

RESEARCH LETTER – Environmental Microbiology

# Microbial detoxification in the gut of a specialist avian herbivore, the Greater Sage-Grouse

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One sentence summary: An avian herbivore that feeds on toxic plants harbors a gut microbial population that is specialized to degrade those toxins.

Editor: Akihito Endo

## ABSTRACT

One function of the gut microbiota gaining recent attention, especially in herbivorous mammals and insects, is the metabolism of plant secondary metabolites (PSMs). We investigated whether this function exists within the gut communities of a specialist avian herbivore. We sequenced the cecal metagenome of the Greater Sage-Grouse (*Centrocercus urophasianus*), which specializes on chemically defended sagebrush (*Artemisia* spp.). We predicted that the cecal metagenome of the sage-grouse would be enriched in genes associated with the metabolism of PSMs when compared to the metagenome of the domestic chicken. We found that representation of microbial genes associated with 'xenobiotic degradation and metabolism' was 3-fold higher in the sage-grouse cecal metagenomes when compared to that of the domestic chicken. Further, we identified a complete metabolic pathway for the degradation of phenol to pyruvate, which was not detected in the metagenomes of the domestic chicken, bovine rumen or 14 species of mammalian herbivores. Evidence of monoterpene degradation (a major class of PSMs in sagebrush) was less definitive, although we did detect genes for several enzymes associated with this process. Overall, our results suggest that the gut microbiota of specialist avian herbivores plays a similar role to the microbiota of mammalian and insect herbivores in degrading PSMs.

**Keywords:** detoxification; gut microbiota; greater sage-grouse; herbivory; plant–animal interactions; plant secondary metabolites

## INTRODUCTION

Recent research has revealed a number of roles that gut microbes play in the ecology and evolution of their hosts (McFall-Ngai et al. 2013). One function that has received recent attention is the microbial metabolism of plant secondary metabolites (PSMs) in the guts of herbivores (Hammer and Bowers 2015).

This microbial processing allows herbivores to consume plants rich in PSMs, but minimize the negative consequences of these defensive compounds. For example, the ability of herbivorous woodrats (*Neotoma lepida*) to feed on a phenolic-rich plant, creosote bush (*Larrea tridentata*), is conferred by their gut microbiota (Kohl et al. 2014). Additionally, the gut microbes of an insect pest of coffee mediate the detoxification of caffeine, thereby

Received: 11 March 2016; Accepted: 24 May 2016

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permitting consumption of coffee plants (Ceja-Navarro et al. 2015). Last, the gut microbial communities of mountain pine beetles (*Dendroctonus ponderosae*) are highly enriched in genes associated with the degradation of terpenes, a class of PSM common in the conifer plants consumed by these beetles (Adams et al. 2013).

Currently, research supporting the notion of microbial detoxification of PSMs is largely limited to work on mammals and insects. Birds are thought to have unique interactions with their gut microbiota compared to mammals in several ways (Kohl 2012; Waite and Taylor 2014), especially since the gut is under strong selective pressure to remain small so as to allow flight (Price et al. 2015). Therefore, it is worth investigating the universality of microbial detoxification by assessing this process in an avian herbivore.

Though herbivory is rare in birds (<2% of species; Olsen 2015), some species do indeed specialize on highly toxic plants. For example, the Greater Sage-Grouse (*Centrocercus urophasianus*; hereafter, sage-grouse), is a specialist avian herbivore that feeds primarily on sagebrush (*Artemisia* spp.; Wallestad, Peterson and Eng 1975). Sagebrush is heavily chemically defended with high concentrations of several classes of PSMs, especially monoterpenes (Shafizadeh, Bhadane and Kelsey 1974; Welch and McArthur 1981; Kelsey, Stephens and Shafizadeh 1982; Wilt and Miller 1992; Wilt et al. 1992), phenolics (Wilt et al. 1992) and sesquiterpene lactones (Kelsey, Morris and Shafizadeh 1976). Interestingly, concentrations of PSMs in the sage-grouse gut are lowest in the caeca (the blind-sac microbial chambers off the gut, just before the rectum), which is suggestive of microbial detoxification in this region (Kohl et al. 2015).

Here, we investigated the genomic potential for microbial detoxification in the gut of an avian herbivore. We performed metagenomic sequencing on the cecal microbiome of sage-grouse collected during late fall and early winter when grouse were feeding almost entirely on sagebrush. We compared the cecal metagenome of sage-grouse to that of the domestic chicken (*Gallus gallus*) and various mammalian herbivores. We predicted that the sage-grouse microbiome would be enriched in genes associated with detoxification compared to the domestic chicken and mammalian herbivores.

## MATERIALS AND METHODS

### Animal collection

Cecal contents were obtained from three sage-grouse (one male, two females) collected in south-central Idaho (E 721093, N 4789063) during November–December 2012. In the winter, sagebrush can be the only component of the diet of sage-grouse (Wallestad, Peterson and Eng 1975) and analysis of crop contents demonstrated that sage-grouse in our study were consuming 100% sagebrush at the time of collection. Our sample size was limited to three individuals because sage-grouse have undergone significant population declines (Connelly and Braun 1997; Garton et al. 2011) and are currently listed as ‘near-threatened’ by the International Union for the Conservation of Nature (Birdlife International 2012). Sage-grouse were collected under approved permits (Idaho permit 110 914 and Boise State University IACUC protocol AC11-022). Carcasses were dissected in the field and cecal contents were immediately stored in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  in the laboratory.

### Metagenomic sequencing and data analysis

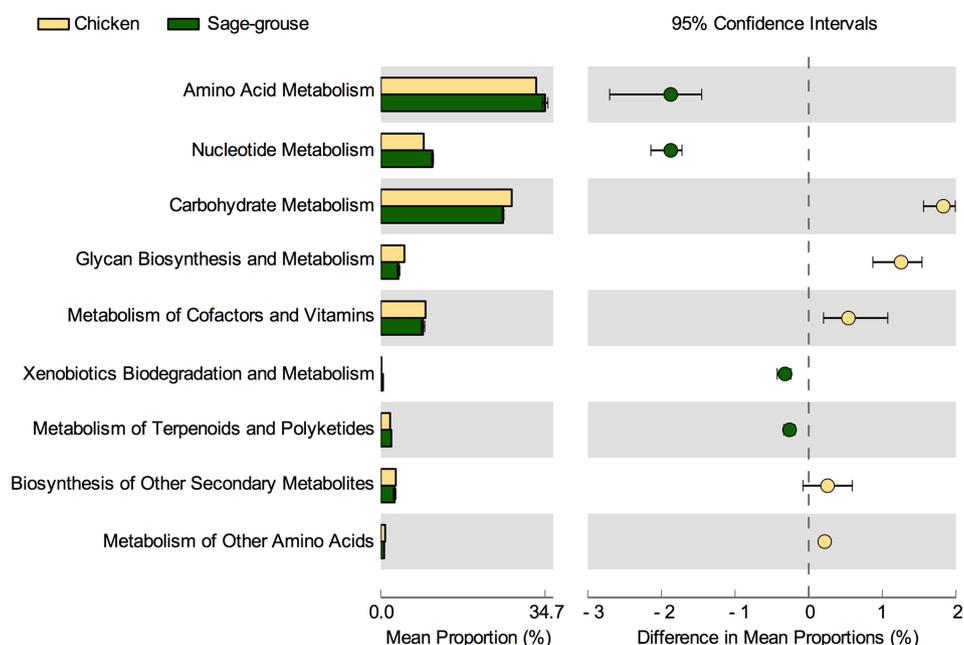
Total DNA was isolated from cecal contents using MoBio PowerFecal DNA extraction kit (MoBio, Carlsbad, CA). Extracted DNA was sent to Argonne National Laboratory for sequencing. Genomic DNA was sheared using a Covaris Sonicator, and metagenomic shotgun libraries were prepared using the Illumina TruSeq DNA preparation kits. We performed size selection on our libraries of roughly 150 bp. Libraries were sequenced on the Illumina HiSeq2000 platform using a  $2 \times 100$  bp run and V3 sequencing by synthesis chemistry, which resulted in overlapping sequences.

Overlapping sequences were joined, and joined reads were uploaded to the MG-RAST website (Metagenomics-Rapid Annotations using Subsystems Technology; 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 five low-quality bases were removed. Sequences were annotated with the KEGG Orthology database (Kanehisa and Goto 2000; Kanehisa et al. 2016) with the following thresholds: (i) e-values  $< 1e-5$ , (ii) a minimum percent identity to database sequences of 60% and (iii) a minimum alignment length of 15 bases. We primarily compared our sequences to a previous study on the chicken cecal metagenome given that this is a closely related host species. Details regarding conditions for this sample can be found in Qu et al. (2008). Chicken B from this experiment was excluded, as it was experimentally infected with *Campylobacter jejuni*. We also compared our findings to those in bovine rumen metagenomes (Brulc et al. 2009) and the fecal metagenomes of 14 species of mammalian herbivores (Muegge et al. 2011). See Table S1, Supporting Information for a list of metagenomes used in our analysis. Metagenomes were not pooled, but rather were used as individual samples in statistical analyses.

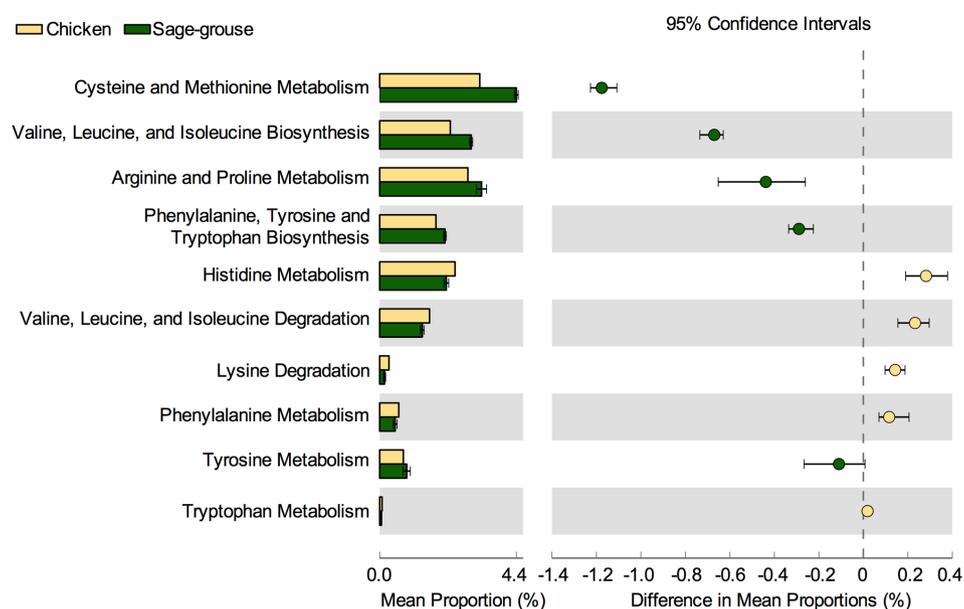
Abundances of functional categories were compared using the program STAMP (Parks et al. 2014). All functional categories were compared with White’s non-parametric t-test (White, Nagarajan and Pop 2009), and corrected using the Benjamini–Hochberg False Discovery Rate correction. We only present differences where the ratio of proportions between the groups were  $\geq 1.05$  (meaning that one group had to have at least  $1.05 \times$  higher abundance of any given functional category). The detection of various enzymes and pathways was investigated using the KEGG pathway viewer (Kyoto Encyclopedia of Genes and Genomes) within MG-RAST (Meyer et al. 2008). Here, we also investigated for the presence of the same detoxification genes in the chicken cecal metagenome (Qu et al. 2008), the bovine rumen metagenome (Brulc et al. 2009) and the fecal metagenomes of 14 species of mammalian herbivores (Muegge et al. 2011). Our data are publically available under MG-RAST Project #10520.

## RESULTS AND DISCUSSION

Our sequencing effort resulted in an average of  $\sim 1930$  000 raw reads per individual. Using the standard quality thresholds within MG-RAST, we could assign roughly 21% of reads to functional categories. This number is comparable with other metagenomic sequencing projects, including previous studies that we used for comparison, which sequenced the metagenome of chicken cecal contents (40% of reads assigned to functional categories; Qu et al. 2008) and the bovine rumen (25% of reads assigned; Brulc et al. 2009).



**Figure 1.** Differential abundances of various KEGG Orthology functions in the cecal metagenomes of sage-grouse (*C. urophasianus*) and the domestic chicken (*G. gallus*). Differences in mean proportions were calculated by subtracting the abundance in grouse samples from abundance in chicken samples. All functional categories were compared with STAMP (Parks et al. 2014), using White's non-parametric t-test (White, Nagarajan and Pop 2009), and corrected using the Benjamini-Hochberg False Discovery Rate correction. Corrected P-values of all functional categories presented in the figure were all <0.00001.



**Figure 2.** Differential abundance of various KEGG Orthology functions associated with 'amino acid metabolism' between the cecal metagenomes of sage-grouse (*C. urophasianus*) and the domestic chicken (*G. gallus*). Differences in mean proportions were calculated by subtracting the abundance in grouse samples from abundance in chicken samples. All functional categories were compared with STAMP (Parks et al. 2014), using White's non-parametric t-test (White, Nagarajan and Pop 2009), and corrected using the Benjamini-Hochberg False Discovery Rate correction. Corrected P-values of all functional categories presented in the figure were all <0.00001.

The cecal microbiome of sage-grouse was largely bacterial ( $97.9 \pm 0.2\%$  of sequences), with fewer reads identified as Archaea (0.8%) and Eukaryota (1.1%). The most abundant bacterial phyla were Firmicutes (42.6% of sequences), Bacteroidetes (32.5%) and Actinobacteria (11.6%). At the level of genera, *Bacteroides* was the most common genus (19.2% of reads), followed by *Prevotella* (9.6%) and *Clostridium* (9.1%). The abundances of these microbial taxa are similar to findings in other avian species (Kohl 2012; Waite and Taylor 2014).

We compared the relative abundances of various functional categories in the metagenomes of sage-grouse and the domestic chicken using the KEGG Orthology database (Kanehisa and Goto 2000; Kanehisa et al. 2016). When we compared functional 'metabolism' categories, we found that the sage-grouse microbiome was enriched in genes associated with 'amino acid metabolism' (Fig. 1, which only presents categories that were statistically significant between sage-grouse and chickens and where one species had at least 1.05 $\times$  higher abundance of

the given functional category compared to the other species). When we investigated deeper functional levels of this category, the sage-grouse metagenome was enriched for genes associated with the biosynthesis of essential amino acids. Specifically, the sage-grouse had 1.3× higher abundance of genes associated with 'valine, leucine and isoleucine biosynthesis', and 1.2× higher abundance of genes associated with 'phenylalanine, tyrosine and tryptophan biosynthesis' than chickens (Fig. 2). These amino acids are considered 'essential amino acids' from a nutritional standpoint, since animals lack the enzymes to synthesize them (Karasov and Martinez del Rio 2007). Obtaining essential amino acids can be a challenge for herbivores, given the relatively low protein content of their food material (Karasov and Martinez del Rio 2007). However, many herbivores harbor gut bacteria that are capable of recycling nitrogenous waste products (such as urea or uric acid) into essential amino acids (Stevens and Hume 2004). These microbes and their products can then be digested and reabsorbed by the animal (Stevens and Hume 2004), and the sage-grouse cecum has a high capacity for absorbing amino acids (Obst and Diamond 1989). Our findings suggest that one function of the sage-grouse microbiome may be microbial production of these essential amino acids to assist the host in maintaining nitrogen balance. This hypothesis warrants further examination.

In agreement with our original prediction, we also found that the sage-grouse microbiome was enriched in genes associated with xenobiotic degradation and the metabolism of terpenoids (Fig. 1). Sage-grouse had 3.3× higher abundances of genes associated with xenobiotic degradation when compared to the domestic chicken, and 1.13× higher abundances of genes associated with the metabolism of terpenoids. Within the functional category of 'xenobiotic degradation', sage-grouse were most enriched in the pathways associated with 'nitrotoluene degradation' and 'benzoate degradation', specifically a number of enzymes that play a role in the degradation of phenols and catechols. While nitrotoluene and benzoate may not necessarily be present in sagebrush, detoxification enzymes often metabolize numerous substrates with similar chemical structures. It is possible that the products of these genes could degrade similar compounds present in sagebrush. The majority of these sequences were from the genera *Bacteroides*, *Eggerthella* and *Clostridium*. These genera play a role in the degradation of a number of PSMs, especially phenolics (Selma, Espin and Tomas-Barberan 2009).

In fact, we detected the presence of genes in a complete pathway to degrade phenols and catechols to pyruvate (Fig. 3). The majority of sequences for each of these genes were identified as originating from the genus *Arthrobacter*. This genus degrades phenolics (Karigar et al. 2006; Unell et al. 2008), and is a core member of the gut community of another avian herbivore, the bar-headed goose (*Anser indicus*; Wang et al. 2016). None of these enzymes were detected in the domestic chicken. Thus, this phenolic degradation pathway is enriched in the cecal metagenome of sage-grouse, and may facilitate the metabolism of phenolics into energy sources that can be used by gut microbes and the host.

Given that the results shown above were based on a single chicken sample, we used additional comparisons to further investigate this pattern. We compared the sage-grouse metagenomes to three bovine rumen metagenomes (Brulc et al. 2009) and 17 fecal metagenomes from 14 species of mammalian herbivores (Muegge et al. 2011). We did not detect any significant differences in the abundance of metabolic pathways between the bovine rumen metagenomes and sage-

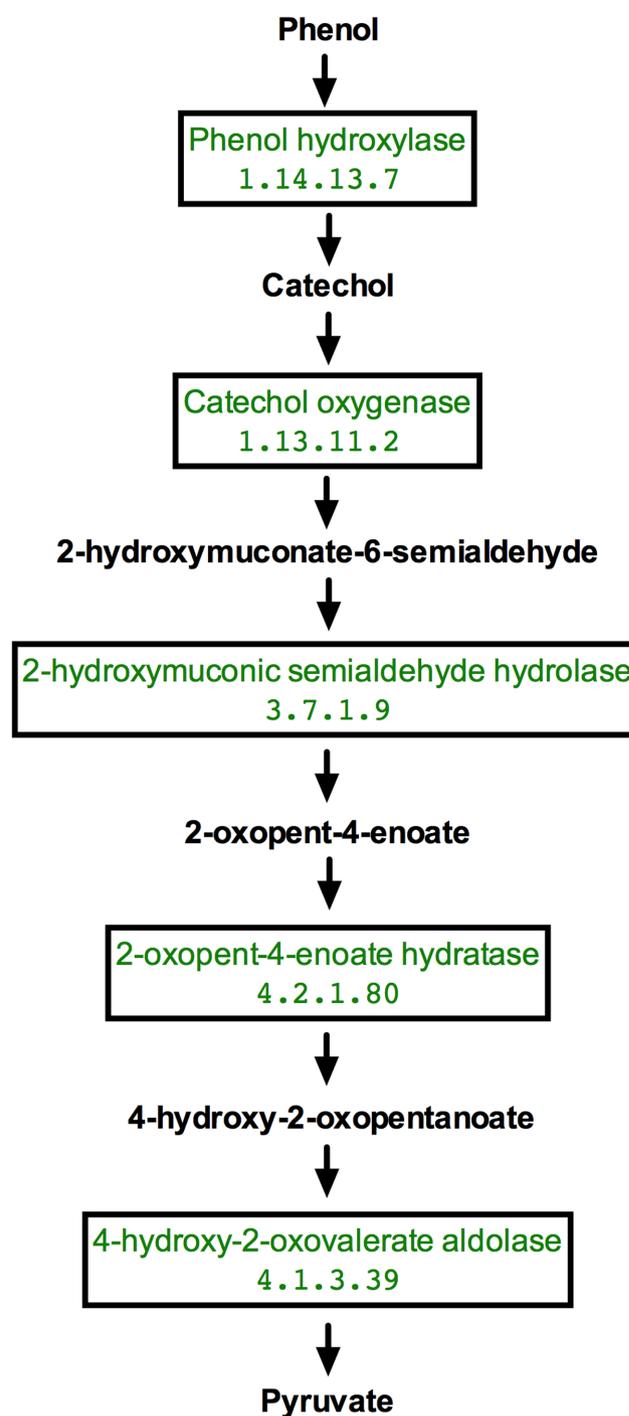
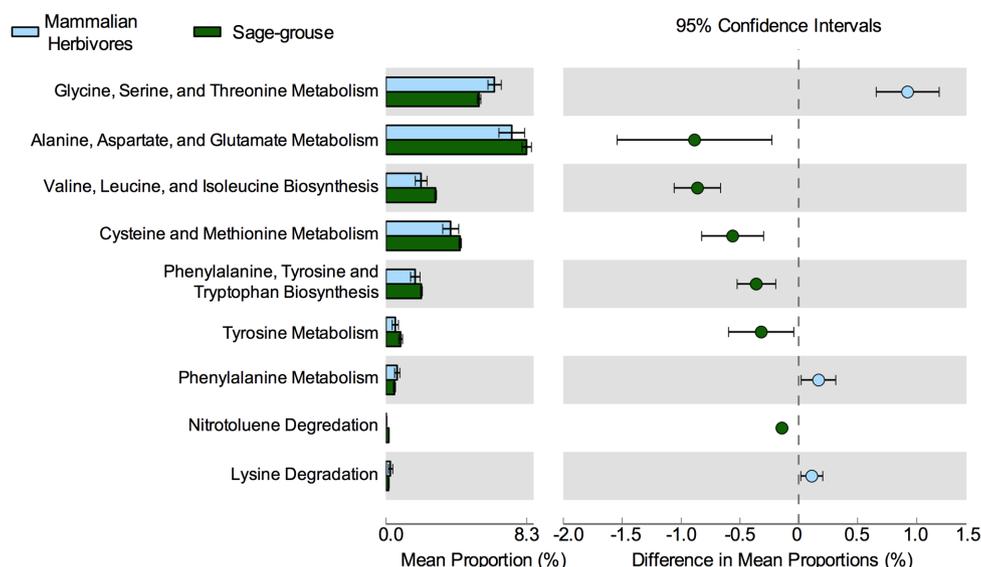


Figure 3. A complete pathway present in the sage-grouse (*C. urophasianus*) cecal metagenome for the degradation of phenol to pyruvate. This pathway was not detected in the metagenomes of the chicken cecum, bovine rumen, or mammalian herbivore feces.

grouse cecal metagenomes. The lack of significant differences is likely due to the large variability across bovine rumen samples, rather than high variability across sage-grouse samples (Fig. S1, Supporting Information; Brulc et al. 2009). Compared to the fecal metagenomes of several mammalian herbivores, the sage-grouse metagenomes had 1.4× higher abundances of genes associated with 'valine, leucine and isoleucine biosynthesis', and 1.2× higher abundances of genes associated with



**Figure 4.** Differential abundance of various KEGG Orthology functions associated with ‘amino acid metabolism’ and ‘xenobiotic degradation’ between the cecal metagenomes of sage-grouse (*C. urophasianus*) and the fecal metagenomes of several mammalian herbivores. Differences in mean proportions were calculated by subtracting the abundance in grouse samples from abundance in mammalian herbivore samples. All functional categories were compared with STAMP (Parks et al. 2014), using White’s non-parametric t-test (White, Nagarajan and Pop 2009), and corrected using the Benjamini–Hochberg False Discovery Rate correction. Corrected *P*-values of all functional categories presented in the figure were all  $<0.04$ .

‘phenylalanine, tyrosine and tryptophan biosynthesis’. Additionally, the sage-grouse metagenomes had 7.7× higher abundances of genes associated with ‘nitrotoluene degradation’ when compared to the fecal metagenomes of several mammalian herbivores (Fig. 4). Only two genes in the degradation pathway from phenol to pyruvate (Fig. 3) were detected in the bovine rumen (catechol oxygenase and 2-oxopent-4-enoate hydratase, both with only a single read). Similarly, in the fecal metagenomes of several mammalian herbivores, only the final enzyme was detected (4-hydroxy-2-oxovalerate aldolase), only in 11 out of 17 samples. Thus, it seems that this degradation pathway is also enriched in the sage-grouse cecal metagenome when compared to other metagenomes. Overall, these comparisons with other taxa support our original findings that the sage-grouse metagenomes are enriched in genes associated with the biosynthesis of essential amino acids and degradation of toxins compared with the chicken metagenome.

We also searched for the presence of genes associated with degrading monoterpenes in the sage-grouse cecal metagenome. We did not identify a complete degradation pathway, but we did detect two genes in the ‘limonene and pinene degradation’ pathway (KEGG pathway 00903). Specifically, we detected reads identified as aldehyde dehydrogenase (E.C. 1.2.1.3) and enoyl hydratase (E.C. 4.2.1.17). Both of these genes are enriched in the gut microbiota of mountain pine beetles (*Dendroctonus ponderosae*), which specialize on terpene-rich conifer trees (Adams et al. 2013). The sequences in the sage-grouse microbiome were most similar to genes present in the genus *Eggerthella*, whereas terpene degradation in the mountain pine beetle gut seems to be performed by *Pseudomonas* (Adams et al. 2013). While both of these genera are known to degrade aromatic compounds and other PSMs (Foght and Westlake 1988; Selma, Espin and Tomas-Barberan 2009), their activity towards monoterpenes has not been demonstrated. Therefore, it is likely that there are unidentified terpene degradation pathways present in the gut microbial communities of terpene-specialist herbivores.

Overall, our findings suggest an enhanced capacity for the degradation of PSMs in the gut microbes of sage-grouse. We

hypothesize that microbial detoxification allows sage-grouse to specialize on chemically defended plants, similar to what has been demonstrated in herbivorous mammals and insects (Kohl et al. 2014; Ceja-Navarro et al. 2015). Our results add an avian herbivore to the growing evidence of detoxification by microbial communities in the guts of herbivores. The independent evolution of herbivory results in unique genes for metabolizing plant compounds (Pope et al. 2010), and the gut metagenomes of herbivores are often targets for bioprospecting for new enzymes (Li et al. 2009). The sage-grouse microbiota may be a rich source for novel enzymes, especially given the interest in finding enzymes capable of biodegradation of aromatic compounds (Haritash and Kaushik 2009), and methods to increase the palatability of terpene-rich forages in agriculture (Utsumi et al. 2013).

## SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

## ACKNOWLEDGEMENTS

We would like to thank S. Vasilchenko, N. Wiggins, B. Robb and Gus, the German short-haired pointer.

## FUNDING

This research was funded by the Idaho Department of Fish and Game, Idaho Governor’s Office for Species Conservation to JSF and the National Science Foundation (Division of Environmental Biology (DEB)-1146194 and Integrative Organismal Systems-1258217 to JSF, DEB-1342615 to MDD, DEB-1210094 to MDD and KDK, and Division of Biological Infrastructure-1400456 to KDK). This is a contribution from Idaho Federal Aid in Wildlife Restoration Project W-160-R.

**Conflict of interest.** None declared.

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