

ORIGINAL ARTICLE

Postprandial remodeling of the gut microbiota in Burmese pythons

Elizabeth K Costello¹, Jeffrey I Gordon², Stephen M Secor³ and Rob Knight^{1,4}

¹Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO, USA; ²Center for Genome Sciences, Washington University School of Medicine, St Louis, MO, USA; ³Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL, USA and ⁴Howard Hughes Medical Institute, University of Colorado, Boulder, CO, USA

The vertebrate gut microbiota evolved in an environment typified by periodic fluctuations in nutrient availability, yet little is known about its responses to host feeding and fasting. As many model species (for example, mice) are adapted to lifestyles of frequent small meals, we turned to the Burmese python, a sit-and-wait foraging snake that consumes large prey at long intervals (>1 month), to examine the effects of a dynamic nutrient milieu on the gut microbiota. We used multiplexed 16S rRNA gene pyrosequencing to characterize bacterial communities harvested from the intestines of fasted and digesting snakes, and from their rodent meal. In this unprecedented survey of a reptilian host, we found that Bacteroidetes and Firmicutes numerically dominated the python gut. In the large intestine, fasting was associated with increased abundances of the genera *Bacteroides*, *Rikenella*, *Synergistes* and *Akkermansia*, and with reduced overall diversity. A marked postprandial shift in bacterial community configuration occurred. Between 12 h and 3 days after feeding, Firmicutes, including the taxa *Clostridium*, *Lactobacillus* and *Peptostreptococcaceae*, gradually outnumbered the fasting-dominant Bacteroidetes, and overall 'species'-level diversity increased significantly. Most lineages seemed to be indigenous to the python rather than ingested with the meal, but a dietary source of *Lactobacillus* could not be ruled out. Thus, the observed large-scale alterations of the gut microbiota that accompany the Burmese python's own dramatic physiological and morphological changes during feeding and fasting emphasize the need to consider both microbial and host cellular responses to nutrient flux. The Burmese python may provide a unique model for dissecting these interrelationships.

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Introduction

The gut microbiotas of terrestrial mammals are among the most widely and intensively surveyed of vertebrate-hosted microbial communities. Indeed, a large and diverse microbiota inhabits the mammalian gastrointestinal tract where its composition is shaped, in part, by host diet and phylogeny (Dethlefsen *et al.*, 2007; Ley *et al.*, 2008a,b) and the majority of bacteria belong to one of the two dominant phyla: the Bacteroidetes or Firmicutes (Ley *et al.*, 2005, 2008a). Among its many important functions, the gut microbiota is involved in degrading otherwise indigestible components of the

host's diet such as plant polysaccharides into compounds that the host can absorb (for example, short-chain fatty acids) (Flint *et al.*, 2008). In the absence of this microbial fermentation, calories present in a diverse array of complex dietary glycans would be lost to the host. Interestingly, when dietary polysaccharides are in short supply (for example, when they are removed from the diet or, ostensibly, after fasting), certain gut-adapted bacteria are capable of alternatively foraging host mucosal glycans (Salyers *et al.*, 1977; Sonnenburg *et al.*, 2005; Bjursell *et al.*, 2006; Martens *et al.*, 2008, 2009). It has been proposed that this flexible foraging strategy may serve to enhance ecosystem stability in a dynamic nutrient environment (Yachi and Loreau, 1999; Sonnenburg *et al.*, 2005).

In humans and mice, increased adiposity has been associated with alterations in the gastrointestinal microbiota that allow it to more efficiently liberate energy from the host's diet, an arrangement that might benefit the host if food resources were only

Correspondence: R Knight, Department of Chemistry and Biochemistry, 215 UCB, University of Colorado, Boulder, CO 80309-0215, USA.

E-mail: rob.knight@colorado.edu

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intermittently available (Backhed *et al.*, 2004; Ley *et al.*, 2005; Turnbaugh *et al.*, 2006, 2009a; Samuel *et al.*, 2008). A corollary hypothesis, that the microbiota assumes a different configuration when nutrients are scarce, and that this state also confers some benefit to the host, has also been posited. Although the latter, nutrient-deprived scenario is less well understood, a recent study has revealed that a 24-h fast in mice produces a marked shift in gut microbial ecology, and that the presence of a gut microbiota while fasting enhances nutrient supply to the heart (Crawford *et al.*, 2009). Further elucidating the interrelationships between the gut microbiota's composition and function, and the host's diet, nutritional status and cellular physiology would benefit from the study of a wider variety of host species, in which each of these parameters could be either intentionally or naturally varied over a broad range.

Many model species, including humans and mice, are adapted to lifestyles of frequent small meals. These species' gastrointestinal tracts typically contain food and are rarely empty and, as a consequence, their postprandial metabolic responses are regulated over relatively narrow ranges (Secor and Diamond, 1998). By contrast, sit-and-wait foraging snakes such as the Burmese python, *Python molurus*, consume enormous meals at long intervals. As an apparent adaptation to extended periods without food, pythons exhibit exceptionally large physiological and morphological responses to feeding and fasting, features that have made them an attractive model species in studies of the regulation of host processes (Secor *et al.*, 1994; Secor and Diamond, 1995, 1998; Starck and Beese, 2001; Secor, 2008). Here, we consider whether changes in the composition and structure of the Burmese python's gut microbiota accompany this reptile host's other, well-documented postprandial responses.

Burmese pythons are native to southeastern Asia (and invasive in Puerto Rico and southern Florida) where they feed largely on birds and mammals (Pope, 1961; Snow *et al.*, 2007). Adult pythons are among the largest snakes, reaching 6.5 m in length and 100 kg; however, juveniles weigh only 0.1–1.0 kg and consume rats and mice making them tractable experimental animals (Pope, 1961; Secor and Diamond, 1998). In the wild, pythons use the sit-and-wait tactic of foraging, which is characterized by long intervals (>1 month) between meals, and by prey that can exceed 50% of the snake's body mass (Pope, 1961; Murphy and Henderson, 1997). Accordingly, the python's gastrointestinal tract is well adapted to long periods of quiescence punctuated by the digestion of extremely large, intact prey (Secor, 2008). Intriguingly, almost nothing is known about the composition of the Burmese python's gut microbiota or, for that matter, the gut microbiota of most reptiles (Hill *et al.*, 2008).

In the fasted state, the Burmese python's gut and other organs exhibit dramatic alterations in structure

and function (Secor, 2008). The ingestion of a meal signals the python's stomach to begin secreting digestive acid and enzymes (Secor, 2003) and for the atrophied small intestine to upregulate brush-border digestive enzymes and nutrient transporters (Secor and Diamond, 1995; Secor, 2008). During the digestion of a meal, the small intestine doubles in mass, reflecting both hyperplastic and hypertrophic responses in its epithelium. These changes within the gut show regional specificity: a comparable increase in mass does not occur in the large intestine. Feeding and fasting also have extra-intestinal manifestations: heart, liver, pancreas and kidney masses increase by 40–106% within 3 days after feeding, although the roles played by hyperplastic and hypertrophic mechanisms in these responses remain undefined (Secor and Diamond, 2000; Andersen *et al.*, 2005).

Peak changes in small-intestinal morphology (a fivefold lengthening of the microvilli, as documented by transmission electron microscopy) and function (a 3–13-fold increase in hydrolase activity and nutrient uptake capacity) occur after 2–3 days of digestion, at which point the unabsorbed material begins to fill the python's large intestine (Secor and Diamond, 1995; Lignot *et al.*, 2005; Cox and Secor, 2008; Secor, 2008). At 6–7 days after feeding, as the last of the meal exits the stomach and traverses the small intestine, downregulation begins and by day 10, enzyme activities, nutrient uptake rates and organ masses have decreased significantly (Secor and Diamond, 1995; Lignot *et al.*, 2005; Cox and Secor, 2008; Secor, 2008). Thus, downregulation with the final passage of the meal appears to occur as quickly as upregulation on ingestion.

In the present study, we surveyed bacterial communities resident within the small and large intestines of fasted Burmese pythons and at eight time points following the ingestion of a rodent meal weighing 25% of the snake's body mass. Prior to experimentation, the 32 juvenile pythons we examined had fasted for a minimum of 30 days. Postfeeding samples were collected at 0.25, 0.5, 1, 2, 3, 4, 6 and 10 days, and three to four snakes were killed at each time point. Contents were harvested from the proximal and distal regions of the small and large intestines and from the cecum, an extension of the large intestine that forms a blind-end pouch at the junction with the small intestine. We also characterized the bacterial assemblages consumed with the python's rodent meal. For each sample, bacterial 16S rRNA genes were PCR-amplified using a primer set with a unique error-correcting barcode (Hamady *et al.*, 2008). Coupling this barcoding approach with high-throughput pyrosequencing, we were able to survey the gut microbiota of a non-mammal vertebrate host at an unprecedented level of detail. We were also able to examine the effects of an extreme fluctuation in nutritional status on the gut bacterial communities of a host species uniquely adapted to this lifestyle.

Materials and methods

Animals

Captive-born hatchling pythons were purchased commercially and housed individually in 20-liter plastic containers at 28–32 °C under a 14 h:10 h light:dark cycle. Snakes were fed laboratory rats every 2 weeks and had continuous access to water. Rats (RodentPro.com 7-wk old CD strain fed proprietary chow) were purchased frozen and thawed in clean warm water before feeding. Prior to experimentation, food was withheld from snakes for a minimum of 30 days to ensure that the snakes were postabsorptive. Burmese pythons are known to complete their digestion within 10–14 days after feeding (Secor and Diamond, 1995). Snakes used in this study were of both sexes, were between 12 and 36 months old, and had body masses averaging 817 ± 38 g (s.d.). The University of Alabama Institutional Animal Care and Use Committee approved all procedures involving the animals used in this study.

Sample collection

Snakes were killed by severing the spinal cord immediately posterior to the head. Following death, a mid-ventral incision was made to expose the gastrointestinal tract, which was removed. The contents of the cecum and the proximal and distal regions of the small and large intestines were emptied into sterile vials, immediately frozen in liquid N₂ and stored at –80 °C. The gut content mass was determined by weighing the organ before and after emptying. The average masses of contents harvested from the large and small intestines at each time point are shown in Figure 1a and Supplementary Figure 3a, respectively. In fasted snakes, the small intestine contains a small amount of clear fluid, presumably secretions from the gall bladder, pancreas and intestine. Here, several milliliters of sterile saline were used to flush the fasted small-intestinal contents into collection vials. After feeding and before day 10, the small intestine contained enough material to be squeezed directly out into vials. The large intestine typically contains some material, including residual feces, fluid and urate originating from the kidneys in fasted snakes. In the present study, material from the meal started entering the large intestine ~1-day postfeeding, with the cecum filling at 2 days. At day 3 and beyond, the large intestine and cecum were fairly filled with the material.

Frozen feeder rats were thawed overnight to 4 °C. Each thawed rat was homogenized for 3–5 min in 750 ml of autoclaved water using a 1-gal commercial blender on the highest speed setting (Waring model CB10, Torrington, CT, USA). Immediately after blending, sterile cotton swabs were dipped into the whole-rat homogenates (five replicates per animal), packaged into sterile tubes and frozen at –80 °C.

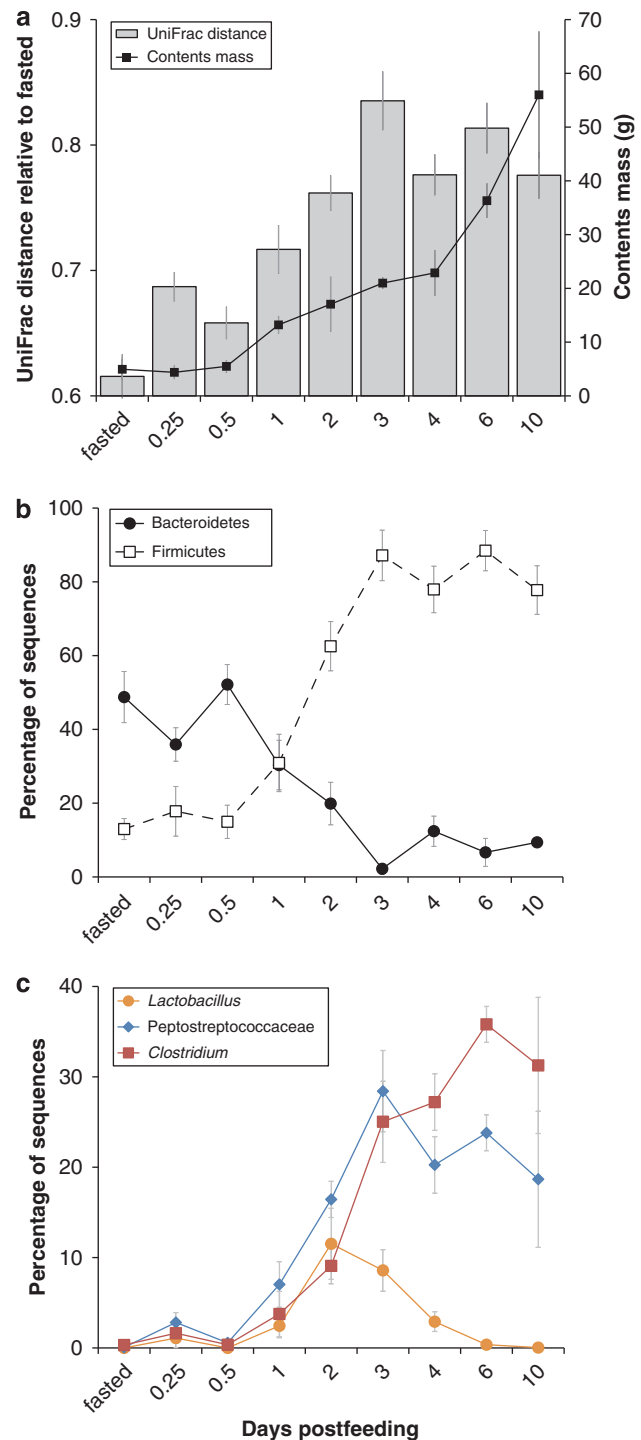


Figure 1 Digestion alters bacterial community membership and taxon relative abundances within the Burmese python large intestine. (a) Average mass of large-intestinal contents harvested at each time point (right axis) and unweighted UniFrac distance between fasted and fed communities and, for the fasted time point, between fasted samples only (left axis). (b, c) Average proportional abundance of sequences classified as (b) Bacteroidetes or Firmicutes or as (c) selected Firmicutes taxa at each time point. Because other lineages can also be present, the proportions need not add up to 100%. Error bars indicate one s.e. of the mean. Fasted snakes were fed a rodent meal equal to 25% of their body mass. Contents masses shown in (a) do not include the cecum, which contained ~3–4 g of material on days 2 through 10.

DNA extraction

Snake gut contents (100 mg) were suspended in 500 μ l of buffer A (200 mM Tris, pH 8.0; 200 mM NaCl; 20 mM EDTA) followed by addition of 210 μ l of 20% SDS and 500 μ l of a 1:1 mixture of phenol/chloroform. A total of 500 μ l of zirconium beads were added to each tube, which were then placed in a bead beater on the highest speed setting for 2 min (Biospec, Bartlesville, OK, USA). The mixture was centrifuged at 6000 *g* at 4 °C for 3 min and DNA was purified from the resulting supernatant via phenol/chloroform extraction, followed by ethanol precipitation.

For rat homogenate samples, genomic DNA was extracted from cotton swabs using the MO BIO PowerSoil DNA Isolation kit (Carlsbad, CA, USA) with modifications. Similar to the extraction protocol described above, this method involves the disruption of cells by bead beating in the presence of a buffered SDS solution. The cotton tips of frozen swabs were broken off directly into bead tubes to which 60 μ l of Solution C1 had been added. Tubes were incubated at 65 °C for 10 min and then shaken horizontally at maximum speed for 2 min using the MO BIO vortex adapter. The remaining steps were performed according to the manufacturer's instructions.

PCR amplification of the variable region 2 of bacterial 16S rRNA genes

We amplified variable region 2 of the 16S rRNA gene using a previously published primer set (Fierer *et al.*, 2008). The forward primer (5'-GCC TTG CCA GCC CGC TCA GTC AGA GTT TGA TCC TGG CTC AG-3') contained the 454 Life Sciences primer B sequence, the broadly conserved bacterial primer 27F, and a two-base linker sequence ('TC'). The reverse primer (5'-GCC TCC CTC GCG CCA TCA GNN NNN NNN NNN NCA TGC TGC CTC CCG TAG GAG T-3') contained the 454 Life Sciences primer A sequence, a unique 12-nt error-correcting Golay barcode used to tag each PCR product (designated by NNNNNNNNNNNN), the broad-range bacterial primer 338R and a 'CA' linker sequence inserted between the barcode and the rRNA primer.

For python samples, PCR reactions were carried out in quadruplicate 20 μ l reactions with 0.3 μ M forward and reverse primers, 100 ng gel purified template DNA (Qiaquick kit, Qiagen, Valencia, CA, USA) and 1X HotMasterMix (5 PRIME, Gaithersburg, MD, USA). Thermal cycling conditions were 95 °C for 2 min, followed by 30 cycles of 95 °C for 20 s, 52 °C for 20 s and 65 °C for 1 min. Replicate amplicons were pooled and cleaned using Ampure magnetic purification beads (Agencourt, Danvers, MA, USA).

For rat samples, PCR reactions were carried out in triplicate 25 μ l reactions with 0.6 μ M forward and reverse primers, 3 μ l template DNA and 1X HotMasterMix. Thermal cycling consisted of 94 °C for 3 min,

followed by 35 cycles of 94 °C for 45 s, 50 °C for 30 s and 72 °C for 90 s, with a final extension of 10 min at 72 °C. Replicate amplicons were pooled and cleaned using the UltraClean-htp 96-well PCR Clean-up kit (MO BIO, Carlsbad, CA, USA).

Amplicon quantitation, pooling and pyrosequencing

Amplicon DNA concentrations were determined using either the bisbenzimidazole H assay (Sigma, St Louis, MO, USA; used for snake samples) or the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen, Carlsbad, CA, USA; used for rat samples). Following quantitation, amplicons were pooled in equimolar ratios and cleaned. Amplicon pyrosequencing was carried out using primer A on a 454 Life Sciences Genome Sequencer FLX instrument (Roche, Branford, CT, USA).

Sequence analysis

Sequences were processed using the QIIME software package (Caporaso *et al.*, 2010). Sequences were removed from the analysis if they had a mean quality score <25, were <200 or >300 nt in length, contained ambiguous characters, did not contain the primer sequence, contained a homopolymer run exceeding 8 nt or contained an uncorrectable barcode. The remaining sequences were assigned to samples by examining the 12-nt barcode. Similar sequences were clustered into operational taxonomic units (OTUs) using cd-hit (Li and Godzik, 2006) with a minimum coverage of 99% and a minimum identity of 97%. A representative sequence was chosen from each OTU by selecting the most abundant sequence. Representative sequences were aligned against the Greengenes (DeSantis *et al.*, 2006) core set using PyNAST (Caporaso *et al.*, 2009) with a minimum alignment length of 150 and a minimum identity of 75%. The PH Lane mask was used to screen out hypervariable regions after alignment. A phylogenetic tree was inferred using FastTree (Price *et al.*, 2009) with Kimura's 2-parameter model. Taxonomy was assigned to each unique sequence (that is, sequences representative of OTUs picked at 100% sequence identity) using the ribosomal database project classifier with a minimum support threshold of 80% and the RDP taxonomic nomenclature (Wang *et al.*, 2007). Statistical analyses were performed in R. The mean (\pm s.e.m.) is reported in the text unless otherwise noted.

α and β diversity

To evaluate the amount of diversity contained within samples (α diversity), we constructed rarefaction plots from both branch length-based phylogenetic diversity measurements (Faith, 1992) and OTU-based measures using QIIME. For each sample, the mean of 10 iterations per sub-sampling interval

was calculated. Sample means were then averaged within each days-postfeeding category (0–0.5, 1–2 and 3–10). The 95% confidence interval was calculated using the critical value for the two-tailed *t* distribution.

To determine the amount of diversity shared between two samples (β diversity), we used the UniFrac metric (Lozupone and Knight, 2005; Lozupone *et al.*, 2007; Hamady *et al.*, 2010) in QIIME. UniFrac distances are based on the fraction of branch length shared between two communities within a phylogenetic tree inferred from the 16S rRNA gene sequences from all the communities being compared. We used unweighted UniFrac, in which only the presence or absence of lineages is considered (community membership) and weighted UniFrac, which also accounts for relative abundance (community structure). Principal coordinates plots were visualized using the KiNG graphics program (<http://kinemage.biochem.duke.edu/software/king.php>). Hierarchical clustering based on UniFrac distances between composite communities was performed using the unweighted pair group method with arithmetic mean (UPGMA).

Data deposition

The bacterial 16S rRNA gene sequences reported in this paper were deposited in the GenBank Short Read Archive (accession number SRA012490).

Results and discussion

Dominance of Firmicutes and Bacteroidetes in the python gut

From 109 gut content samples collected from 32 juvenile Burmese pythons (Supplementary Table S1), we obtained ~280 000 high quality, classifiable 16S rRNA gene sequences with an average read length of 230 nt and an average count per sample of 2560 ± 480 (s.d.; ~9000 sequences per animal). To our knowledge, this constitutes the most comprehensive survey of a reptilian microbiota performed to date. We detected members of 12 bacterial phyla in the python gastrointestinal tract. Surprisingly, as observed in humans, mice and a wide variety of other mammals (Eckburg *et al.*, 2005; Ley *et al.*, 2005, 2008a), most of the sequences detected in the python gut were classified as either Firmicutes (61.8%) or Bacteroidetes (20.6%). Proteobacteria (10.1%) and Deferribacteres (3.9%) were also abundant, and less abundant phyla included the Actinobacteria (0.6%), Verrucomicrobia (0.6%), Fusobacteria (0.5%) and Lentisphaerae (0.01%). When sequences were classified to the highest taxonomic level to which they could be confidently assigned, the following taxa were abundant in the overall pool of python gut sequences: the class Clostridiales (Firmicutes, 4.2%); the families Clostridiaceae and Peptostreptococcaceae (Firmicutes, 19.0 and 18.5%, respectively) and the genera

Bacteroides and *Rikenella* (Bacteroidetes, 7.0 and 5.6%, respectively), *Lactobacillus* (Firmicutes, 5.6%) and *Synergistes* (Deferribacteres, 3.9%). Thus, the Burmese python seems to have in common with mammals several gross taxonomic features of its gut microbiota. These results raise the question of whether the last common ancestor of the amniotes (reptiles, birds and mammals) also harbored a gut microbiota dominated by Firmicutes and Bacteroidetes, or whether this trait has evolved more than once. Notably, 16S rRNA-based profiling of the gastrointestinal bacteria of several wild-captured pit vipers (three individuals from two species) also detected the genera *Lactobacillus*, *Bacteroides* and *Fusobacterium* (Hill *et al.*, 2008). Later in this paper we examine whether the mammal-like membership of the python's gut microbiota stems from an input of bacteria from the snake's rodent meal.

Fasting is associated with expanded Bacteroidetes and reduced diversity in the large intestine

After fasting for >30 days, Burmese pythons harbored gut bacterial communities dominated by members of the phylum Bacteroidetes ($48.8 \pm 7.0\%$ ($N =$ seven samples from three individuals; different regions, including the cecum, were similar; hence, we averaged their values and refer to them jointly as large intestine)). Due, in part, to the difficulty in obtaining adequate quantities of microbial biomass from the fasted small intestine, only a single sample was available for analysis (distal region; 34.4% Bacteroidetes). Thus, our analysis of the fasted microbiota is focused on the large intestine. Abundant Bacteroidetes-related taxa included the genera *Bacteroides* ($16.0 \pm 1.1\%$) and *Rikenella* ($12.7 \pm 2.5\%$), as well as lineages that could not be confidently assigned beyond the phylum level ($15.7 \pm 3.7\%$). In addition to the Bacteroidetes, the fasted communities also featured abundant Proteobacteria ($14.3 \pm 6.7\%$), a relatively small proportion of Firmicutes ($13.2 \pm 2.6\%$), and Deferribacteres and Verrucomicrobia of exclusively two genera, *Synergistes* ($6.5 \pm 1.5\%$) and *Akkermansia* ($4.6 \pm 1.9\%$), respectively.

In addition to our findings, an expanded representation of Bacteroidetes has been observed in mouse ceca following a 24-h fast (Crawford *et al.*, 2009) and, as noted in the Introduction, host-derived glycans can present a consistent nutritional foundation for certain members of this phylum (for example, *Bacteroides thetaiotaomicron*) in the absence of dietary glycans (Sonnenburg *et al.*, 2005; Bjursell *et al.*, 2006; Martens *et al.*, 2008). Our results raise the interesting possibility that *Bacteroides* spp. may function in a similar manner in the Burmese python, a host that is distantly related to mammals and a strict carnivore. Notably, *Akkermansia muciniphila* is also capable of subsisting on host mucus as a sole source of carbon and nitrogen (Derrien *et al.*, 2004), and this genus

has recently been found to proliferate in the ceca of active (that is, non-hibernating) hamsters subjected to a 96-h fast (Sonoyama *et al.*, 2009).

Finally, we found that fasted snakes harbored large-intestinal bacterial assemblages with significantly lower overall phylogenetic diversity (17.0 ± 0.6 in fasted vs 20.5 ± 0.6 in fed at 1400 sequences per sample; $P = 0.001$; two-tailed *t*-test with unequal variance) and a significantly lower number of 'species'-level OTUs defined at $\geq 97\%$ sequence similarity (253 ± 18 in fasted vs 375 ± 12 in fed at 1400 sequence per sample; $P < 0.001$) when compared with the recently fed snakes. Taken together, these results support the notion that in the absence of dietary nutrients, such as after extended periods of fasting, the Burmese python's gut microbiota, similar to that of mammals, alters its structure.

Temporal analysis of the postprandial reconfiguration of the large-intestinal microbiota

Next, we examined whether the Burmese python gut microbiota remodels itself when the host digests a meal. Fasted snakes were fed rats weighing 25% of the snake's body mass (see Introduction and Material and methods for details). We assessed changes in overall bacterial community composition, as a function of time, postfeeding using a phylogeny-based metric, UniFrac (Lozupone and Knight, 2005; Lozupone *et al.*, 2007). A relatively small UniFrac distance implies that two communities are similar, consisting of closely related lineages. UniFrac analysis confirmed that relative to the fasted state, a substantial postprandial shift in the large-intestinal bacterial community membership (Figure 1a and Supplementary Figure S1b) and structure (Supplementary Figure S2) occurred. Alteration in bacterial community composition appeared to begin at ~ 1 day postfeeding with the entry of material from the meal, and to peak at ~ 3 days postfeeding, corresponding with the peaks in the python's digestive form and function (Secor, 2008) and the accumulation of unabsorbed material (for example, hair) in the large intestine. Changes in the overall community membership and structure were underpinned by a large postprandial shift in the proportional abundances of Bacteroidetes and Firmicutes in the python large intestine (Figure 1b). In the fasted state and up to 12 h postfeeding, Bacteroidetes were dominant ($45.6 \pm 5.6\%$ of the community; $N = 24$ samples from 10 individuals) whereas Firmicutes were less abundant ($15.3 \pm 4.7\%$). Beginning at 12 h postfeeding, the proportional representation of the Firmicutes increased significantly over time ($R^2 = 0.65$, 35 d.f. (degrees of freedom), $P < 0.001$) at the expense of the Bacteroidetes, which decreased significantly ($R^2 = 0.48$, 35 d.f., $P < 0.001$), until 3 days postfeeding. From 3 days onward, Firmicutes were dominant ($82.8 \pm 6.3\%$ of the community; $N = 20$ samples from 14 individuals)

whereas Bacteroidetes were less abundant ($7.7 \pm 2.5\%$). Firmicutes taxa that increased in relative abundance during digestion included *Lactobacillus* spp. (maximum of 11.5% on day 2), Peptostreptococcaceae (maximum of 28.4% on day 3) and *Clostridium* spp. (maximum of 35.8% on day 6) (Figure 1c). We also observed a modest postprandial increase in the average overall phylogenetic diversity of the large-intestinal microbiota (Figure 2a), coupled with a significant increase in the average number of OTUs defined at $\geq 97\%$ sequence similarity (Figure 2b). This suggests that the dominant lineages (primarily Firmicutes) that appeared in the digesting python's large intestine were more diverse at the 'species' level than those present during fasting (primarily Bacteroidetes).

By contrast, over time in the small intestine (between 6 h and 6 days postfeeding), the initially

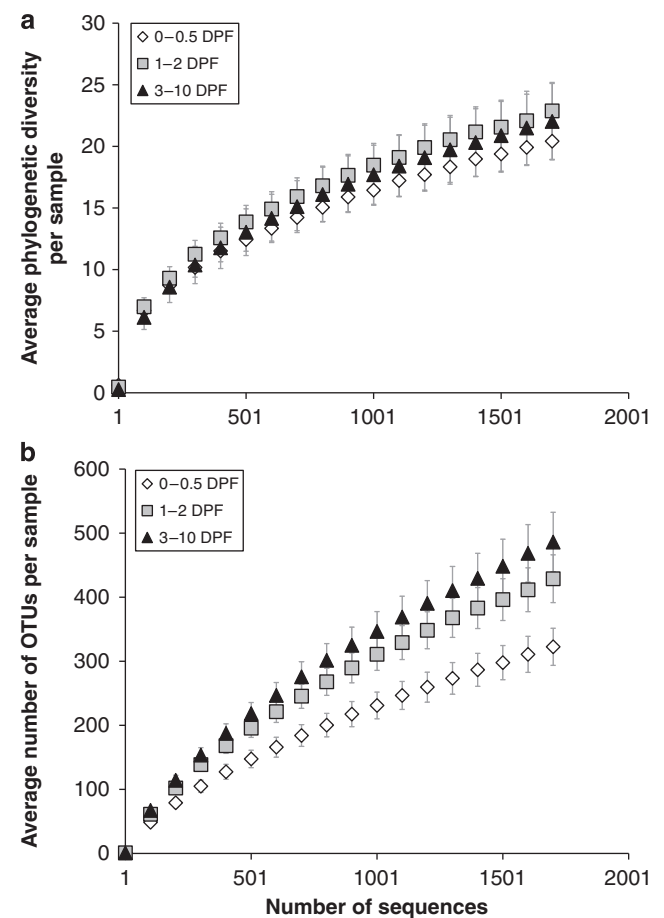


Figure 2 Digestion increases bacterial community diversity and 'species' richness within the Burmese python large intestine. (a) Rarefaction curves for phylogenetic diversity measured by the average total branch length in a phylogenetic tree per sample after a specified number of sequences were observed. (b) Rarefaction curves for 'species' richness measured by determining the average number of operational taxonomic units (OTUs) defined at $\geq 97\%$ sequence similarity per sample. Samples were partitioned into three postfeeding time intervals on the basis of the results shown in Figure 1b. Bars indicate 95% confidence intervals. DPF, days postfeeding.

dominant Firmicutes increased only modestly in proportional abundance (Figure 3). Yet, like in the large intestine, dominant taxa in the small intestine during digestion included the Firmicutes family Peptostreptococcaceae (30.8%) and the genera *Clostridium* (19.6%) and *Lactobacillus* (8.3%), and these taxa fluctuated in their proportional abundances over time (Supplementary Figure S3b). A small number of available samples from fasted snakes and from 10 days postfeeding had similar membership (Supplementary Figure S1a) and suggest that Bacteroidetes may increase in proportional abundance in the nutrient-deprived small intestine as well (Figure 3). If this is indeed the case, postprandial shifts in small-intestinal microbial community structure may occur in less than 6 h. However, this may not be surprising given that the python's small intestine also doubles its microvillus length, amino-acid uptake rates and aminopeptidase-N activity within 6 h of feeding, even before it receives a significant amount of chyme from the stomach, which contains the amino acids and peptides known to trigger upregulation (Secor *et al.*, 2002; Secor, 2008). Within 24 h of feeding ~25% of the meal has entered the small intestine and within 2 to 3 days only ~25% of the meal remains in the stomach, including portions of the trunk vertebrae, hind limbs, tail and hair (Secor and Diamond, 1995). The latter time point corresponds with maximal changes in small-intestinal anatomy (a fivefold lengthening of the microvilli) and physiology (a 3–13-fold increase in hydrolase activity and nutrient uptake capacity), and the accumulation of unabsorbed material in the python's cecum and large intestine where,

unlike in the small intestine, large-scale changes in morphology and function are not apparent during digestion (Secor and Diamond, 1995; Ott and Secor, 2007; Cox and Secor, 2008). Unabsorbed material continues to fill the cecum and large intestine until the last remains of the meal (mainly hair) pass from the stomach and through the small intestine, usually by 6–7 days postfeeding. Pythons typically defecate within 2–3 weeks of consuming a meal, and by day 30 the stomach, small intestine and large intestine are fairly empty (Secor and Diamond, 1995). It is interesting to note that the observed changes in large-intestinal bacterial community composition at 6 h postfeeding (Figure 1a) seem to precede the entry of unabsorbed material into the large intestine and, subsequently, occur at several transition points including the onset of the Firmicutes bloom at 1 day postfeeding and later peaks in *Lactobacillus* (2 days postfeeding), Peptostreptococcaceae (3 days postfeeding) and *Clostridium* (6 days postfeeding) relative abundance (Figure 1). The underlying causes of these shifts are unknown, but may relate to changes in the quantity and quality of unabsorbed nutrients passing into the large intestine, as well as to the timing and rate of their transit, or to other physiological changes in the host.

Overall, our results indicate that large-scale alterations in the Burmese python's gut microbiota accompany its extensive physiological and morphological responses to feeding and fasting. Although many aspects of the host environment change during digestion, bacterial community modification may be driven, in part, by a shift from host glycan foraging to the degradation of unabsorbed material derived from the prey. The snake's carnivorous diet dictates that much of this material will be soluble or insoluble animal protein, but it may also include small amounts of carbohydrate. In the case of the Burmese python, postfeeding increases in the relative abundance of the Firmicutes may depend on the bacteria's ability to rapidly exploit unabsorbed, labile amino acids and peptides from the python's diet. Indeed, many of the lineages with increased relative abundance during digestion are closely related to isolates with known proteolytic activity, including *Clostridium*, *Lactobacillus*, and *Peptostreptococcus*. Firmicutes may also contribute to the degradation of recalcitrant animal protein, such as hair, which has a relatively long transit time in the python gut. Keratin is a main component of hair and several keratinolytic Firmicutes have been isolated (although most of them are from the genus *Bacillus*). The metabolic impact of the observed postprandial increase in the ratio of Firmicutes to Bacteroidetes on python biology remains unclear. It has been suggested that the python's large intestine plays a relatively limited role in taking up nutrients that have passed unabsorbed through the small intestine, and a larger role in absorbing water, salts and trace nutrients synthesized by

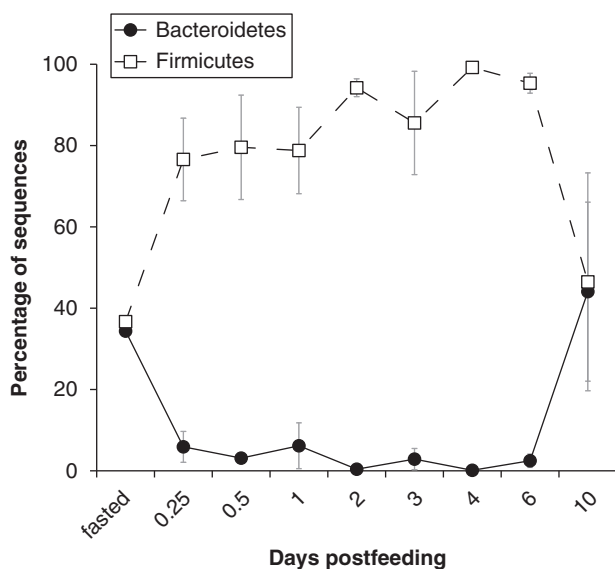


Figure 3 Average proportional abundance of sequences classified as Bacteroidetes or Firmicutes from the Burmese python small intestine at each time point. Because other lineages can also be present, the proportions need not add up to 100%. Error bars are one s.e. of the mean. For fasted, $N = 1$.

intestinal bacteria (Secor and Diamond, 1995); however, Firmicutes-dominated gut microbiotas have been found to enhance energy harvest from the diets of other animals (Ley *et al.*, 2006; Turnbaugh *et al.*, 2006, 2009a).

The rodent meal is an unlikely source of most python gut bacteria

The gut is not a closed system and animals rarely consume microbe-free meals. In principle, the python provides a model for determining the extent to which an established host microbiota can be invaded by microbial consortia associated with consumed prey. Therefore, we investigated the degree to which bacteria observed in the python's gut after feeding were derived from the mammal it ingested. Because the composition of the vertebrate gut microbiota is influenced by host phylogeny (Ley *et al.*, 2008b), we predicted that the python and rat would contain relatively distinct bacterial assemblages; however, it is also feasible that particular indigenous lineages could be conserved (or convergently acquired) in the two hosts.

To determine whether the potentially large bacterial loads associated with intact prey could be detected in the python's intestine, we first obtained >30 000 16S rRNA gene sequences from 23 aliquots of

whole-rat homogenate prepared from 5 individuals. Aliquots yielded an average of 1320 ± 210 (s.d.) sequences (~ 6000 sequences per animal), with an average read length of 250 nt. The direct transfer of bacteria from the rat meal to the python gut should result in shared identical 16S rRNA gene sequences. To find these exact matches, all sequences from the rat and python samples were pooled and de-replicated (that is, nearly identical sequences were clustered by picking OTUs at 100% sequence similarity after prefix-based pre-clustering at 200 nt). This procedure resulted in 90 461 OTUs of which, 85 354 were exclusive to the python gut, 4370 were exclusive to the rat and 737 were found in both hosts. To be deemed present in both hosts, the OTU had to contain at least one sequence from a python sample and one sequence from a rat sample. Over 50% of the shared OTUs were classified as *Lactobacillus* (Supplementary Table S2). Next, we used lineage-specific unweighted UniFrac and principal coordinates analysis to compare the memberships of the whole rat and python gut microbiotas. We found that rats and pythons harbored compositionally distinct assemblages at each of the several key taxonomic levels (Figure 4). Notably, *Lactobacillus*, which was abundant in whole-rat assemblages ($51.4 \pm 2.8\%$), did not appear to be host specific (Figure 4h) and also exhibited a modest

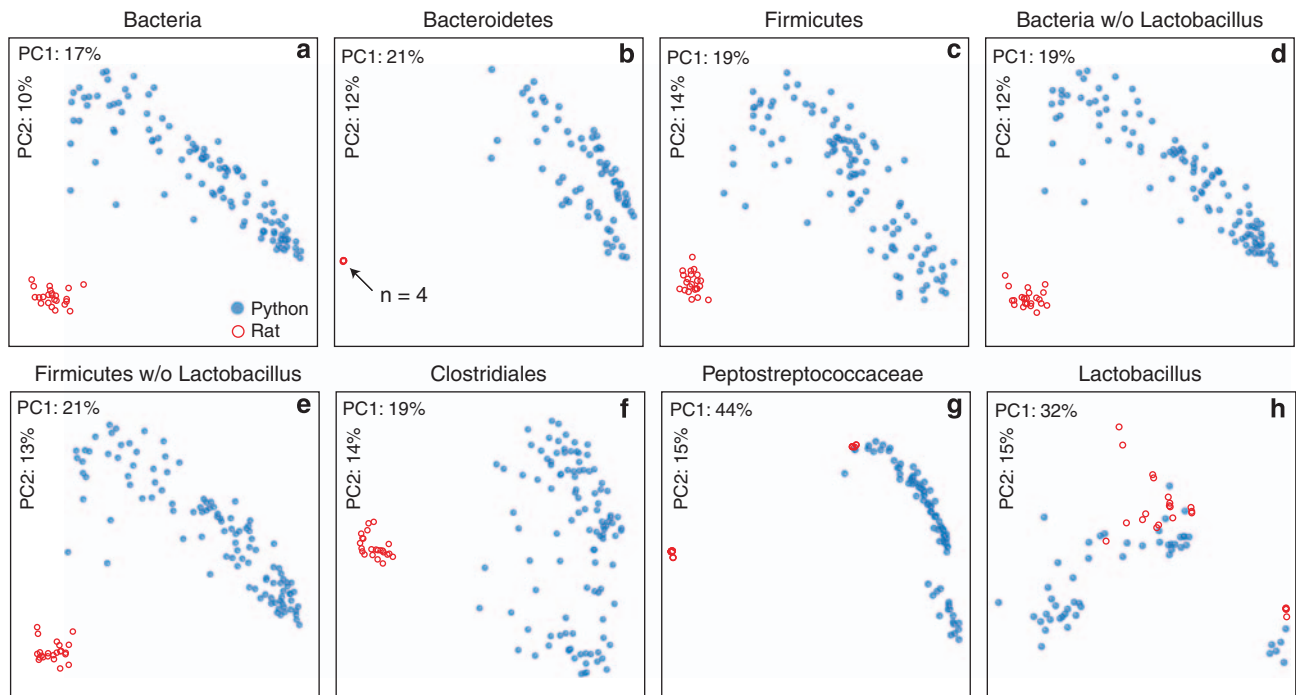


Figure 4 Python intestine and rodent meal (i.e., whole rat) bacterial communities appear to have largely divergent membership. Communities clustered using principal coordinates analysis of unweighted UniFrac distance matrices. Each circle corresponds to a sample colored according to host species. The percentage of the variation explained by the plotted principal coordinates is indicated on the axes. Each panel shows a different taxon-specific analysis. These taxa are: (a) all Bacteria, (b) all Bacteroidetes (note that the four rat samples appear superimposed in this panel), (c) all Firmicutes, (d) all Bacteria but excluding *Lactobacillus*, (e) all Firmicutes but excluding *Lactobacillus*, (f) Clostridiales, (g) Peptostreptococcaceae and (h) *Lactobacillus*. Comparisons of panels a and d, and c and e, show that the *Lactobacillus* component, which was not host specific, did not greatly influence the clarity of separation in the snake and rat communities. Samples were excluded if they contained fewer than 30 sequences from the specified taxon.

postprandial spike in the python's large intestine (Figure 1c). Although we did not detect *Lactobacillus* in the fasted large intestine, it did appear in the fasted small intestine (0.9% of the community; $N=1$), leaving open the possibility that this common gut inhabitant is indigenous to both the python and the rat. Similarly, members of the Peptostreptococcaceae, which were also shared between a subset of the rat and python samples (Figure 4g), were also present in the fasted python's small and large intestine, averaging 4.9 and 0.06% of the communities, respectively. In summary, although difficult to ascertain, owing to the potential for conserved or convergent community membership and carry over from past meals, our results suggest that most of the python gut bacteria observed in this study were indigenous to the snake rather than input with the meal. However, we cannot rule out that certain lineages, such as *Lactobacillus*, were delivered to the python's intestine via the diet.

Prospectus: the Burmese python as a model for host-microbe interactions during feeding and fasting

The Burmese python presents an attractive model organism for investigating the interplay between gut microbial communities, diet, nutritional status and host postprandial responses. While the latter are well characterized in the python (Secor, 2008), the present study provides an initial foundation for understanding the gut microbiota's response to feeding and fasting in a host specifically adapted to consuming large intact prey after long periods without food. The results presented here indicate that python gut assemblages are dominated by bacteria belonging to the phyla Bacteroidetes and Firmicutes and are thus broadly comparable to mammalian assemblages. We also find that the python gut microbiota is highly responsive to feeding and fasting, exhibiting wide-range alterations in composition and diversity in concert with the host's own dramatic postprandial physiological and morphological changes.

Previous animal models, notably gnotobiotic mice, have been used to show that gut microbial communities have a marked effect on organ size: for example, transplantation of a gut microbiota from conventionally raised mice to germ-free mice produces an increase in the mass of the heart and adipose tissue (Backhed *et al.*, 2004; Crawford *et al.*, 2009), stimulates cell proliferation in the gut and alters the small intestine morphology, including the density of the submucosal capillary network (Stappenbeck *et al.*, 2002; Backhed *et al.*, 2007; Turnbaugh *et al.*, 2009b). Similarly, gnotobiotic mouse models have shown that the gut microbiota is rapidly responsive to diet shifts and to fasting (Crawford *et al.*, 2009; Turnbaugh *et al.*, 2009b). However, because colonization of a germ-free animal with a microbiota is irreversible, the snake model

could, provided non-lethal procedures are developed, allow us to track the same individual across multiple feeding cycles. As such, this model organism provides a unique opportunity for dissecting the interrelationships between nutrient load and availability, microbiota configuration, and host cellular proliferative and hypertrophic responses both within and outside the gastrointestinal tract. Moreover, the python responses are consistent even between genetically heterogeneous individuals, both at the level of the microbiota and of the host. These observations prompt the question of whether the microbiota has a key role in integrating the impact of feeding and fasting on host cellular functions, including cell hyperplasia and hypertrophy, across a broad range of vertebrates.

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