ECOLOGY LETTERS

Ecology Letters, (2014) doi: 10.1111/ele.12329

LETTER

Gut microbes of mammalian herbivores facilitate intake of plant toxins

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Abstract

The foraging ecology of mammalian herbivores is strongly shaped by plant secondary compounds (PSCs) that defend plants against herbivory. Conventional wisdom holds that gut microbes facilitate the ingestion of toxic plants; however, this notion lacks empirical evidence. We investigated the gut microbiota of desert woodrats (*Neotoma lepida*), some populations of which specialise on highly toxic creosote bush (*Larrea tridentata*). Here, we demonstrate that gut microbes are crucial in allowing herbivores to consume toxic plants. Creosote toxins altered the population structure of the gut microbiome to facilitate an increase in abundance of genes that metabolise toxic compounds. In addition, woodrats were unable to consume creosote toxins after the microbiota was disrupted with antibiotics. Last, ingestion of toxins by naïve hosts was increased through microbial transplants from experienced donors. These results demonstrate that microbes can enhance the ability of hosts to consume PSCs and therefore expand the dietary niche breadth of mammalian herbivores.

Keywords

Detoxification, herbivory, host–microbe interactions, mammalian herbivore, microbiome, plant secondary compounds, plant–herbivore interactions, symbiosis.

Ecology Letters (2014)

INTRODUCTION

Mammalian herbivores comprise the most abundant feeding group within mammals (Price *et al*. 2012), play critical roles in shaping ecosystem structure (Martin & Maron 2012) and serve as essential resources to humans as livestock. A major determinant of the dietary niche breadth of mammalian herbivores is tolerance to plant secondary compounds (PSCs), many of which are toxic (Dearing *et al*. 2000; Moore & Foley 2005). While tolerance has many ecological definitions, in plant–animal interactions it is defined as the ability to consume high doses of toxins while maintaining body mass and normal growth, and can be mediated through various mechanisms such as enhanced detoxification or behaviorial strategies (Mangione *et al*. 2000). Tolerance to plant toxins allows animals access to nutrients in the absence of competition from other herbivores. Nearly four decades ago, renowned ecologists Freeland and Janzen described a suite of strategies employed by mammalian herbivores to overcome challenges posed by PSCs (Freeland & Janzen 1974). One of those strategies, the use of microbial detoxification in the gut, has been generally overlooked in research on plant–herbivore interactions (Dearing *et al*. 2005). The few documented examples of microbial detoxification in mammalian herbivores are restricted to highly artificial systems, i.e. agricultural animals feeding on a single toxic compound (Jones & Megarrity 1986; Sundset *et al*. 2010). Experiments investigating the mechanism of microbial adaptation in an ecological and evolutionary context are lacking. Furthermore, native mammalian herbivores represent unique study organisms, since they often forage on plants producing myriad chemical defences as opposed to a single compound (Moore & Foley 2005).

To advance our knowledge in this area of mammal–microbe interactions, we conducted a series of experiments to understand the function of the gut microbiota with respect to detoxification of PSCs. We focused this investigation on the gut microbes of the desert woodrat (*Neotoma lepida*; Fig. 1a). In the Mojave desert of the USA, populations of *N. lepida* feed primarily on creosote bush (Karasov 1989). The leaves of creosote bush are covered in a phenolic-rich resin composed of a complex mixture of hundreds of chemical products, including phenolics, *O*-methylated flavones and flavonols, catechols, vinyl ketones, and saponins, which, together comprise up to 25% of the dry mass of the plant (Mabry *et al*. 1977). The majority of creosote resin is comprised of nordihydroguaiaretic acid (Fig. 1b), a phenolic compound that causes kidney cysts and liver damage in laboratory rodents (Goodman *et al*. 1970; Lambert *et al*. 2002). Strikingly, in the Mojave desert, woodrats consume daily doses of resin that would be lethal to laboratory mice (Rios *et al*. 2008).

Creosote bush is a relatively recent addition to the diet of *N. lepida*. It was introduced *c*. 17 000 years ago when natural climate change facilitated the invasion of creosote bush into the southern portion of the range of the desert woodrat (Van Devender & Spaulding 1979). Due to the length of ecological and evolutionary exposure, we refer to the Mojave population as the 'experienced' population. In contrast, creosote bush did not invade the adjacent Great Basin desert, resulting in 'naïve' populations of *N. lepida* that lack ecological and evolutionary experience with creosote and feed on the ancestral diet of juniper. In the laboratory, woodrats from experienced (Mojave) populations can consume roughly 25% more creosote resin than those from naïve (Great Basin) populations (Mangione *et al*. 2000) and are still able to feed on their ancestral diet of juniper (Magnanou *et al*. 2009). Thus, the experienced population exhibits an expanded dietary niche breadth. The experienced population has higher expression of a greater number

(b)

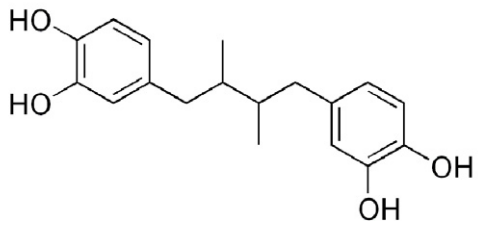

Figure 1 The woodrat system. (a) The desert woodrat and the toxic shrub, creosote. A schematic of the woodrat gut can be found in (Kohl *et al.* 2011). (b) Nordihydroguaiaretic acid (NDGA), the most common plant secondary chemical in creosote resin.

of hepatic detoxification enzymes, which partly explains differential tolerance to PSCs (Magnanou *et al.* 2009). However, the experienced and naïve populations also harbour unique foregut microbial communities (Kohl & Dearing 2012). This observation led us to hypothesise that the microbiota of experienced woodrats facilitates ingestion of dietary toxins. The potential for microbes to allow herbivores to consume dietary toxins represents an uncharted frontier in our understanding of plant–herbivore interactions.

Using this study system, we tested the hypothesis that gut microbes facilitate the ingestion of creosote resin by *N. lepida*. We first investigated mechanisms of microbial detoxification by conducting metagenomic sequencing on the foregut contents of animals fed control diets or diets containing creosote resin. Next, we disrupted the microbiota using broad-spectrum antibiotics and monitored changes in toxin tolerance. Last, we transplanted the microbiota from experienced individuals into naïve individuals to investigate conference of toxin tolerance by the microbiota. Together, these experiments present a thorough investigation into the role of gut microbes in allowing mammalian herbivores to consume toxic plants.

METHODS

Animals and diets

Animals were collected using Sherman live traps. Woodrats for the metagenome experiment were collected from a Mojave Desert habitat in Lytle Ranch, Washington Co., UT (37°07′ N, 114°00′ W) in October 2010. Animals for the antibiotic treatment experiment were also collected from Lytle Ranch in July 2011. Experienced donors for the microbial transplant experiment were collected from Lytle Ranch in May 2012. Naïve donors and recipients were collected from the Great Basin desert in White Rocks, Tooele Co., UT (40°19′ N, 112°54′ W) in November 2012. No creosote is present at this site. All woodrats were transported to the University of Utah Department of Biology Animal Facility and housed in individual cages (48 × 27 × 20 cm) under a 12 : 12-h light : dark cycle, with 28 °C ambient temperature and 20% humidity.

Prior to experimentation, animals were maintained on a diet of high-fibre rabbit chow (Harland Teklad formula 2031). During experimentation, animals were fed the same chow except in a powdered form to prevent caching of food. To prepare diet treatments containing creosote resin, creosote leaves were collected from trapping sites and frozen at −20 °C prior to resin extraction. We performed surface extractions by soaking leaves in acetone (1 : 6, wet leaf mass : volume solvent) for 45 min. Solvent and resin were filtered (Whatman filter paper grade 1) to remove large particles and evaporated using a rotary evaporator until the resin was highly viscous, at which point it was transferred to a vacuum pump for 48 h to remove any remaining acetone. Extracted resin was stored at −20 °C prior to use.

Creosote diet treatments were prepared by dissolving the appropriate amount of resin in a volume of acetone equal to 25% of the dry weight of ground rabbit chow to which it was added. The concentrations of resin varied depending on the specific experiment (see below). Control diet (0%) was prepared by adding an identical ratio of acetone, without creosote resin. Acetone was evaporated from all diets in a fume hood, and complete evaporation was confirmed gravimetrically.

Metagenomics

Four individuals from the experienced population served as control animals and were fed powdered rabbit chow in cages for 8 days. Another four individuals were fed the control diet for 3 days, followed by the same diet with increasing amounts of creosote resin (1 and 2% creosote resin for 2 and 3 days respectively). Following diet treatments, animals were euthanised under CO_2, and immediately dissected. Contents of the foregut were removed and frozen at −80 °C prior to DNA isolation.

Foregut contents were thawed and a small amount (~25 mg) was incubated with 180 μL enzymatic lysis buffer (20 mM Tris·Cl; pH 8.0, 2 mM sodium EDTA, 1.2% Triton X-100 and 20 mg mL^{-1} lysozyme) at 37 °C for 30 min. DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA). These samples are the same ones that were used to compare microbial inventories between control- and creosote-fed animals using 16S rRNA sequencing (Kohl & Dearing 2012). Here, we sequenced the metagenomes of these samples to investigate changes in the relative abundances of genes with specific functions.

Total genomic DNA was sequenced on an Illumina HiSeq 2000 platform at the University of Utah Microarray and Genomic Analysis Core Facility to yield 100 base paired-end reads. Unassembled metagenomic data were compared

between treatment groups using MG-RAST (Meyer *et al.* 2008). Using unassembled sequences allowed us to retain frequency data for investigating the relative abundances of various metabolic functions (Meyer *et al.* 2008). Sequences were screened against the genome of *Arabidopsis thaliana* to remove potential contamination from the plant-based diet. The reads were then filtered using dynamic trimming with a quality threshold of 15, such that any sequences with more than 5 low-quality bases were removed. Sequences were annotated with the SEED Subsystems (Overbeek *et al.* 2005) with the following thresholds: (1) e-values < 1e-5, (2) a minimum per cent identity to database sequences of 60% and (3) a minimum alignment length of 30 bases. Abundances of functional categories were normalised, and a heatmap and dendrogram were generated using a clustering algorithm within MG-RAST. Abundances of functional categories were also compared between control- and creosote-fed animals using t-tests.

The metagenomes were also assembled using the IDBA-UD assembler (Peng *et al.* 2012) with standard parameters, including '–pre_correction'. We assembled contigs to determine the taxonomic identities of woodrat microbiome genes based on sequence identity. We searched the metagenomes for aryl-alcohol dehydrogenase genes by creating a database of 42 aryl-alcohol dehydrogenase protein sequences from the SEED database, and searching translated metagenomic sequences using TBLASTN. Hits were parsed using a minimum e-value of 1e-70 and a minimum translated length of 300 amino acids. Normalised 'reads per million assembled reads' were calculated for each resulting candidate gene, and compared between control- and creosote-fed animals using a one-way ANOVA. Phylogenetic placement of candidate aryl-alcohol dehydrogenase (AAD) genes was determined by aligning candidate AAD genes using MUSCLE (Whelan & Goldman 2001), and creating a phylogenetic tree of select sequences from the SEED database and translated metagenomic contigs using PhyML v3.0 (Guindon *et al.* 2010) with the WAG amino-acid substitution model (Whelan & Goldman 2001) with 25 random starting trees and 100 bootstrap replicates.

Antibiotic treatment

The antibiotic experiment was conducted 1 week after capture. Ten animals were fed powdered rabbit chow, and another 10 animals were fed rabbit chow supplemented with 2% extracted creosote resin. A diet of 2% creosote resin represents a low dose of PSCs for experienced animals, and is tolerable even for naïve animals (Mangione *et al.* 2000). Within each diet treatment, five animals were given neomycin in their water (0.5 g L^{-1}) with sucralose (2.5% w/v) to encourage drinking. Neomycin is a broad spectrum antibiotic that is poorly absorbed across the gut tissue and reduces gut bacterial density by ~90% (Vijay-Kumar *et al.* 2010). Five animals were given water containing only sucralose as a control. Body mass was monitored for 2 weeks, and animals were removed from the trial if they lost more than 10% of their original body mass. Following the trial, animals were given at least 2 weeks to recover body mass. Afterwards, antibiotic treatments were switched and the trial was repeated. Most animals recovered their microbiota relatively quickly after the antibiotic treatment (Fig. S1A). Some animals could not be used in the second trial due to excessive weight loss, and the resulting sample sizes in each group were: No AB, control diet: 7; No AB, creosote diet: 8; AB, control diet: 10; AB, creosote diet: 10. Persistence curves were compared using a Log-Rank Kaplan–Meier survival analysis and adjusted for multiple comparisons using the Bonferroni correction.

Microbial transplant

We transplanted the faeces from experienced woodrats in to woodrats that were naïve to creosote. Naïve woodrats were collected from the Great Basin desert site and allowed to acclimate to captivity for 10 days. Donors (experienced and naïve) were kept in metabolic cages and fed powdered rabbit chow containing 2% creosote resin to prime their microbiota. We collected and ground faeces from these animals daily. Ground faeces (15% w/w) from either experienced or naïve donors were added to the food of naïve recipients for 6 days. Because woodrats naturally cache their faeces and are coprophagic (Kenagy & Hoyt 1980), this experiment mimics an ecologically relevant route of transmission. All recipients in the transplant experiment were from the naïve population, and so they should share somewhat similar hepatic detoxification machinery (Magnanou *et al.* 2009). The 'experimental recipients' ($N = 7$) received faeces from experienced individuals, while the 'control recipients' ($N = 9$) received faeces from other naïve individuals. All recipient animals were kept in metabolic cages and fed an increasing amount of creosote resin in their diet (0, 1, 2, 3, 4, 6, and 8%, increasing every 3 days). We measured food intake and body mass daily. Animals were removed from the trial if they lost more than 10% of their original body mass. Animals were then fed a 0% creosote diet for at least 4 days to facilitate recovery, and we collected an additional urine sample. Persistence curves were compared using a Log-Rank Kaplan–Meier survival analysis. Urine and faeces were collected daily. Urine pH was measured with an Omega Soil pH electrode (PHH-200). Urine pH is an indicator of hepatic detoxification; animals excreting more toxin metabolites tend to produce more acidic urine (Foley *et al.* 1995; Mangione *et al.* 2001). Faeces were dried at 45 °C overnight and weighed. We calculated dry matter digestibility as the [(grams food intake – grams faeces output)]/grams food intake. Body mass, food intake and dry matter digestibility were all compared using repeated measures ANOVA.

16S rRNA inventories

We collected faeces for microbial analysis from both the antibiotic and transplant experiments. For the antibiotic trial, we collected faeces on the final day of each treatment. For the transplant experiment, we collected faeces before the trial, as well as on the last day of 2% diet, which was 4 days after the conclusion of the transplant. Whole DNA was extracted from all faeces using a QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD, USA). A previously established technique was used to amplify and sequence the V4 region of the 16S rRNA gene with primers 515F and 806R (Caporaso *et al.*

2012). Sequences were analysed using previously described techniques (Kohl & Dearing 2012, 2014).

We calculated estimated species richness, or Chao1, which estimates the asymptote on a species accumulation curve. We compared community memberships (presence or absence of lineages, and not their relative abundances) of treatment groups. We also compared diversity between samples (β diversity) by calculating unweighted UniFrac scores, which measures the fraction of branch length shared between two samples in the phylogenetic tree created from all representative sequences. We then conducted Principal Coordinates Analysis (PCoA) on unweighted UniFrac scores to investigate similarities. Similarities were tested using the ANOSIM function within QIIME using 999 permutations.

Urine extraction and GC-MS analysis

We conducted metabolomic analysis on urine samples from the transplant experiment on the third day of the 1% creosote diet, as well as on day 4 of the 0% creosote recovery diet at the end of the trial. This collection schedule allowed us to conduct metabolomics on a 0% diet after the transplant. A methanol extraction was used to remove protein from urine prior to analysis. In brief, 900 μL of −20 °C 90% methanol (aq.) was added to 40 μL of urine. The samples were incubated for 1 h at −20 °C followed by centrifugation at 30 000 × ***g*** for 10 min using a rotor chilled to −20 °C. The supernatant containing the extracted metabolites was then transferred to fresh tubes and completely dried *en vacuo*.

All GC-MS analysis was performed with a Waters GCT Premier mass spectrometer fitted with an Agilent 6890 gas chromatograph and a Gerstel MPS2 autosampler. Dried samples were suspended in 40 μL of a 40 mg mL^{-1} O-methoxylamine hydrochloride in pyridine and incubated for 1 h at 30 °C; 25 μL of this solution was added to autosampler vials. A quantity of 10 μL of *N*-methyl-*N* trimethylsilyltrifluoracetamide (MSTFA) was added and incubated for 60 min at 37 °C with shaking. After incubation, 3 μL of a fatty acid methyl ester standard solution was added, and then 1 μL of the prepared sample was injected to the gas chromatograph inlet in the split mode with the inlet temperature held at 250 °C using a 50 : 1 split. The gas chromatograph had an initial temperature of 95 °C for 1 min followed by a 40 °C min^{-1} ramp to 110 °C and a hold time of 2 min. This process was followed by a second 5 °C min^{-1} ramp to 250 °C, a third ramp to 350 °C, then a final hold time of 3 min. A 30 m Phenomex ZB5-5 MSi column with a 5-m-long guard column was employed for chromatographic separation. Helium was used as the carrier gas at 1 mL min^{-1}.

Data were collected using MassLynx 4.1 software (Waters, Milford, MA, USA). A two-step process was employed for data analysis, a targeted followed by non-targeted analysis. For the targeted approach, known metabolites were identified and their peak area was recorded using QuanLynx. Concentrations of 95 known metabolites were compared using two-way ANOVAs with diet and recipient type (experimental vs. control) as variables. We corrected p-values using the False Discovery Rate correction. For the non-targeted approach, peak picking and analysis was performed using MarkerLynx. Principle component analysis and partial least squares-discriminate analysis were performed using SIMCA-P 12.0 (Umetrics, Kinellon, NJ, USA). Potential metabolite biomarkers were further investigated by comparing their peak area using similar two-way ANOVAs. For metabolites that were found at high concentrations in the 10 : 1 analysis, particularly proline, aspartic acid and glutamic acid, we used the 100 : 1 data set to record accurate data. These data were normalised for extraction efficiency and analytical variation by mean centring the area of D4-succinate.

Data deposition

Foregut metagenomes have been deposited in MG-RAST under Project 9847. Microbial inventories were deposited in the Sequence Read Archive under accession code SRP027399.

RESULTS

Metagenomic sequencing resulted in an average of 52 078 268 raw reads per sample. MG-RAST was able to annotate function to an average of 8 034 844 reads per sample using the SEED Subsystems. PSC ingestion definitively shaped the microbiota at the gene level; creosote-fed animals harboured a microbiota with higher abundances of genes associated with the metabolism of aromatic compounds and the stress response (Fig. 2). When investigating deeper levels of functional classification within the category 'metabolism of aromatic compounds', we found several functions that exhibited differential abundances that were driven by cumulative differences across several or many genes. However, at the gene level we found that the microbiota of creosote-fed animals only exhibited significantly higher abundance of a single gene: aryl-alcohol dehydrogenase (Fig. S2).

Assembly of the metagenome resulted in an average of 188 733 contigs per sample with an average n50 value of 1687 bp. Detailed assembly statistics can be found in Table S1. This analysis revealed 58 unique candidate AAD genes that resemble those isolated from bacteria in the family Lactobacillaceae (Fig. S3).

Antibiotic treatment (AB) significantly altered the microbial community composition and decreased microbial diversity by roughly 50% (Fig. S1B). At the phylum level, antibiotics decreased the relative abundance of Spirochaetes (0.08% of community in controls, 0.02% of community in antibiotic treated; Bonferonni-corrected $P = 0.027$) and Tenericutes (0.98% of community in controls, < 0.01% of community in antibiotic treated; Bonferonni-corrected $P = 0.004$). We could not detect any significant differences in relative abundances of identified genera.

Antibiotics did not impair the ability of woodrats to feed on a control diet and maintain body mass (Bonferonni-corrected *P*-values; No AB, control diet vs. AB, control diet, $P = 0.20$; Fig. 3). However, antibiotic-treated animals fed the creosote diet did not ingest enough food to maintain body mass (No AB, creosote diet vs. AB, creosote diet, $P < 0.001$; AB, control diet vs. AB, creosote diet, $P = 0.009$; Fig. 3). No individual given the AB treatment and fed creosote PSCs lasted more than 13 days in the trial.

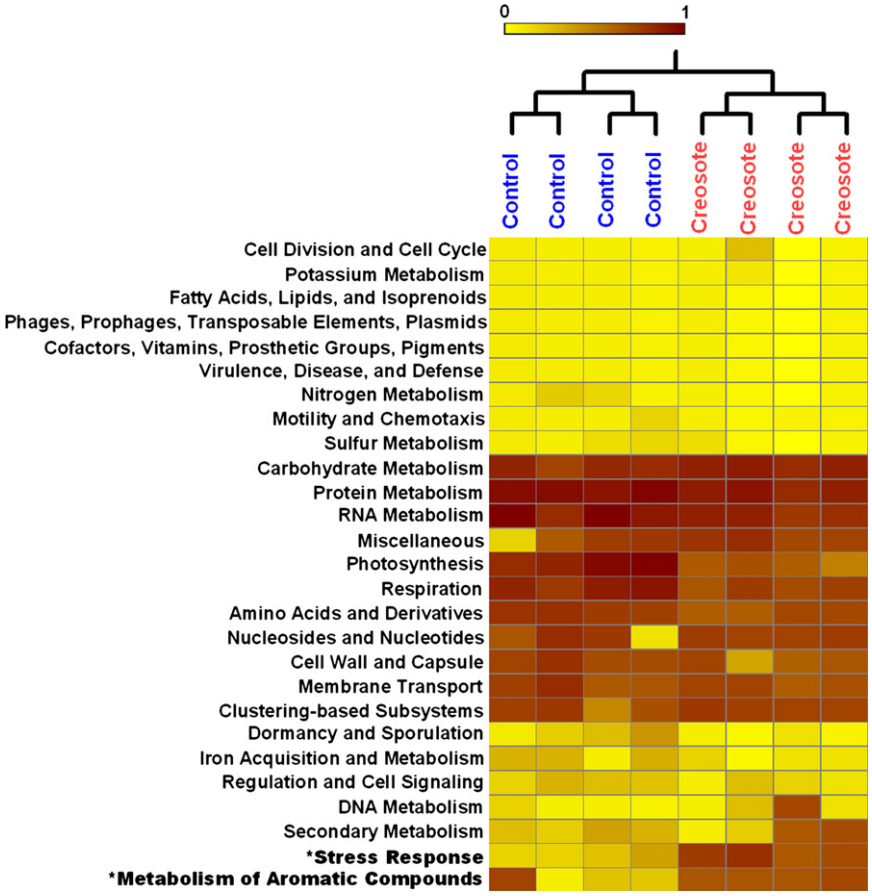

Figure 2 Heat map showing normalised abundances of genes in various functional categories in the foregut metagenomes of animals fed a control diet, or one with 2% creosote resin. Each column represents an individual animal, and columns are clustered according to similarity in functional profiles. Each row represents a functional category. Those in bold with an asterisk are significantly more abundant in animals fed creosote resin.

Our microbial transplant was effective at transmitting the microbiota to each of our recipient groups. Prior to the transplant, both groups exhibited similar microbial communities (pre-experiment animals: ANOSIM, $P = 0.67$; Fig. S4). Following the transplant, experimental recipients maintained a microbiota that closely resembled the experienced donors and differed from control recipients (ANOSIM, $P = 0.001$; Fig. 4a).

The gradual increase in creosote concentration of the diet revealed differences in upper thresholds between the treatments. When fed a diet lacking creosote resin, there was no difference in body mass between groups (Days 0–3; Fig. 4b). However, when creosote was added to the diet, the experimental recipients maintained a higher body mass ($P = 0.008$; Fig. 4b). Moreover, the experimental recipients were able to persist in the trial significantly longer than the control group ($P = 0.038$; Fig. 4c).

Various physiological parameters also differed between the control and experimental recipients. The experimental recipients produced less acidic urine (Quadratic mixed effects model, Donor effect: $P = 0.035$; Fig. 5a) with unique metabolite signatures compared to the control group fed a 1% creosote diet (Fig. 5b). This difference was not observed in animals fed a creosote-free diet following the end of the trial (Fig. S5). We identified several urinary metabolites that were produced either by experimental or control recipients (Fig. 5c

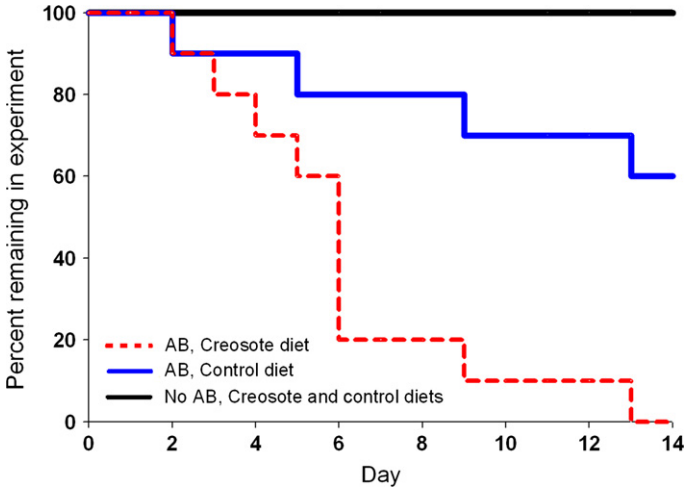

Figure 3 Persistence analysis of experienced animals fed either a control diet, or one containing 2% creosote resin, either with or without antibiotics (AB). Animals were removed from the trial when they lost more than 10% of their original body mass. Sample sizes (N) for each group are as follows: AB, creosote diet: 10; AB, control diet: 10; No AB, control diet: 7; No AB, creosote diet: 8.

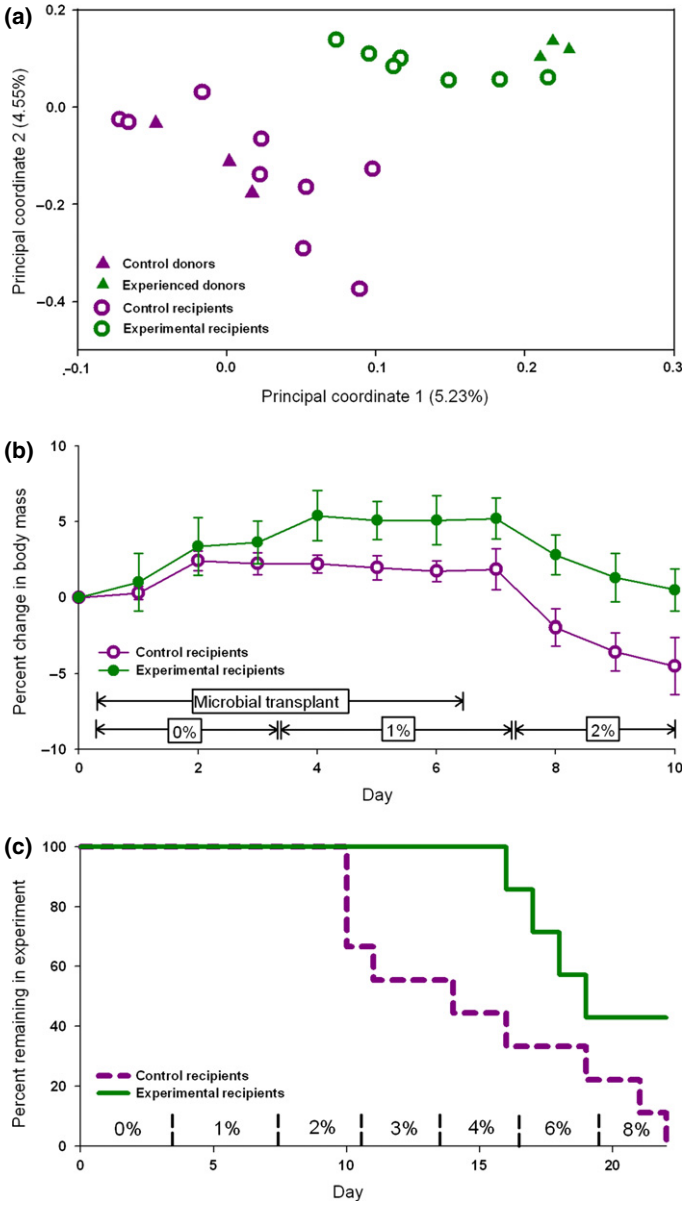

Figure 4 Microbial transplants improve performance on toxic diets. (a) Principal coordinate analysis of unweighted UniFrac distances generated from 16S rRNA microbial inventories of faeces from donors and recipients. Circles represent microbial communities on day 10 (b) Percent change in body mass (mean ± SEM) of control and experimental recipients over time. Labels above the *x*-axis denote when the microbial transplant was conducted and the percentage of creosote resin in the diet. (c) Persistence analysis of control and experimental recipients throughout the trial. Labels on the *x*-axis correspond to the percentage of creosote resin in the diet. All recipients were originally from the naïve population, but differed in the type of microbiota they were given during the transplant. For comparison, animals from the experienced population normally consume diets of 3–7% resin (Mabry *et al.* 1977; Karasov 1989; Mangione et al. 2000). Sample sizes (N) for all panels are as follows: control recipients: 9; experimental recipients: 7.

& d, Table S2, Fig. S6). We did not detect differences in the relative concentrations of the 95 known metabolites. There were also no differences in food intake or dry matter digestibility between groups (Fig. S7).

DISCUSSION

Forty years ago, microbes were hypothesised to play a key role in the abilities of mammalian herbivores to feed on toxic plants (Freeland & Janzen 1974). Our study presents comprehensive evidence that gut microbes enhance tolerance to plant toxins and can expand the dietary niche breadth of mammalian herbivores. Our study advances understanding by going beyond a description of the microbiota and including several assays designed to address implications for the host, such as whole-organism feeding trials, microbiome removal and microbial transplants, as well as metagenomic and metabolomic approaches.

Dietary toxins sculpt microbial functions

Feeding on dietary toxins significantly altered the metagenome of the woodrat foregut at the functional level. Most strikingly, genes associated with the metabolism of aromatic compounds and the stress response exhibited higher abundance when hosts were fed creosote resin. The human gut microbiota shows similar transcriptional changes in these same functional categories in response to pharmaceuticals (Maurice *et al.* 2013). These results suggest that microbes with genes associated with the metabolism of aromatic compounds are selected for within the community when woodrats feed on creosote. The toxins in creosote are known to be antimicrobial (Verástegui *et al.* 1996), and so they likely impart a selective effect on the microbial community. This notion is supported by the fact that ingestion of creosote resin significantly alters the microbial community structure of the woodrat gut at the taxonomic level (Kohl & Dearing 2012).

Specifically, creosote resin caused an increase in the abundance of aryl-alcohol dehydrogenases, which degrade aromatic alcohols and lignins (Muheim *et al.* 1991). We predict that these enzymes play a role in the degradation of the numerous components of creosote resin, such as benzyl acetate, benzyl butanoate and a number of lignins because of their structural similarity to the substrates acted on by aryl-alcohol dehydrogenases (Mabry *et al.* 1977). However, further work is required to test the substrate specificities of microbial aryl-alcohol dehydrogenases from the woodrat gut.

It is worth noting that one animal maintained a high abundance of genes associated with the metabolism of aromatic compounds even when feeding on a control diet. This individual variation is common in gut microbial communities and is similar to the variation in the abundance of drug metabolising genes observed in the human gut microbiota (Maurice *et al.* 2013). We hypothesise that this standing variation is acted on by natural selection. Additional work is needed to determine whether animals that maintain a microbiota enriched in toxin-degrading genes even on a control diet exhibit higher tolerance to plant secondary compounds.

Disruption of the microbiota impairs animals' abilities to consume toxins

Antibiotic treatment significantly decreased microbial diversity of the woodrat gut and likely lowered bacterial density (Vijay-Kumar *et al.* 2010). Specifically, it reduced the abun-

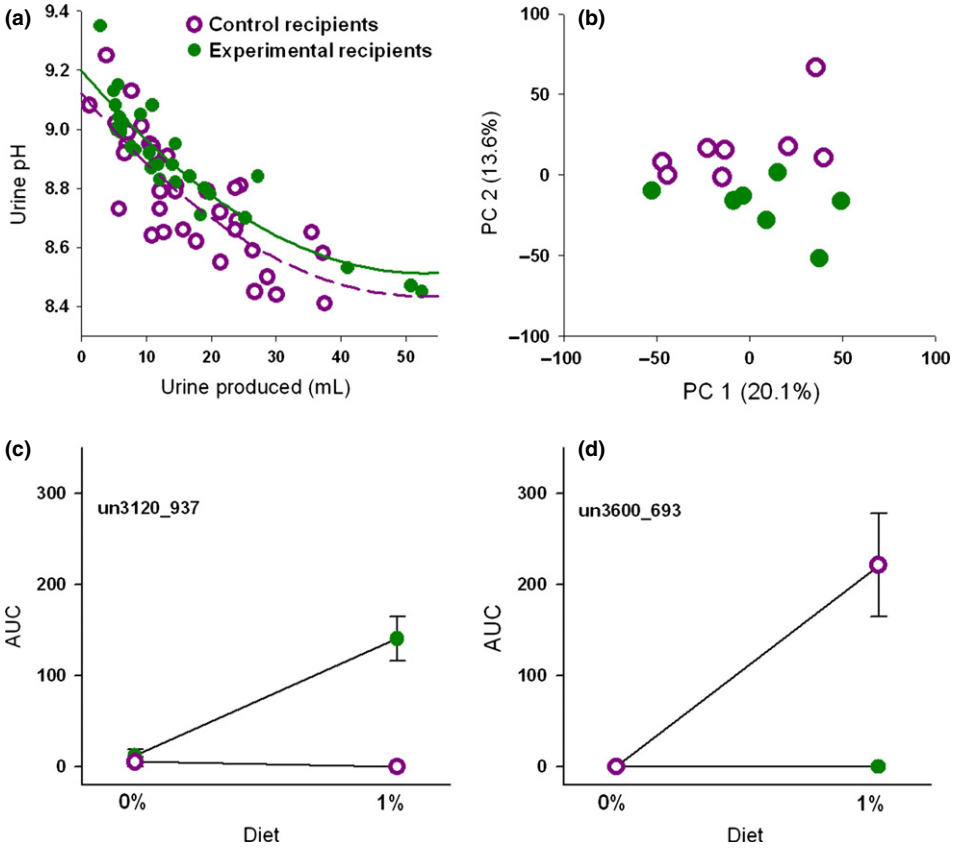

Figure 5 Microbial transplants alter detoxification routes. (a) Urine pH as a function of urine produced. (b) Principal component analysis of over 800 urinary metabolites from animals fed a 1% creosote resin diet. (c and d) Examples of two unknown urinary metabolites produced from either experimental (c) or control (d) recipients fed a 1% creosote resin diet. Numbers under the panel label refer to the retention index of the metabolite. Metabolites were not detectable when animals were returned to a 0% resin diet at the end of the trial. The amounts of each metabolite detected are expressed as area under the curve (AUC; mean ± SEM). Statistics can be found in Table S2. All recipients were originally from the naïve population, but differed in the type of microbiota they were given during the transplant. Sample sizes (N) for all panels are as follows: control recipients: 9; experimental recipients: 7.

dances of Spirochaetes and Tenericutes. While these phyla are not specifically known to aid in the degradation of phenolics, they may be providing unique functions in the woodrat gut. Disruption of the microbiota did not significantly impair animals fed the control diet. However, all animals fed a diet containing 2% creosote resin lost > 10% of their original body mass within 2 weeks. This finding is striking given that it was observed in animals from the experienced population, which can consume diets of 3–7% resin (Mabry *et al*. 1977; Karasov 1989; Mangione *et al*. 2000). Even naïve individuals can ingest a 2% resin diet for weeks while maintaining body mass (Mangione *et al*. 2000). Thus, depletion of the microbiota effectively removed 17 000 years of ecological and evolutionary experience with creosote PSCs, and caused experienced animals to be unable to maintain mass when feeding on creosote compounds. These results have implications for animals bred through captive rearing programs, which may exhibit decreased microbial diversity and thus be unable to consume PSCs.

Gut microbes confer toxin tolerance to naïve hosts

Transplantation of the microbiota from experienced woodrats caused experimental recipients to maintain higher body mass when feeding on creosote resin. Interestingly, this difference in body mass was not coupled with changes in food intake or digestibility. These results are consistent with earlier studies also demonstrating that naïve individuals lose body mass when fed a 2% resin diet with no changes in food intake or digestibility (Mangione *et al*. 2000). We hypothesise that the maintenance of body mass in the experimental recipients fed low-resin diets was due to lower costs of hepatic detoxification, which can be as high as 50% of basal metabolic costs in some herbivores (Sorensen *et al*. 2005). Differences in urine pH and the concentrations of urinary metabolites suggest that the inoculation of woodrats with an experienced microbiota altered detoxification routes (Foley *et al*. 1995; Mangione *et al*. 2001). This outcome may have been the result of direct microbial degradation of toxic compounds, perhaps by aryl-alcohol dehydrogenase enzymes. In addition, the gut microbiota can influence gene expression and xenobiotic metabolism in the liver (Björkholm *et al*. 2009), which could alter tolerance to PSCs.

Overall, the transplanted microbiota from experienced animals substantially increased the tolerance to dietary toxins of naïve animals. The experimental recipients were able to consume higher concentrations of resin and persist in the trial significantly longer. These results suggest that differences in

gut microbial communities underlie the interpopulational differences in toxin tolerance observed within this species (Mangione *et al.* 2000). In an ecological setting, the acquisition of new microbes would allow herbivores to feed on otherwise toxic plants, giving them access to nutrients in the absence of competition from other herbivores. Remarkably, this acquisition of microbes and increase in tolerance was accomplished within an individual, and not over the course of generations. Thus, this represents a rapid mechanism for expanding an animal's dietary niche.

However, it should be noted that host adaptation is also likely to play a role in allowing herbivores to consume high doses of toxins. Animals collected from the experienced population regularly consume concentrations of 3–7% resin in nature (Mabry *et al.* 1977; Karasov 1989; Mangione *et al.* 2000) and can consume concentrations of 5% resin without losing excessive body mass (Mangione *et al.* 2000). While recipients of the microbial transplant in our study were able to consume more creosote resin, they did not reach the level of these experienced individuals. Thus, the microbiota may offer a rapid expansion in dietary niche breadth, while host adaptations in hepatic detoxification machinery are expected to enhance specialisation on toxic diets (Magnanou *et al.* 2009).

It is still unknown how herbivores may acquire novel detoxifying microbes in nature. The microbes may come directly from plants, since 24% of microbes found in the woodrat gut are also found on creosote leaves (Kohl & Dearing 2014). In addition, herbivores faced with toxic challenges will often engage in geophagia, or the consumption of soil, to adsorb toxins (Krishnamani & Mahaney 2000). Indeed, soil has been detected in the gut contents of woodrats (Vorhies & Taylor 1940). Soil contains dense populations of microbes that are often able to degrade phenolic compounds (Blum & Shafer 1988). Last, woodrats, also known as packrats, frequently collect and store faeces from jackrabbits, coyotes and cows (Betancourt *et al.* 1990). These unique behaviours may allow woodrats to obtain novel microbes.

Implications

Our results hold promise for other experimental designs to conduct microbial transplants in non-model systems. Feeding ground faeces to recipients was effective at transmitting the population-specific microbial signatures of donors. We did not pre-treat animals with antibiotics for this experiment, since treatment causes lasting effects that interfere with colonisation by a novel microbial community (Manichanh *et al.* 2010). Although animals were housed individually, our experiments were conducted with open-top cages in a single room, without sterilised food or water. This design is in contrast to the multitude of recent microbial transplant experiments in the human-health field that use highly sterilised and germ-free methods (Manichanh *et al.* 2010; Markle *et al.* 2013). While the use of germ-free animals provides clear evidence for the function of the gut microbiota, these types of experiments may not be economically or logistically feasible for most researchers studying the ecology or evolution of microbes in non-model species.

Our work documents that gut microbes play an important role in facilitating the ingestion of dietary toxins by wild herbivores. Likewise, the gain of such microbes can facilitate the consumption of toxic plants. Microbial probiotics are of interest to farmers aiming to increase the feed efficiency of livestock (Krehbiel *et al.* 2003), and detoxifying microbes might provide a means to engineer livestock that can be reared in an environment containing toxic plants. Moreover, symbiotic interactions such as those reported in our study may have influenced the foraging ecology and evolution of many herbivorous mammalian species, thus greatly influencing ecosystem structure worldwide. In the future, herbivores may be faced with novel and more potent plant toxins due to changes in land use, introduction of exotic species, or global climate change (Verhoeven *et al.* 2009; Dearing 2013). Although past rates of niche evolution lag far behind the pace at which humans are expected to alter ecosystems (Quintero & Wiens 2013), microbial detoxification may represent a mechanism for accelerated niche expansion in herbivores.

ACKNOWLEDGEMENTS

We thank Dr. Fred Adler and Dr. Kelly Oakeson for their statistical help. We also thank Johanna Varner for giving helpful comments on previous drafts of the manuscript. Research was supported by the National Science Foundation (Graduate Research Fellowship to K.D.K., Dissertation Improvement Grant, DEB 1210094 to M.D.D. and K.D.K., and DEB 1342615 to M.D.D.).

AUTHORSHIP

KDK conducted experiments and participated in data interpretation. RBW, JC, CD and MDD participated in experimental design and data interpretation. MDD oversaw the project. KDK wrote the first draft of the manuscript and all other authors contributed substantially to revisions.

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Editor, Nicole van Dam
Manuscript received 17 March 2014
First decision made 14 April 2014
Manuscript accepted 18 June 2014