” if only fecal samples are considered, even though characterizing the endogenous microbiome through fecal sampling is a common practice with humans and other mammals (Ley *et al.* 2008; Arumugam *et al.* 2011a; Keenan *et al.* 2013).

## *Birds*

Avian reptiles (birds) are the most diverse group of amniotic vertebrates with 10,425 described species and more than 20,000 subspecies varieties (avibase.org). Birds are endothermic, feathered amniotes with a global distribution and many species undergo lengthy seasonal migrations across great distances. While birds feed on a variety of diets, dietary preferences are often related to body size, with smaller species (e.g. hummingbirds) tending to be herbivorous and larger species (e.g. eagles) being carnivorous, with the exception of flightless birds (Stevens & Hume 1998). Birds exhibit parental care to a greater degree than other vertebrates with the exception of mammals, and these factors could be presumed to have important relationships to their associated microbial communities.

Compared to other non-mammalian vertebrates, our understanding of the enteric microbiome of birds is greater, although the majority of avian microbiome studies have focused on economically important species such as chicken and turkey. The impacts of diet, probiotic treatment, kinship and captive rearing conditions on the endogenous microbiomes of poultry has been reviewed elsewhere (Brisbin *et al.* 2008; Cisek & Binek 2014) and given the highly artificial conditions in which poultry are reared are likely of little benefit in understanding the evolution and ecology of avian microbiomes in general. The wide variety of diets and life history strategies employed by birds make them of particular interest to microbiome research, but although recent studies have capitalized on the NGS revolution (Benskin *et al.* 2010; Hird *et al.* 2014; Waite & Taylor 2014), as with other taxa, most published studies of avian endogenous microbiomes have used culture-dependent approaches or limited to Sanger sequencing of 16S rRNA gene clone libraries (Figure 1, Table 1, Table S1 Supplementary Information). Those studies do reveal the dominance of bacteria belonging to the Bacteroidetes and Proteobacteria in



the avian GI tract, and while members of the Firmicutes are typically present in any avian sample, samples from captive poultry contain higher proportions of Firmicutes than do those acquired from wild birds (Waite & Taylor 2015). Vultures may be an exception, and have high proportions of Clostridia (a class of Firmicutes) in their hindgut, presumably an adaptation to feeding on carrion (Roggenbuck *et al.* 2014). Ordination analyses of the earlier avian microbiome sequence data has revealed that gastrointestinal microbial communities group by sampling region (crop, ceca, cloaca, fecal; Waite & Taylor 2014), a result shared with reptiles (Costello *et al.* 2010; Colston *et al.* 2015) and mammals (Ley *et al.* 2008). The same data also separated into gut communities sampled from captive vs. wild individuals (reinforcing the idea that microbiome studies of captive vertebrates may be of limited use in extrapolating to wild populations) and into microbiome assemblages from carnivorous vs. omnivorous bird species (Waite & Taylor 2014).

#### *Endogenous microbiomes of birds*

One of the first culture-independent studies to investigate the gut microbiome of wild birds, examined seasonal changes in the gut microbial community of capercaillie (*Tetrao urogallus*) examining both wild and captive individuals (Wienemann *et al.* 2011). Wild birds showed differences in bacterial community composition in summer vs winter months, likely because of drastic dietary shifts, which the captive individuals did not experience (Wienemann *et al.* 2011). Differences in the proportions of specific taxa between wild and captive individuals were also observed, with members of the Clostridiales, Synergistetes, and Actinobacteria being abundant in wild birds, and significantly reduced in captive individuals, whose gut microbiome was dominated by members of the Gammaproteobacteria. The finding that Actinobacteria are abundant in the GI tract of wild birds was further supported by a study on the cloacal

microbiomes of barn swallows (*Hirundo rustica*), where Proteobacteria, Firmicutes and Actinobacteria were the dominant bacterial phyla (Kreisinger *et al.* 2015). Once again these findings highlight the necessity to use caution when drawing inferences from microbiome studies of captive animals, and also raise the question as to whether conservation planners should incorporate microbiome analyses into management plans, particularly for endangered species that may be raised in captivity for re-introduction into the wild.

The only study to characterize the gut microbiome of volant seabirds, a highly divergent group of birds that are unique in their ability to produce stomach oils through partial digestion of prey which aids in trans-oceanic dispersal, found GI bacterial communities to be dependent on host species (and thus the hosts' ability to produce stomach oils; Dewar *et al.* 2014). Firmicutes and Bacteroidetes dominated the bacterial community across all host species, although communities also contained high proportions of Proteobacteria (5-30%). The significant differences found between gut microbial communities of oil-producing and non-oil producing seabirds was likely not just a reflection of host species or digestive physiology, but rather average retention time of ingesta (Dewar *et al.* 2014). Retention time of food within the GI tract could have implications for the endogenous microbiome of many vertebrate taxa, and is an intriguing concept that has yet to be explored in other taxa which feed infrequently. A combination of qPCR (to measure overall bacterial abundance) and NGS analyses have been used to examine the gut microbiota of penguins (Dewar *et al.* 2013) which also have the ability to store food for long periods. The four penguin species investigated (king, *Aptenodytes patagonicus*; Gentoo, *Pygoscelis papua*; macaroni, *Eudyptes chrysolophus*; and little penguin, *Eudyptula minor*) shared the same dominant bacterial phyla (Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria) but the proportion of these bacterial phyla varied greatly by

species (Dewar *et al.* 2013). Each penguin species harbored a distinct GI microbiome with overlap between host species ranging from as little as 10% (king and all other species) to 50% (between Gentoo and macaroni penguins). As well as harboring the most distinct gut microbial community, king penguins also showed the lowest diversity in their microbiomes. The distinctness of the king penguin microbiome from that of the other penguin species examined could be a reflection of host phylogenetic distance or trophic position, as higher predators often have more prey-associated microbiota and lower overall diversity (Dewar *et al.* 2013, Nelson 2006).

The facial microbiomes of two species of wild vulture (*Coragyps atratus* and *Cathartes aura*) showed greater bacterial diversity than gut microbiomes of the same species, although both facial samples and hindgut samples were dominated by Clostridia and Fusobacteria (Roggenbuck *et al.* 2014). This finding was most attributed to the ecology of vultures, which primarily feed on carrion and often open carcasses from anal orifices (Roggenbuck *et al.* 2014). Facial and hindgut samples were also collected from captive individuals of the same vulture species, as well as from several additional predatory bird species. Although all predatory birds were fed similar diets in captivity, bacterial communities were found to be host-species specific, with only captive vultures having the abundance of Clostridia and Fusobacteria that characterized the microbiomes of wild vultures. This finding is in contrast to other studies on captive vs. wild bird microbiomes (Kreisinger *et al.* 2015), or the overwhelming support for differences between the microbiomes of wild and captive individuals for other vertebrates. It suggests that for certain host lineages, the phylogenetic signal in their associated microbiomes may be greater than an environmental or dietary signal that is subject to change during captivity.

Unlike other vertebrate taxa (mammals, fish and reptiles) where host genetics or phylogeny have shown a clear influence on gut microbial community structure (Ley *et al.* 2008; Arumugam *et al.* 2011b; Wong & Rawls 2012), the evidence for this association is generally lacking in birds (Waite & Taylor 2015). Avian host-specific gut bacterial communities appear to be more of a reflection of diet and geography (Hird *et al.* 2014; Waite & Taylor 2014) rather than phylogeny, which may reflect differences in reproductive physiology and/or offspring rearing in birds compared to other vertebrates, although this idea remains unexplored. It is assumed that birds acquire their gut microbiota from the nest environment or from food consumed after hatching, although few studies have tested this (Kohl 2012). In cowbirds (*Molothrus ater*), a brood parasite which relies on other species to hatch and raise their young, gut microbial community composition is not related to host species or ecology, but rather to geographic location, lending support to the theory that birds primarily acquire their gut microbiota from their immediate surroundings post-hatching (Hird *et al.* 2014). However, many hatchling birds feed on regurgitated food items from their parents, which potentially provides a mechanism for vertical transmission of gut microbiomes across generations (van Dongen *et al.* 2013). Gastrointestinal microbiota can also be transferred between individual birds during sexual copulation, providing another avenue for the acquisition of both beneficial bacteria as well as potential pathogens (White *et al.* 2010). This was shown to be the case in barn swallows, where nesting pairs had significantly more similar microbiomes within pairs than between non-breeding individuals (Kreisinger *et al.* 2015). The potential exchange of components of the endogenous microbiome during reproduction has not been explored in other terrestrial vertebrates (reptiles, amphibians) that have cloacae that are used for both reproductive and excretory functions.

## Ecological and Physiological Factors Influencing the Vertebrate Microbiome

### *Diet*

Studies on mammals suggest that host diet can have a profound effect on the gut microbiome, with recognized carnivore and herbivore mammalian gut types and an increase in microbiome diversity from carnivores through omnivores through herbivores (Ley *et al.* 2008; Muegge *et al.* 2011). These findings are also supported by surveys of human populations, and differences in the microbiome associated with a typical high fat, high protein “Western” diet compared to those of more agrarian cultures have been reported (Yatsunenko *et al.* 2012). Indeed, the development of agricultural practices and associated dietary change may be one of the most important drivers in the recent evolution of the human-microbiome symbiosis (Walter & Ley 2011). Based on human studies, it is clear that differences in dietary preferences and practices within a species can result in the development of substantially different gut microbiomes. How this translates to comparisons between different species of vertebrates has rarely been addressed. Do different species that share the same general dietary preferences also share some components of their microbiome?

At least at a very broad level this seems to be the case, and the GI tract of most vertebrates is dominated by members of the Firmicutes, Bacteroidetes and Proteobacteria regardless of whether the host is herbivorous or omnivorous, although the proportion of each of these groups varies substantially (Ley *et al.* 2009; Hird *et al.* 2014; Colston *et al.* 2015). As well as the general patterns seen for mammals between carnivorous and herbivorous diets (Ley *et al.* 2008), phylogenetically distant mammals which have converged on highly specialized diets (e.g. ants) have been found to have highly similar gut microbiomes (Delsuc *et al.* 2014). Whether or not similar convergence occurs at a broader phylogenetic range of vertebrates has not been

addressed with data from wild hosts. Human studies suggest that the members of the Bacteroidetes that are present in the GI tract are often responsible for carbohydrate fermentation, degrading plant-derived material and potentially producing short chain fatty acids that can be absorbed by the host and even contribute to its nutrition (Walter & Ley 2011). However, this is not so clear in birds, and two herbivorous foregut fermenting species (the South American hoatzin, *Opisthocomus hoazin*, and New Zealand's kakapo (a presumed foregut fermenter), *Strigops habroptilus*) have been found to have significantly different endogenous microbiomes despite similar dietary strategies (Godoy-Vitorino *et al.* 2012; Waite *et al.* 2012). In contrast, members of the Firmicutes may be more responsible for protein degradation, and the gut bacterial communities of terrestrial carnivorous mammals contain greater proportions of Firmicutes than those of terrestrial herbivorous mammals (Nelson *et al.* 2013). However, both of these bacterial phyla contain many different subgroups of bacteria, including species with a variety of properties so that these distinctions are not absolute (e.g. there are members of the Firmicutes that ferment plant polysaccharides and whose numbers increase when on an herbivorous diet; David *et al.* 2014).

There has been little interest in examining how changes in diet can influence the gut microbiome in vertebrates other than humans, or how changes to one part of the microbiome might influence that of another anatomical region. Cutaneous and mucosal microbiomes play an important role in disease resistance of the host (The Human Microbiome Consortium 2012) and changes in the gut microbiome of humans can be correlated with changes in the microbiome of other body regions (Cho & Blaser 2012; Clemente *et al.* 2012). It is unclear as to whether similar shifts occur in the microbiomes of other organisms, or how linked these communities are. Humpback whales (*Megaptera novaeangliae*) show significant shifts in their skin microbiomes

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during long periods of fasting vs active feeding, possibly reflecting stress or reduced health during fasting periods (Apprill *et al.* 2014). Whether such changes occur in non-mammalian vertebrates that feed intermittently (e.g. many reptile species) has yet to be explored. The gut microbiome of Burmese pythons changes during periods of fasting and feeding (Costello *et al.* 2010) suggesting that changes to other compartments of the host microbiome are possible. An active area of research in human microbiome studies are the links between the gut microbiome and the endocrine system, and ultimately host behavior (Lyte 2013; Foster & McVey Neufeld 2013). Given the importance of hormonal cues in the behavior of many vertebrate taxa, it's certainly possible that diet-induced changes in the gut microbiome could have far-reaching impacts for many non-mammalian species.

Temporal patterns in the microbiome of animals that feed intermittently is another area that is poorly understood. Many vertebrates undergo cycles of feeding and fasting, a feeding pattern that is common in reptiles but also seen in amphibians and fish. Studies on mammals have shown changes in endogenous microbial community structure following fasting (Morishita & Miyaki 1979; Sonoyama *et al.* 2009) and the same has been suggested for fish (Xia *et al.* 2014) and reptiles (Colston *et al.*; Costello *et al.* 2010; Keenan *et al.* 2013). Extended periods of fasting are likely to lead to substantial reductions in nutrient availability to the endogenous microbiome, potentially leading to changes in both overall diversity and phylogenetic composition. In a comparison across different classes of vertebrates, Kohl *et al.* (2014) showed that fasting increased diversity in the colon microbiome of fish (tilapia, *Oreochromis niloticus*) and amphibia (southern toads, *Anaxyrus terrestris*), but decreased diversity in the colon microbiome of birds (quail, *Coturnix coturnix*), and had no effect on a reptile (leopard geckos, *Anaxyrus terrestris*). While that study suggested some common responses of the vertebrate gut

microbial community to food availability (decreases in the relative abundance of genera such as *Ruminococcus* and *Coprobacillus*), comparisons across different vertebrate classes are of limited value without knowledge of the variation in the response between species within each class. Each class of vertebrates show substantial variation in species that feed often or occasionally, suggesting that class-scale comparisons are of only minimal value. This is compounded by the effects of dietary composition; and if diet influences the composition of the vertebrate microbiome then lab-reared animals (generally on a defined and restricted diet) are not likely to be at all representative of those in a natural setting. That said, the finding that geckos, organisms that have a more opportunistic diet, showed only minimal changes in their gut microbial community compared to other vertebrates (Kohl *et al.* 2014) does suggest possible evolutionary adaptation of the endogenous microbiome to host feeding strategy. Regardless, care must be taken when sampling wild individuals to note (if possible) their feeding state as this could profoundly impact their gut microbiome composition.

#### *Life History and Ontogeny*

Vertebrates go through a variety of physiological transformations throughout ontogeny which influence microbiome composition (Stevens & Hume 1998). These changes may be gradual, as in the case of placental mammals, or extreme as in amphibian metamorphosis. Little attention has been given to how non-mammalian vertebrates acquire their microbiome, or how ontogenetic shifts affect its composition. Even in adults, dietary shifts because of migration or other life history strategy (i.e. sneaker male toads, fishes) are accompanied by a suite of physiological and hormonal changes (Plaistow *et al.* 2004) which could influence microbiome structure. The extent to which the microbiome could influence these changes and vice versa has not been investigated outside of mammals. In humans it is generally thought that it takes long



term dietary changes to influence the core gut microbiome (Walter & Ley 2011) although significant shifts have been observed in as little as five days (David *et al.* 2014). As humans age, decreased immunity and other physiological changes may be related to shifts in the microbiome (Heintz & Mair 2014). There are non-mammalian vertebrates with lifespans that equal or significantly exceed that of humans (e.g. tortoises >100 years). It would be interesting to investigate the microbiome composition across a variety of age classes in other long-lived species.

Ecological speciation is often accompanied by shifts in life history traits (Rundle & Nosil 2005; Schluter 2009), and could be accompanied by or even driven by shifts in the microbiome (Brucker & Bordenstein 2012). Yet few studies have investigated shifts in the vertebrate microbiome in the context of ecological speciation. In one such study, the kidney-associated bacterial communities of sympatric species pairs (dwarf and normal) of lake whitefish (*Coregonus clupeaformis*) were investigated across five lakes, to test whether ecologically divergent forms differed in their microbiomes (Sevellec *et al.* 2014). While an effect of lake/locality was significant, the differences in bacterial composition between lakes were not the same for the two ecological species, suggesting form-level variation (Sevellec *et al.* 2014). Speciation in poeciliid fishes provides another example of shifts in physiological function and ecology, as species have diverged along a continuum of freshwater to water containing high levels of hydrogen sulfide (toxic to most other fish species) as well as both freshwater and toxic caves (Tobler *et al.* 2008). This has led to a multitude of shifts in life history and physiology including fecundity, offspring size, feeding performance and behavioral adaptations (Riesch *et al.* 2010). It would be interesting to investigate the microbiome composition of the different host

ecotypes in this system and how it relates to host fitness, as well as changes to the microbiome structure along this natural toxicity gradient.

Reptiles and amphibians regularly experience ecdysis or sloughing of their skin throughout their lifetime (Vitt & Caldwell 2013). Although the amphibian skin microbiome has been extensively investigated for its role in disease resistance, only cursory attention has been paid to the turnover in skin-associated microbiota during sloughing. Culturable bacteria present on the skin of marine toads (*Rhinella marina*) changed significantly after sloughing; reductions in culturable numbers of up to 100% occurred in some individuals post-sloughing (Meyer *et al.* 2012). That would imply an almost cleansing of the skin microbiome during sloughing events, which has important implications not only for natural biological resistance against pathogens, but also for the effective administration of probiotics to combat emergent disease.

Many mammals, birds, amphibians and reptiles are known to actively suppress their metabolism during winter or other periods of inactivity in order to gain energetic benefits during non-feeding periods (Lyman *et al.* 1982; Vitt & Caldwell 2013; Ruf & Geiser 2015). While this reduction in metabolism is less widespread in fish, suppression of metabolism during winter inactivity has been documented (Campbell *et al.* 2008). A reduction in metabolism could have substantial influence on the composition of the microbial community anywhere in the host, but this has yet to be explored in detail. The effects of short term fasting on the gut microbiome have been investigated across several vertebrate classes (Kohl *et al.* 2014), but studies investigating temporal changes to the microbiome throughout hibernation or torpor have been limited to mammals. Seasonal reductions in the microbial diversity of the gut lumen and gut mucus associated community have been documented in ground squirrels (*Ictidomys tridecemlineatus*), which was coupled with a decrease in the proportions of Firmicutes and increase in Bacteroidetes

and Verrucomicrobia (Carey *et al.* 2013; Dill-McFarland *et al.* 2014). Despite those seasonal shifts, a core microbiome comprised of OTUs present in all seasons was identified in the gut mucosa, the region of the GI tract that is more closely associated with the host's epithelial cells and has a stronger influence on the hosts immune response (Dill-McFarland *et al.* 2014). Although changes to Toll-like receptors (TLRs) in response to shifts in the gut microbiome were not explicitly tested, increases in TLR5 receptors during hibernation suggests that shifts in the microbiome may contribute to a decreased inflammatory response during hibernation (Dill-McFarland *et al.* 2014). The role and persistence of a core gut microbiome throughout hibernation is an avenue of research that has yet to be thoroughly explored outside of mammals.

The skin microbiome plays an important role in host defense and changes to the bacterial composition on the skin of hibernating bats have been investigated (Hoyt *et al.* 2015). Cultured bacteria from four species of bats in hibernacula were shown to have inhibitory effects on the fungal pathogen *Pseudogymnoascus destructans*, the causative agent of white-nosed syndrome, a disease which is causing widespread population extinctions in hibernating bats (Hoyt *et al.* 2015; Frick *et al.* 2015). Again, outside of mammals, we are not aware of studies investigating changes to the skin microbiome of other hibernating vertebrates or their implications for host disease resistance during prolonged periods of torpor.

## Conclusions

We have presented a survey of the literature on non-mammalian vertebrate microbiomes. Much of this discussion has focused on the evolution of the gut microbiome, and the evidence of microbial interactions with ancient host lineages leading to convergent microbial assemblages across a wide range of taxa (Rawls *et al.* 2004; Ley *et al.* 2008, 2009; Costello *et al.* 2010).

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However, these studies drew largely from captive animals in zoos or laboratories, and we question whether these relationships are as clearly partitioned in the natural world. The hypothesis that animals harbor a “core” microbiome that is reflective of phylogeny or ecology is intriguing nonetheless and there is mounting evidence that this is the case in natural populations, although significant variations exist across vertebrate classes. Whether the core microbiome is more reflective of ecology or phylogenetic history is likely linked to how an organism acquires its microbiome, via vertical transmission as in mammals or largely from the environment as appears to be the case in fishes. However, this area is largely understudied and unknown. Through NGS technologies it is now becoming relatively inexpensive to characterize host associated microbiomes. We no longer need to rely on culture-based methods to characterize the microbiome. Researchers need to work together to develop standardized methods that aim to reduce taxonomic bias introduced from variation in sample type and collection method, as well as in DNA extraction and sequencing protocols, in order to accumulate datasets that are complementary in order to facilitate reliable meta-analyses of the vertebrate microbiome in natural populations.

### **Box 1 – Variation in Non-Mammalian Vertebrate Digestive Tracts and the “Gut”**

#### **Microbiome**

Variation in the vertebrate digestive tract and its relevance to physiological function has been reviewed elsewhere (Stevens & Hume 1998), but given that such variation may impact the structure of the gut microbial community, it is important to highlight key differences both between and within major vertebrate classes and how “gut” microbiome sampling varies. A number of studies have investigated the variation in gut microbiome along different regions of

the GIT (Waite *et al.* 2012; Kohl *et al.* 2013; Colston *et al.* 2015; Lowrey *et al.* 2015) and although representatives of all major classes of vertebrates have now been investigated, these studies have not investigated the breadth of GIT variation found within these taxa (Figure 1).

The variation in pH, particle retention time, and nutrient absorptive function of each region along the GIT will influence the microbiota that can survive and inhabit that environment. As digesta passes through the GIT there is an expected turnover in microbial species and abundance, and the final excrement of feces will have an expected environmental influence on the fecal microbiome.

*Fish* – Nearly one half of all described vertebrate species are classified as fish. Fishes may be carnivorous, omnivorous, detritivorous, herbivorous and may vary their diet seasonally or through ontogeny. As such fishes have a wide range of digestive physiologies but generally, fish have a short esophagus that leads to a straight, U or Y shaped stomach (if present) that is lined with gastric mucosa. The midgut or intestine of fish is either short and straight or long with many loops which may form a lumen encapsulated spiral valve that may store food and delay digestion. The hindgut of fish is generally short. Herbivorous fish may have specialized gizzard like stomachs and/or pharyngeal teeth present to assist in the grinding of food. Most studies of the fish “gut” microbiome characterize the hindgut or whole intestinal tract, but some studies include or limit the microbiome to feces (Table S1 Supplementary Information). The variation in the region of the 16S rRNA gene sequenced has been substantial, with most studies utilizing the V1-V3 region, but with more recent work emphasizing the V4-V5 region. Within fish gut microbiome studies there is also variation as to whether intestinal contents, mucosa or tissue are used to characterize gut microbiota. Each of these sampling methods would yield expectedly different results.

*Amphibians* – Most amphibians begin life as free-living aquatic larvae that may be carnivorous, omnivorous or herbivorous. Larval amphibians generally lack a stomach but rather the mouth immediately leads to a long looped, mucus-lined intestine with low pH and no distinct regions. The GIT of amphibians undergoes restructuring during metamorphosis and adult amphibians have a mucosa-lined stomach, shortened intestine, and defined hindgut. The few studies on the amphibian gut microbiome have used cloacal swabs, swabs of the different gut regions, gut tissue, whole GIT and feces (Table S1 Supplementary Information). There is less variation in the region of the 16S rRNA gene sequenced for amphibian gut microbiota, with studies typically utilizing the V4 region.

*Reptiles* – Most species of reptiles are either carnivorous or omnivorous, but a few species are herbivorous. Most reptiles, like birds and mammals, have salivary glands which aid in the deglutition of food as it travels the esophagus from the mouth. Reptile stomachs are tubular, and lack a separate pylorus with the exception of crocodylians. The mucosal surface of the stomach is divided into gastric, pyloric and occasionally cardiac regions. The midgut of carnivorous reptiles tends to be longer than that of herbivores, with the opposite being true of the hindgut.

Herbivorous reptiles usually have a cecum and proximal colon which are defined by mucosal folds. Reptile gut microbiomes are often characterized with fecal samples that have been exposed to the environment, although section GIT tissue, swabs and cloacal swabs have been employed (Table S1 Supplementary Information). The portion of the 16S rRNA gene sequenced has typically been the V1-V4 region, with more recent studies focused on the V4 region.

*Birds* – Birds may be carnivorous, omnivorous or herbivorous. Functions typically carried out in the stomachs of other vertebrates, such as food storage, acid secretion and trituration are divided amongst the crop, proventriculus and gizzard in birds. The relative sizes and mucosal properties

of these organs vary with diet, with herbivores typically having larger crops and muscular gizzards. The midgut of most birds is short, and the hindgut consists of a short straight colon and typically paired ceca. Within herbivorous bird species the site of microbial fermentation is known to vary substantially and may be the crop (rare), midgut, ceca or colon. Typically the site of fermentation is enlarged relative to other organs (e.g. the emu has a relatively short ceca and colon but a long midgut). The bird gut microbiome has overwhelmingly been characterized via fecal samples with the occasional use of cloacal swabs or intestinal tissue (Table S1 Supplementary Information). While the microbiome along the GIT of domestic poultry has been investigated thoroughly, only a few studies have longitudinally sampled the GIT of other species. The variation in the region of the 16S rRNA gene sequenced has been substantial, with most studies utilizing the V3-V4 region.

### **Box 2 – Major Bacteria of the Vertebrate Gastrointestinal Tract**

The microbiome of the vertebrate GIT is likely dominated by bacteria which aid in nutrient absorption and maintaining homeostasis. The various regions of the GIT are inhabited by a wide range of bacteria, many of which are poorly known and not culturable using standard microbiological techniques. Here we summarize the diversity and function of the major bacterial phyla common to the vertebrate GIT. Functional information is largely derived from human microbiome studies, with an excellent overview of that topic and the role of bacteria in host health provided by the Human Microbiome Consortium (2012).

*Actinobacteria* – Actinobacteria (formerly the “High GC Gram-positive bacteria”) are typically thought of as soil bacteria but are found in most environments, including associated with animals. All members of the phylum are heterotrophs, but it includes both aerobic and anaerobic

species. The phylum includes some pathogenic genera (*Corynebacterium*, *Mycobacterium*, *Propionibacterium*), and is typically a minor (<5%) component of the gut microbiome, but is much more prevalent on the skin where it can account for 50% of the human skin microbial community. The phylum has been detected in the guts of fishes and birds, as well as on the mucosa and skin of fishes. The majority of gut-inhabiting Actinobacteria are species of *Bifidobacterium*, which have been shown to aid in maintaining host homeostasis, inhibition of Gram-negative pathogens and lactic acid fermentation. In humans, *Bifidobacterium* are dominant members of the gut microbiome during infancy where they likely help metabolize milk sugars.

*Bacteroidetes* – Members of the Bacteroidetes are heterotrophic bacteria which carry out a range of metabolisms ranging from aerobic respiration to fermentation. The phylum was formerly named the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, which represent genera in its three dominant classes (Cytophagia, Flavobacteriia, Bacteroidia). Bacteroidetes are one of the most abundant bacterial phyla found in the vertebrate GIT, with the genus *Bacteroides* typically being the most common. These organisms are strictly anaerobic, and have the ability to degrade complex molecules (polysaccharides, proteins) in the intestine, making them important for both herbivorous and carnivorous diets. Increased presence of *Bacteroides* has been linked to obesity in mammals, potentially from their ability to release extra energy from otherwise indigestible food (Turnbaugh *et al.* 2006), and differences between a microbiome that is predominantly *Bacteroides* with one that is predominantly *Prevotella*, a related genus, may reflect differences between a protein/fat-based and carbohydrate-based diet (Wu *et al.* 2011). Bacteroidetes also likely aid in the development of host mucosal and systemic immunity. While not commonly seen in the GIT, species of both *Flavobacterium* and *Cytophaga* are known pathogens of fishes, typically causing diseases of the skin or gills.



*Firmicutes* – The Firmicutes (or “Low GC Gram-positive bacteria”) are typically the most abundant bacterial phylum present in the vertebrate GIT, particularly in herbivores, and are also one of the dominant phyla found on skin. Firmicutes in the GIT are typically members of the class Clostridia, obligate anaerobes utilizing fermentation as their sole metabolism, and important in the breakdown of carbohydrates and nutrient absorption. While some GIT-associated species can become pathogenic in humans following gut microbiome disturbance (e.g. *Clostridium difficile* after antibiotic use), most are commensal and have been found to be important in the maintenance of gut homeostasis and the development of immunity (Lopetuso *et al.* 2013). Other Firmicutes include the lactic acid bacteria, which while found in the GIT are typically more common on the skin. This clade (the Lactobacillales) is also the most prevalent group of bacteria in the human vaginal tract, where it is thought to play an important role in pathogen reduction through the production of lactic acid. Interestingly, lactic acid bacteria have also been detected in the cloaca of amphibians.

*Fusobacteria* – Fusobacteria are one of the less abundant phyla in the typical vertebrate GIT but can account for approximately 5% of bacteria in the human oral cavity (Cho & Blaser 2012). The normal role for these organisms in the GIT tract is unknown, but most species are anaerobic and metabolize amino acids rather than sugars, suggesting a potential role in protein degradation. An increase in the prevalence of Fusobacteria within the human colon has been linked to the presence of cancer cells (Gao *et al.* 2015), but it is unclear as to whether Fusobacteria are involved in tumor formation or simply make use of tumors as attachment sites for growth. Of relevance to their protein-degrading capability is that an increased prevalence of Fusobacteria has been reported in the microbiomes of vertebrates that commonly feed on carrion (alligators, vultures).

*Proteobacteria* – As with the Firmicutes and Bacteroidetes, the Proteobacteria are abundant in the GIT of most vertebrates, and these three phyla essentially make up the core vertebrate GIT microbiome. While the Proteobacteria may be the third most abundant phylum in the GIT of a typical mammal, they have been found to be the dominant phylum in the GIT of some fish, reptiles and birds. The Proteobacteria is the largest bacterial phylum in terms of the number of culturable bacteria, and has been the most extensively studied. While all of its members are Gram-negative, they are metabolically diverse and include heterotrophs and autotrophs, with metabolisms including respiration, fermentation, photosynthesis and chemoautotrophy. The Proteobacteria are typically classified into the Alpha-, Beta-, Gamma-, Delta- and Epsilon-Proteobacteria, of which the Gammaproteobacteria are the most common in the vertebrate GIT.

These bacteria typically break down and ferment complex sugars, and include the well-studied bacteria *Salmonella* and *Escherichia*, the latter of which may be important in production of vitamins for the host. Members of the Betaproteobacteria and Epsilonproteobacteria can also inhabit the vertebrate GIT, and the Epsilonproteobacteria includes one genus (*Helicobacter*) that is a natural inhabitant of the mammalian stomach. These and other Epsilonproteobacteria have also been found in the GIT of birds and reptiles.

*Tenericutes* – The Tenericutes have typically been grouped as the Mollicutes, an unusual group of Firmicutes, but are more correctly identified as their own phylum. They are characterized by a lack of cell wall and are typically of very small physical size and genome size. Many are parasitic (of hosts ranging from plants to vertebrates) and all appear to require a host, making them difficult to culture and therefore study. Within the vertebrate GIT, members of the Tenericutes have been identified as important members of the gut communities of fish and juvenile amphibians, where they may aid in nutrient processing, particularly for detritivorous

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hosts. They have also been found to be dominant members of the microbiomes of corals (Kellogg *et al.* 2009; Gray *et al.* 2011) suggesting that they may be particularly important for aquatic organisms.

### **Box 3 – Future Directions**

#### *Broader, deeper sampling*

Compared to humans, our current knowledge of the microbiomes of other vertebrates, especially non-mammals, is extremely limited. For some anatomical regions we have essentially no information at all on the microbiome present, even for entire vertebrate classes (e.g. the skin microbiome of reptiles). Similarly, there are entire taxonomic groups that have never been sampled for any associated microbial community (e.g. amphisbaenids, sphenodontids). There have been recent calls for broader sampling of the global microbiome (Alivisatos *et al.* 2015; Dubilier *et al.* 2015) and the same effort is needed for non-human vertebrates. If evolutionary and ecological patterns in vertebrate microbiomes are to be examined effectively then a much broader sampling effort is needed. NGS technology has developed to the point where a single microbiome sample can be sequenced for less than US\$10 (as of 2016), yielding tens of thousands of 16S rRNA gene sequences. Analyses of hundreds of samples, potentially many different vertebrate species (broad sampling) or many individuals within a species (deep sampling), are therefore affordable for many research groups. Rather, we are at a point where collecting the samples, rather than analyzing them, is the limiting factor. Thus, coordination and cooperation between scientists in different fields is likely to be essential, with field zoologists and ecologists collecting samples for lab-based microbial ecologists and microbiologists, and bioinformatics specialists working with the subsequent data. Sampling is easier if the

microbiome can be sampled non-invasively: skin samples can be taken by simple swabbing, and the gut microbiome could be determined from feces (although there are problems with that approach) or cloacal samples for some vertebrates, which we have found to be effective in elucidating differences between individuals (Colston *et al.* 2015). Making microbiome sampling a default process when collecting tissue samples from any vertebrate taxa for phylogenetic/phylogeographic studies would require not much more than researchers carrying sterile swabs and tubes into the field, and vastly increase our knowledge of the vertebrate microbiome, as well as potentially link microbiome composition to phylogeny. Additionally, it has become commonplace for natural history collections to store genetic samples (muscle tissue, blood, fin clips, feathers etc) associated with voucher specimens. Many of these samples are stored in ultracool freezers, the same equipment necessary for the storage of microbiome samples, and we propose that the host's microbiome is of no less importance to collect and maintain than tissue samples.

As well as broadening our knowledge of the microbiomes of different vertebrate groups, deeper knowledge in individual to individual variation within species is desperately needed. This will be difficult for many species, for which field surveys may only detect a few individuals, but focusing on common taxa, especially those that can be easily collected en masse (e.g. many fish) would be a place to start. Studies on captive animals could contribute to this area, and while the microbial communities of captive vertebrates may not be a reflection of those in the wild, they do provide a relatively easy mechanism to sample multiple individuals of the same species. It's surprising that most of the microbiome studies on captive vertebrates have still just sampled a limited number of individuals, but greater coordination between different groups could be encouraged (for example, between different zoos or aquariums that house the same species).

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Sampling the microbial communities associated with many individuals of captive species would help determine the extent of individual variation within a more controlled setting, but also allow us to more clearly elucidate the influence of age, growth rate, diet, and even genetic relatedness on microbiome composition.

*The biogeography of the vertebrate microbiome*

Concurrent with efforts to sample more broadly and deeply, more extensive sampling across the ranges of species is needed. With a few exceptions, studies on wild vertebrates have tended to focus on animals sampled at one or a few specific locations, so that we have little knowledge of biogeographic patterns in microbiome composition and how much it may be driven by environmental variation or differences in diet or behavior on different parts of the range. Microbial biogeography has emerged as a field in and of itself (Dolan 2005; Martiny et al. 2006; Fuhrman et al. 2008) but the majority of studies on spatial patterns in microbial community structure or diversity have focused on microbial assemblages in aquatic or terrestrial environments, or on the microbiomes of plants rather than animals. How the gut or skin microbiome varies across the range of a vertebrate host is an intriguing question, particularly for ectothermic organisms which may be at different temperatures in different parts of their range, or for any organism that may shift its diet depending on location or because of seasonal variation in food availability.

The human gut microbiome has been found to be substantially different in different parts of the world (De Filippo *et al.* 2010; Yatsunenکو *et al.* 2012), and such differences are likely attributable to diet as studies have typically compared urban individuals in Western countries to agrarian societies elsewhere. In a broad study of microbiomes from over 200 individuals within a single country (USA), the Human Microbiome Project found that geographic location was not a

strong factor influencing microbiome composition, and that ethnic/racial origin was a stronger correlate to microbiome structure and function (The Human Microbiome Project Consortium 2012). Thus, genetics would appear to be a stronger influence on the human microbiome than geographical location. How this finding might be extended to other vertebrates that are less individually mobile and for which individuals within a specific geographic part of the range are likely to be genetically related is unknown.

While advances in technology have made humans more mobile at the individual level than other vertebrates, whole populations of non-mammalian vertebrate species (especially birds) show extensive mobility during migratory events. How the microbiome of such species changes either during migration events or from one (likely seasonal) range to another is largely unknown. In a migratory fish species (Atlantic salmon, *Salmo salar*), gut community composition was influenced by life-cycle stage rather than geography (Llewellyn *et al.* 2015), but this may not be typical given the reproductive basis for salmon migration. Collaboration between scientists in the different ranges of seasonally migratory species will be critical to understand the effects of shifting range on the microbiome, which may reflect changes in environmental influence as well as diet.

Invasive species may present an experimental system to examine biogeographic patterns in the animal microbiome but we know of no studies to date that have compared the microbial communities of vertebrates in their native and expanded invasive range. A few studies on invasive invertebrates have suggested that hosts in native and invaded regions show similar microbiome structure, with only minor differences in community composition (e.g. Bansal *et al.* 2014), but even that area is underexplored. Given the importance of invasive species to issues

regarding biodiversity, assessing the relationships between invasive species and their microbiome would seem to be critical.

#### *Functional aspects of the vertebrate microbiome (metagenomics)*

Most NGS studies of the microbiomes of non-mammalian vertebrates have focused solely on community composition, typically using partial 16S rRNA gene sequencing to describe the structure of the gut or skin assemblage. Studies of the human microbiome have incorporated a metagenomics approach to also characterize the functional genes present in the microbiome of different individuals and body habitats. Such studies have found that while the composition of the microbiome changes from body region to body region, or from individual to individual, many microbial metabolic pathways are prevalent across most individuals and body habitats (Human Microbiome Project Consortium 2012, Lozupone *et al.* 2012). The human gut microbiome, for example, always contains the genes for central pathways in carbohydrate and amino acid metabolism, regardless of the individual bacterial species present (Turnbaugh *et al.* 2009). Such functional similarity might also occur in comparisons between taxa, with, for example, animals that are phylogenetically distant but overlap in diet, potentially having endogenous microbial communities that have similar properties (Hanning and Diaz-Sanchez 2015). Herbivorous mammals that rely on their gut microbiome for cellulose digestion are an obvious example, and comparative metagenomics has been used to compare the functional capability of the gut microbial communities of agriculturally important mammals (Lamendella *et al.* 2011).

The application of functional approaches to the microbiomes of non-mammalian vertebrates is sorely lacking even though techniques such as shotgun metagenomics could be used to answer questions relating to the influences of diet, biogeography, and phylogeny on the host microbiome. Functional comparisons across taxonomic groups that show similar properties,

such as between herbivorous fish, reptiles, and amphibia; or between different groups of fasting reptiles; or even between different taxa inhabiting the same environment (e.g. comparing the functions of the skin and gut-associated microbial communities of co-existing amphibians and fish in the same pond) could reveal important findings in regards to how structurally distinct microbial assemblages can show overlapping function. Costs for such metagenomics analyses exceed those for 16S rRNA gene surveys of community structure, but if care is taken during the initial sampling and DNA extraction process, any microbiome sample taken could be preserved for potential future metagenomic analysis. A greater sampling effort of non-mammalian microbiomes coupled with careful archiving of samples that can be used for multiple levels of analyses, and potentially by different groups, would be highly beneficial.

*The influence of the microbiome on host evolution and diversification*

Studies of the microbial communities associated with eukaryotic organisms have an overwhelming tendency to emphasize the impacts of host ecology (diet, age, growth rate, location, genetics) on the microbiome. The reverse – the influence of the microbiome on the ecology and evolution of the host – has rarely been considered. Research on the human microbiome is beginning to elucidate the role of our associated microbial community in our physiology, immunity, and even neurological and thought processes (Cho & Blaser 2012; Foster *et al.* 2013; Lyte 2013; Stilling *et al.* 2014)). As such, it's becoming clear that the ecology of the host is highly dependent on their associated microbiome, and that the host and microbiome are likely in a hologenomic state of coevolution to maximize the success of both (Brucker & Bordenstein 2012; Amato 2013). But to what extent does the microbial community associated with vertebrates actually drive diversification?



Addressing such a question will require more extensive analyses of the microbiomes of closely related species, as well as of analyses of differing individuals within a species (essentially, the broader, deeper sampling that we highlight above). For example, surveying the gut microbial communities associated with a broad taxonomic range of vertebrates such as the squamate reptiles or the perciform fish, and linking the composition of the microbiome to ecological factors such as diet or the environment as well as to evolutionary phylogeny and diversification rate may help understand whether the endogenous microbial community can itself be thought of as an ecological trait; potentially influencing both host diversification and community assembly. Dietary changes can reflect shifts in the niche, one of the classic traits used to characterize ecological processes affecting speciation (Kozak & Wiens 2010; Jeraldo *et al.* 2012), and clearly affects, and is potentially affected by, the gut microbial community. Similarly shifts in parity mode are associated with diversification in reptiles (Sites *et al.* 2011; Pyron & Burbrink 2014), and may represent another ecological trait that influences or is influenced by the endogenous microbiome. To what extent changes in microbiome structure have been coupled with or even driven diversification patterns in different branches of the vertebrate tree of life is an intriguing question for future work.

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**Data Accessibility:**

Data not presented in manuscript is available in supplementary information file.

**Author Contributions:**

TJC & CRJ developed the concept of the paper. TJC performed the literature review. TJC & CRJ wrote the manuscript.

**Tables**

Table 1. Summary of host organism, technology used: next generation sequencing (NGS), traditional molecular methods (TMM; including Sanger sequencing, DGGE, TGGE, PCR, qPCR) culture or microscopy (including florescent imagery and SEM), wild vs. captive, and number of publications from 229 published studies since 1990. A more detailed table which further categorizes the studies according to both technology used, sample type and genetic marker sequenced is available in Supplementary Table S1.

<b>Organism</b>	<b>Technology used</b>	<b>Captive (C) or wild (W) host</b>	<b>Number of published studies</b>
Fish	NGS	C	19
Fish	NGS	W	3
Fish	NGS	W&C	2
Fish	Cloning & TMM	C	2
Fish	Cloning & TMM	W	4
Fish	Culture & TMM	C	35
Fish	Culture & TMM	W	6
Fish	Culture & TMM	W&C	1
Fish	Culture only	C	23
Fish	Culture only	W	8
Fish	Culture only	W&C	1
Fish	Culture, cloning, TMM	W	1
Fish	Cloning, microscopy &		
Fish	TMM	C	2
Fish	TMM	C	22
Fish	TMM	W&C	1
Fish	TMM & NGS	C	2
Fish	Microscopy	C	4
Fish	Microscopy	W	3
Frog	NGS	C	2
Frog	NGS	W	3
Frog	NGS	W&C	2
Frog	Culture NGS	W	1

Frog	Culture & TMM	C	3
Frog	Culture & TMM	W	7
Frog	Culture & TMM	W&C	1
Frog	Culture only	C	6
Frog	Culture only	W	5
Frog	TMM	C	1
Frog	TMM	W&C	2
Frog (tadpole)	NGS	C	1
Salamander	NGS	W	4
Salamander	NGS	W&C	2
Salamander	Culture & NGS	W	1
Salamander	Culture & TMM	C	1
Salamander	Culture & TMM	W	4
Salamander	Culture only	W	1
Salamander	TMM	C	1
Salamander	TMM	W&C	1
Lizard	NGS	C	1
Lizard	NGS	W	1
Lizard	TMM	C	1
Snake	NGS	C	1
Snake	NGS	W	1
Snake	Cloning & TMM, NGS	W	1
Snake	Culture only	W	1
Snake	TMM	C	1
Snake	TMM	W	2
Tortoise	NGS	W	1
Alligator	NGS	W&C	2
Bird	NGS	C	2
Bird	NGS	W	8
Bird	NGS	W&C	4
Bird	Cloning and TMM	W	11
Bird	Culture only	W	1
Bird	Culture only	W&C	1
Bird	PCR	W&C	1

Figures

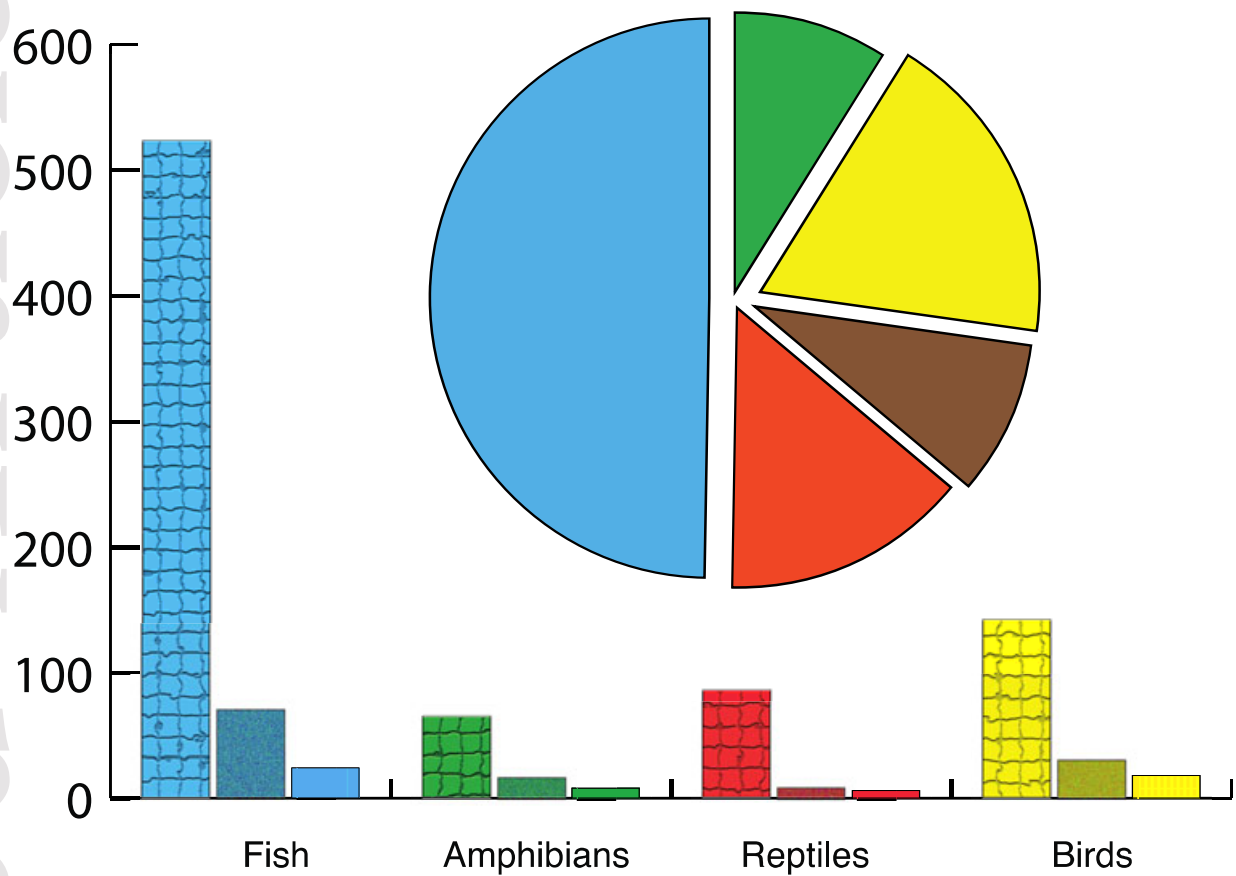


Figure 1. Column chart describing the number of families (tiled bar), number of families whose microbiome has been studied (hatched bar), and number of families whose microbiome has been investigated using NGS methods (plain bar) for fish (blue), amphibians (green), reptiles (red) and birds (yellow). Inset pie chart displays percentage of vertebrate species described and includes mammals (brown).

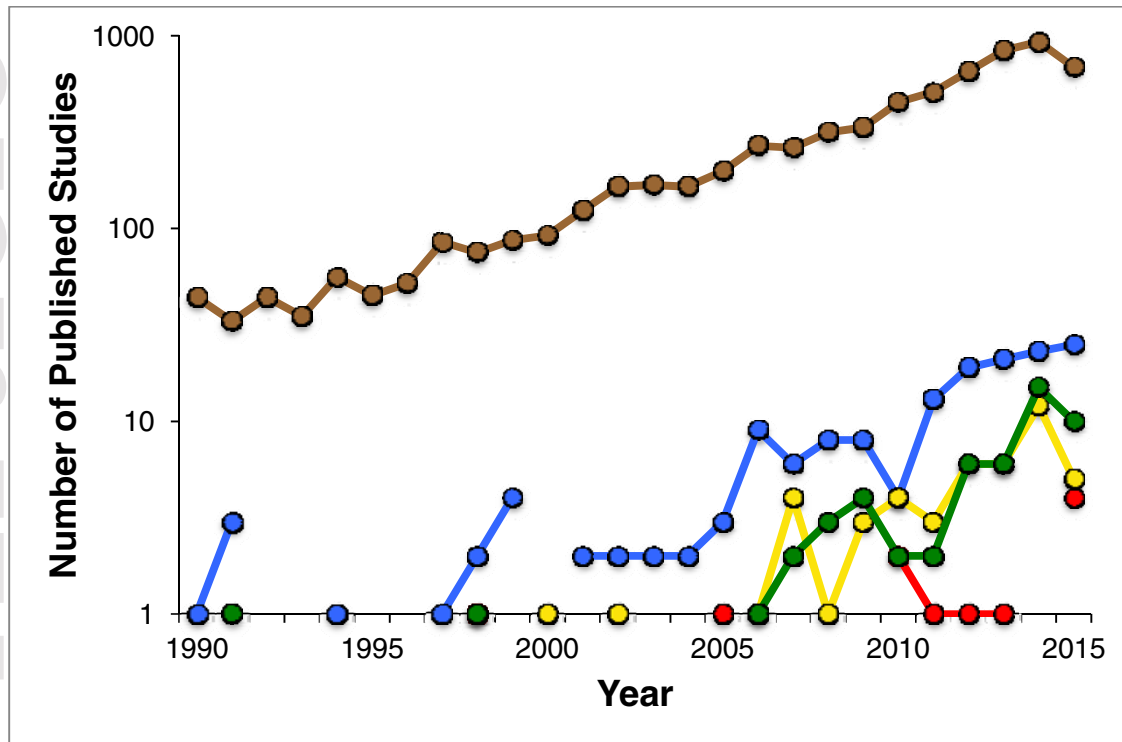


Figure 2. Increase in the number of microbiome studies for different classes of vertebrates over a 25 year period from 1990 to 2015. Numbers were derived from a search of the Scopus database ([www.scopus.com](http://www.scopus.com)) for each of amphibians (green), birds (yellow), fish (blue), reptiles (red), and mammals (brown) using the search terms “microbiome” and/or “bacteria” and “gut”, and not “mouth” and not “blood”. Mammals included humans while for birds we excluded strictly domesticated poultry studies focused on pathogens. Because of an increased interest in that area, amphibians included the search term “skin”. Publication number is shown on a log scale. Microbiome studies of mammals have increased at a fairly consistent exponential rate each year, whereas increases in studies of non-mammalian vertebrates have been more erratic and even combined fall substantially below those of mammals.