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# Strategies in herbivory by mammals revisited: The role of liver metabolism in a juniper specialist (*Neotoma stephensi*) and a generalist (*Neotoma albigula*)

Teri J. Orr[1] | Smiljka Kitanovic[1] | Katharina M. Schramm[1,2] | Michele M. Skopec[3] |
P. Ross Wilderman[4] | James R. Halpert[4] | M. Denise Dearing[1]

[1]School of Biological Sciences, University of Utah, Salt Lake City, UT, USA

[2]Department of Botany, Weber State University, Ogden, UT, USA

[3]Department of Zoology, Weber State University, Ogden, UT, USA

[4]School of Pharmacy, University of Connecticut, Storrs, CT, USA

**Correspondence**
Teri J. Orr, New Mexico State University, Las Cruces, NM, USA.
Email: TeriOrr@nmsu.edu

## Abstract

Although herbivory is widespread among mammals, few species have adopted a strategy of dietary specialization. Feeding on a single plant species often exposes herbivores to high doses of plant secondary metabolites (PSMs), which may exceed the animal's detoxification capacities. Theory predicts that specialists will have unique detoxification mechanisms to process high levels of dietary toxins. To evaluate this hypothesis, we compared liver microsomal metabolism of a juniper specialist, *Neotoma stephensi* (diet >85% juniper), to a generalist, *N. albigula* (diet ≤30% juniper). Specifically, we quantified the concentration of a key detoxification enzyme, cytochrome P450 2B (CYP2B) in liver microsomes, and the metabolism of α-pinene, the most abundant terpene in the juniper species consumed by the specialist woodrat. In both species, a 30% juniper diet increased the total CYP2B concentration (2–3×) in microsomes and microsomal α-pinene metabolism rates (4-fold). In *N. stephensi*, higher levels of dietary juniper (60% and 100%) further induced CYP2B and increased metabolism rates of α-pinene. Although no species-specific differences in metabolism rates were observed at 30% dietary juniper, total microsomal CYP2B concentration was 1.7× higher in *N. stephensi* than in *N. albigula* ($p < .01$), suggesting *N. stephensi* produces one or more variant of CYP2B that is less efficient at processing α-pinene. In *N. stephensi*, the rates of α-pinene metabolism increased with dietary juniper and were positively correlated with CYP2B concentration. The ability of *N. stephensi* to elevate CYP2B concentration and rate of α-pinene metabolism with increasing levels of juniper in the diet may facilitate juniper specialization in this species.

**KEYWORDS**

biotransformation, diet switching, enzyme assay, herbivory, mammalian herbivores, metabolism, *Neotoma*, terpenes, trade-offs, woodrat

## 1 | INTRODUCTION

Although most herbivores are generalists and consume a wide variety of plants, only a dozen or so species have evolved the ability to specialize on a single or few plant species. Specialists typically ingest high concentrations of a limited suite of plant secondary metabolites (PSMs; Shipley, Davila, Thines, & Elias, 2006; Shipley, Forbey, & Moore, 2009; Torregrossa & Dearing, 2009). Over 40 years ago, Freeland and Janzen (1974) proposed that liver biotransformation enzymes mediate interactions between

mammalian herbivores and the plants they ingest, and that there are fundamental physiological constraints in the detoxification systems of generalists compared to those of specialists. In this key synthesis, the generalist feeding strategy used by most mammalian herbivores was surmised to result from a hepatic detoxification system that had evolved to process a wide range of substrates with low catalytic activity towards any specific substrate. In contrast, nearly a dozen mammalian dietary specialists were predicted to have evolved biotransformation pathways with enhanced abilities to catalyze a restricted set of PSMs in their diet. Thus, the biochemical trade-offs hypothesis by Freeland and Janzen (1974) describes the molecular costs and benefits associated with these different foraging strategies (Dearing, Foley, & McLean, 2005; Marsh, Wallis, Andrew, & Foley, 2006) and predicts that the enhanced biotransformation capabilities of the specialist may result from either an increased concentration of specific enzymes and/or a fundamental structural change of a biotransformation enzyme that increases its efficiency in metabolizing dietary PSMs. Detailed studies of the quantities and efficiencies of biotransformation enzymes have been undertaken in insect herbivores (Berenbaum, 1991; Gavrilović et al., 2017; Li, Berenbaum, & Schuler, 2003; Marsh et al., 2006; Saha, 2016; Schuler, 2011; Wen, Pan, Berenbaum, & Schuler, 2003; Wybouw, Pauchet, Heckel, & Van Leeuwen, 2016), and although not as extensive, similar work in mammalian herbivores has focused on the identification of biotransformation enzymes critical for dietary specialization (Boyle, McLean, Foley, & Davies, 1999; Foley & Moore, 2005; Haley, Lamb, Franklin, Constance, & Dearing, 2007; Lamb, Sorensen, & Dearing, 2001; Skopec, Haley, & Dearing, 2007; Sorensen, McLister, & Dearing, 2005).

To advance our understanding of the mechanisms underlying dietary specialization in mammalian herbivores, we conducted a study on two related species of herbivorous woodrats that differ in their dietary breadth. Our investigation focused on Stephens' woodrat (*Neotoma stephensi*), a specialist that consumes almost exclusively foliage from one-seeded juniper (*Juniperus monosperma*, 80%–95% of diet, Dial, 1988; Vaughn, 1982) across its range. Juniper has high concentrations of terpenes, such as α-pinene, which comprises over 61% of its monoterpene concentration (Adams, Skopec, Kohl, & Dearing, 2014). Terpenes elicit neurotoxic and hepatoxic effects in mammals (Falk, Hagberg, Lof, Wigaeus-Hjelm, & Zhiping, 1990; Savolainen, 1978; Sperling, Marcus, & Collins, 1967). Stephen's woodrat ingests a daily dose of terpenes that exceeds the lethal dose of terpenes in humans when adjusted for body size (Dearing, Mangione, & Karasov, 2000; Gscheidmeier & Fleig, 1996; Saeidnia, 2014; Troulakis et al., 1997). Stephen's woodrat is often sympatric with the white-throated woodrat (*Neotoma albigula*), a generalist that consumes some juniper (18%–34% of diet; Dial, 1988) with the balance of its diet consisting of other species of plants (e.g., *Yucca*, Dial, 1988). In laboratory feeding trials, the specialist can maintain and even gain body mass on a diet of 95% juniper, whereas the generalist regulates toxin intake and cannot maintain body mass on a diet of 50% juniper (Sorensen et al., 2005;

Torregrossa, Azzara, & Dearing, 2011). Previous work in this system has shown that cytochromes P450 2B (CYP2B), a subfamily of liver enzymes, play a critical role in the oxidation of the PSMs in juniper (Haley et al., 2007; Lamb, Marick, Sorensen, Haley, & Dearing, 2004; Skopec et al., 2007). More recent work characterized variants of CYP2B in these two woodrat species and identified differences in key amino acid residues likely to alter the substrate preference and function of these enzymes (Kitanovic et al., 2018). Moreover, this study also documented a difference in the number of copies of the CYP2B gene with the specialist having five more copies of CYP2B than the generalist per haploid genome (Kitanovic et al., 2018). However, it is not known whether the increased number of gene copies directly increases CYP2B concentrations.

We investigated whether CYP2B protein was induced by juniper feeding in both woodrat species, and whether induction of CYP2B was correlated with the amount of juniper in the diet of the specialist. We compared the induction of CYP2B by juniper ingestion in woodrats to CYP2B induction by a pharmacological agent (phenobarbital) in the laboratory rat. We also quantified the abilities of these two woodrats to metabolize the most abundant monoterpene in *J. monosperma*, α-pinene, by measuring its metabolism by liver microsomes. Finally, we explored the relationship between CYP2B protein concentration and metabolism of α-pinene. Furthermore, we confirmed that CYP2B metabolizes α-pinene by using engineered CYP2B proteins from woodrats. In the context of the biochemical trade-offs hypothesis, we predicted that the specialist would have higher CYP2B concentration and higher rates of α-pinene metabolism when compared with the generalist on a similar dietary level of juniper.

## 2 | MATERIALS AND METHODS

### 2.1 | Capture, husbandry and juniper collection

Woodrats were captured using Sherman live traps (7.6 × 89 × 23 cm). Stephens' woodrats (*Neotoma stephensi*) were collected on Woodhouse Mesa, Coconino County, Arizona, near Flagstaff, Arizona, USA (35°30′N, 111°27′W). White-throated woodrats (*Neotoma albigula*) were captured in Castle Valley, Grand County, Utah, USA (38°30′N, 109°18′W).

Woodrats were transported to the animal facility in the School of Biological Sciences at the University of Utah. A subset of *N. stephensi* was fed juniper (details below). All other animals were given water and fed high-fibre rabbit chow ad libitum (Harlan Teklad formula 2031). Individuals were housed singly in shoebox cages (48 × 27 × 20 cm, Techniplast) with shavings and a plastic tube. The animal room was maintained at 28°C with humidity between 15% and 20% and a constant light/dark cycle (12L/12D, Kohl, Miller, Marvin, Mackie, & Dearing, 2014; Skopec, Kohl, Schramm, Halpert, & Dearing, 2015). All procedures were approved by the University of Utah Institutional Animal Care and Use Committee (IACUC 10-01013 and 12-12010).

*Juniper monosperma*, used in the induction trials was collected at the same localities as *N. stephensi*. Juniper was immediately placed in plastic bags, sealed and kept in a cooler with ice to prevent volatile compounds from dispersing until reaching the lab, where it was stored at −20°C prior to diet preparation.

## 2.2 | Induction of CYP2B

To determine whether CYP2B was induced by juniper ingestion, we fed animals a series of juniper diets. Both species of woodrats were fed control (0% juniper) and treatment (30% juniper) diets (four animals per treatment with; two females and two males in the 30% juniper diet treatments for both species as well as the specialists on 30% juniper, and three females and one male for the generalists on the control diet and the specialists on the 60% juniper diet, and finally all of the specialists on the 100% juniper diet were female). The 30% juniper diet was selected because it represents the natural diet for the generalist, whereas higher doses of juniper result in depressed food intake in the generalist, but not the specialist (Dial, 1988; Sorensen et al., 2005). In addition to being fed 30% juniper (two females, two males), the specialists were fed higher levels of juniper to better represent their diet in nature (Dial, 1988). Four animals were fed treatment diets of 60% juniper (three females: one male), and four additional animals (four females) were maintained on an exclusively juniper diet (100%) for 1 day. With the exception of the animals fed exclusively juniper diets, all animals were in captivity starting 6 months prior to diet trials, so we are confident that liver enzyme induction levels had returned to baseline, as that process takes 2–6 weeks (Yamashita, Kazawa, Minatogawa, Ebisawa, & Yamauchi, 2002). Sample sizes of four animals are standard for these types of biochemical assays and consistently demonstrate sufficient power for detecting the desired effect, should the effect exist (Boyle et al., 1999; Haley et al., 2007; Lamb et al., 2004; Skopec & Dearing, 2011; Sorensen & Dearing, 2006).

Diets were prepared by grinding frozen juniper foliage in a Waring blender (model CB-5) with dry ice until homogenous to prevent sorting and caching by animals during experiments. With the exception of the 100% juniper diet, ground juniper was then mixed with the appropriate amount of ground rabbit chow (Skopec et al., 2015). Diet treatments were prepared daily and offered in feeder hoods (Lab Products Inc.) attached to individual cages to accurately measure food intake. Each treatment was provided for 3 days, except the 100% juniper diet, which was provided for 1 day after capture. Daily measurements of body mass confirmed that all subjects maintained their starting body masses throughout the diet treatments.

## 2.3 | Microsome preparation

On the last day of each diet treatment, animals were dispatched with isoflurane. Their livers were perfused in situ via the hepatic portal vein with cold isotonic solution to remove blood and associated heme. Approximately 2.5 g of liver per individual was used for microsome preparation as described in Franklin and Estabrook (1971). Purified microsomes were resuspended in a sucrose buffer and frozen as 100 μl aliquots at −80°C until use. Protein concentration of liver microsomes was measured with the Bradford assay (Sigma). We prepared liver microsomes from four animals on each diet treatment with the exception of the 100% juniper treatment, for which microsomes were made from eight animals. Microsomes were used for both the enzyme assays and the quantification of CYP2B concentration.

## 2.4 | P450 and CYP2B concentration

We determined total P450 concentration from the reduced CO difference spectrum following Omura and Sato (1964) in both species and across all induction treatments except those of the *N. stephensi* on 100% juniper as these samples were lost prior to analysis. We compared P450 concentrations in both species at shared treatment levels using a two-way ANOVA.

We estimated CYP2B concentration in the liver microsomes obtained from all diet treatments, as well as microsomes from Sprague Dawley (SD) laboratory rats injected with phenobarbital, a strong inducer of CYP2B (XenoTech, LLC, R1078) using semiquantitative western blotting (Taylor, Berkelman, Yadav, & Hammond, 2013). Microsomes stored at −80°C were thawed on ice and lightly vortexed in 1 M Tris buffer (pH 7.4), and then mixed with equal volumes of 2× Laemmli sample buffer. After incubation at 80°C for 10 min, 10 μl of each microsome sample was loaded per well of Mini-Protean TGX precast gels (Bio-Rad). This sample volume contained 0.15 or 0.3 μg of microsomes from woodrats on juniper diets or the control diet, respectively, ensuring that woodrat microsomal CYP2B amounts loaded per well were in the linear range (Taylor et al., 2013). Serial dilutions of purified woodrat CYP2B37 enzyme, also in the linear range (0.06, 0.125, 0.25, and 0.5 pmol), were loaded as standards onto each gel. Additionally, for comparison, we included pooled microsomes from Sprague-Dawley (SD) laboratory rats injected with phenobarbital (Supplier: XenoTech, LLC, R1078). Phenobarbital (Pb) is a strong inducer of CYP2B, and Pb-induced rats are used to define the upper limits of CYP2B induction (Correia, 1996). Microsomal proteins were separated in the Mini-Protean Tetra Cell (Bio-Rad) for 1 hr at 200 V. Protein gels were blotted using the Trans-Blot Turbo apparatus and Mini Trans-Blot Turbo transfer packs for 7 min at 25 V (Bio-Rad). Blots were gently agitated in blocking buffer for 1 hr at room temperature, and then transferred into blocking buffer with anti-rat CYP2B rabbit polyclonal antibody (Duignan, Sipes, Leonard, & Halpert, 1987; Stevens & Halpert, 1988) at 1:2,000 dilution.

Blots were next washed with Tris-buffered saline and Polysorbate (Tween) 20 (TBST) three times for 10 min and then gently agitated for 45 min at room temperature with peroxidase-labeled goat anti-rabbit IgG antibody (KPL) in blocking buffer at 1:10,000 dilution. After three 10 min washes in TBST at room temperature, and a 5 min incubation in Clarity Western

ECL substrate (Bio-Rad), chemiluminescent blots were imaged with the ChemiDoc MP imager (Bio-Rad). The densities of manually selected bands containing CYP2B were determined using IMAGELAB (software version 5.2.1, Bio-Rad) and density data were then exported and analysed in Excel. Serial dilutions of purified woodrat CYP2B37 enzyme (see Wilderman et al., 2014) were used to generate standard curves and determine amounts of microsomal CYP2B protein loaded on the same gel. Microsomes were run in duplicate on separate gels. Microsomal CYP2B concentration in both species was analysed using a two-way ANOVA with species and dietary induction treatment as independent variables. We used linear regression to determine the relationship between CYP2B concentration and P450 concentration in both woodrat species.

## 2.5 | α-pinene metabolism assays

We used gas chromatography head-space analysis to estimate the microsomal metabolism of α-pinene. Substrate loss is an indicator of detoxification enzyme activity (or metabolism). The same microsomal preparations used in the determination of CYP2B concentration were analysed in these experiments. For each microsomal sample, a reaction mixture of 1 ml was prepared in duplicate, the values of which were averaged. Reactions were conducted in 10 ml glass headspace vials (Agilent). Vials contained 1 mM NADPH, 300 μg microsomes, and 200 μM α-pinene in 1 ml reaction volume (pH 7.4, phosphate buffer). The substrate was added from a stock solution of 20 mM α-pinene that had been prepared in dimethyl sulfoxide (DMSO). Negative control reactions contained the same components, with the exception of NADPH, and were adjusted for volume using buffer. Buffer, NADPH and microsomes were preincubated in vials at 37°C for 5 min and substrate was then added. Vials were then immediately crimped and incubated at 37°C for 0, 15, 30, or 45 min, with gentle agitation two to three times. All reactions were terminated by placing vials on a hotplate (~300°C) for 90 s. Vials were allowed to cool to room temperature and either immediately analysed in the gas chromatographer (GC) or frozen (−20°C) to prevent the loss of volatiles prior to their subsequent analysis. Our initial analysis indicated that the rate of metabolism slowed after 30 min; therefore we only present data for 0, 15, and 30 min. This slowing of substrate metabolism is likely due to the depletion of substrate available for reaction.

Headspace analysis was performed using a Tekmar 7000 HT autosampler connected to a Trace GC Ultra (Thermo Fisher Scientific). Our parameters were adjusted after those used by Shipley, Davis, Felicetti, McLean, and Forbey (2012). The headspace platen was heated to 70°C, the sample loop and transfer lines to 85°C and the GC inlet temperature was set to 100°C. Then, 1 ml of headspace volume was transferred from the auto sampler via the transfer line to the GC for analysis. Times were set to 0.6 min for vial pressure time, 0.5 min for loop fill time and 0.5 min for inject time. Analytes were detected using a flame ionization detector (FID). Carrier gas (helium) had a flow rate of 140 ml/min. Gas used for the FID was hydrogen. GC outputs were manually analysed in XCALIBUR (version 2.1.0, Thermo Fisher Scientific) where the area under the curve (AUC) of α-pinene peaks was integrated. AUC values were exported into Excel and analysed further in SPSS (version 20). To calculate α-pinene concentrations we considered decreases in peak size relative to the starting peak size for samples at timepoint zero that had no NADPH. These baseline starting peak values were also compared to negative controls to ensure that excess α-pinene was not lost over time due to the poor crimping of headspace vials.

We compared the metabolism rates of α-pinene between species and across induction treatments with an ANOVA and Bonferroni post-hoc tests on the amount of substrate metabolized at the 15 min time point. Because substrate availability may become limited after 15 min, we focused on this time point for our analyses. For each species, we evaluated the relationship between CYP2B concentration and α-pinene metabolism at the 15 min time point using regression. All data were tested for normality using Kolmogorov-Smirnov (KS) tests (all data were normally distributed). All values below are reported as: means ($\bar{x}$) ± standard error (*SE*) unless otherwise noted.

## 2.6 | Engineered proteins and enzymatic assays

Because microsomes contain a variety of P450s other than CYP2B, we also used engineered proteins of CYP2B1 (SD rats), CYP2B35 and CYP2B37 (both from *Neotoma*) to test whether α-pinene is a substrate for CYP2B in particular. The recombinant enzymes were *N*-terminally truncated and contain a *C*-terminal tetrahistidine tag for purification (Wilderman et al., 2014). Engineered CYP2B proteins were expressed in C43 (DE3) *E. coli* containing the pGro7 plasmid and purified from the membrane fraction by detergent extraction. This process was followed by Ni2+-NTA affinity chromatography and cation exchange chromatography as previously described (Scott, Spatzenegger, & Halpert, 2001; Wilderman et al., 2014).

For recombinant protein assays, a standard NADPH-dependent assay was performed (Kumar, Chen, Waxman, & Halpert, 2005; Shah et al., 2016). For engineered proteins, the reconstituted system contained the following proteins at a molar ratio of 1:4:2–CYP2B enzyme, rat NADPH-cytochrome P450 reductase (Harlow & Halpert, 1997), and rat cytochrome $b_5$ (Holmans, Shet, Martin-Wixtrom, Fisher, & Estabrook, 1994) previously optimized for maximum activity (Scott et al., 2001). A 100 μl aliquot of the reconstituted system containing 0.25 nmol of CYP2B enzyme was added to phosphate reaction buffer and preincubated in the presence of NADPH for 10 min as for the headspace reactions described above. The reaction was initiated by addition of α-pinene and was allowed to proceed for 10 min in a 37°C water bath, as these enzymes were more delicate than those in woodrat microsome preparations. The reaction was quenched by heating, and substrate elimination analysed as described above.

## 3 | RESULTS

### 3.1 | Total P450 and CYP2B concentrations

Total P450 concentration increased with level of juniper in the experimental diets but did not vary by species (Figure 1; two-way ANOVA $F_{3,15}$ = 2.3, $p$ = .13, Species $F_{1,15}$ = 1.4, $p$ = .27 Diet $F_{1,15}$ = 5.6, $p$ = .36, Interaction $F_{1,15}$ = 0.009; $p$ = .93). P450 concentration increased by 40%–50% with the addition of 30% juniper to the diet for both species and doubled in the *Neotoma stephensi* feeding on 60% juniper compared to the control diet.

CYP2B concentration varied between species (Figure 1; two-way ANOVA, $F_{3,15}$ = 41.7, $p$ < .0003, Species $F_{1,15}$ = 22.3, $p$ = .003) and by diet ($F_{1,15}$ = 9.4, $p$ < .0001) with a species by diet interaction ($F_{1,15}$ = 9.3; $p$ = .01). *Neotoma stephensi* had 1.3× higher constitutive concentration of CYP2B than the generalist, *Neotoma albigula*. Juniper induced CYP2B protein expression in both species, with the specialist exhibiting almost twice the CYP2B concentration of the generalist, (Figure 1). In *N. stephensi*, 86% of the variation in CYP2B concentration was explained by dietary juniper ($p$ < .0001). The microsomal CYP2B concentration when *N. stephensi* ingested a diet of 100% juniper was 4.6× that of the constitutive concentration of CYP2B (Figure 1). Microsomes from Pb-induced SD rats contained less CYP2B (0.968 ± 0.052 nmol/mg) compared with those from *N. stephensi* fed any of the juniper diets. The Pb-induced SD rat microsomes were used for a reference only and were not statistically compared to the woodrat samples because they were induced by injection versus diet and were pooled versus individual samples.
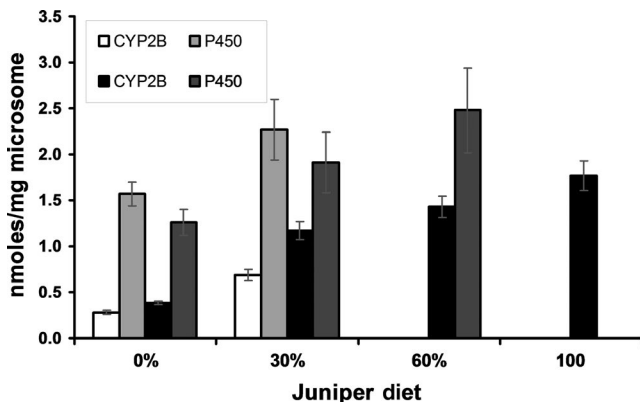


**FIGURE 1** CYP2B and P450 concentration by species and diet. CYP2B and P450 protein concentration (nmol CYP2B/mg of microsomal protein) relative to level of juniper induction. Bars represent the means (±1 *SE*) for four individuals in each juniper induction treatment. Data for the specialist are indicated by darker bars, with solid black bars indicating concentration of CYP2B and bars with dark grey hatching indicating P450 concentration. The generalist is indicated by lighter bars with solid white bars denoting concentration of CYP2B and bars with light grey hatching denoting P450 concentration. The SD rat had P450 concentration values of 0.65 nmol/mg (as measured by XenoTech) and CYP2B values of 0.97 nmol/mg. Note that the SD rat was induced through an injection of phenobarbital

CYP2B concentration was a function of P450 concentration. P450 concentration explained 54% of the variation in CYP2B concentration ($p$ = .014).

### 3.2 | α-pinene metabolism

Microsomal metabolism of α-pinene was positively related to levels of dietary juniper (Figure 2; ANOVA, $F_{5,23}$ = 18.8, $p$ < .0001). There was little metabolism of α-pinene by microsomes from either *N. albigula* or *N. stephensi* on the 0% juniper diet (Figure 2). Relative to the control diet, consumption of a 30% juniper diet increased metabolism of (α-pinene) in both the generalist and specialist but did not differ between species (Figure 2, Bonferroni post-hoc between diets $p$ < .024, and $p$ > .60 between species on the two diets). In the specialist, the rate of α-pinene metabolism was higher with increasing levels of juniper in the diet (Figure 2).

### 3.3 | Relationship between CYP2B concentration and α-pinene metabolism

α-pinene metabolism at 15 min was also explained by CYP2B concentration (Figure 3). CYP2B concentration explained 87% of α-pinene metabolism for *N. albigula*, and 79% of α-pinene metabolism for *N. stephensi* (independent linear regressions, $p$ = .001, $p$ < .0001 respectively).

Using our optimized assay conditions, CYP2B1 (11.8 nmol α-pinene/min/nmol P450), CYP2B35 (14.0 nmol α-pinene/min/nmol P450) and CYP2B37 (12.1 nmol α-pinene/min/nmol P450) metabolized α-pinene at similar rates. The metabolic rates from these purified CYP2B enzymes were similar to those observed in the microsomal assays, which ranged from 10.4–19.0 nmol α-pinene/min/nmol CYP2B.

## 4 | DISCUSSION

The mechanisms underlying dietary specialization in mammalian herbivores are largely unknown. A recent study using this same specialist-generalist pair of species provided support for the biochemical trade-offs hypothesis with respect to diversity of CYP2B variants and gene copy number in that the specialist had more similar copies of CYP2B than the generalist (Kitanovic et al., 2018). Here we extended that work to further test aspects of this hypothesis. Our results provide partial support for the biochemical trade-off hypothesis. We found that the level of induction of CYP2B proteins was associated with the concentration of juniper in the diet, and that α-pinene metabolism was a function of CYP2B concentration, indicating the importance of CYP2B in juniper digestion. However, at similar levels of induction the specialist and generalist did not differ in the rate of α-pinene metabolism (Figure 2). We discuss these
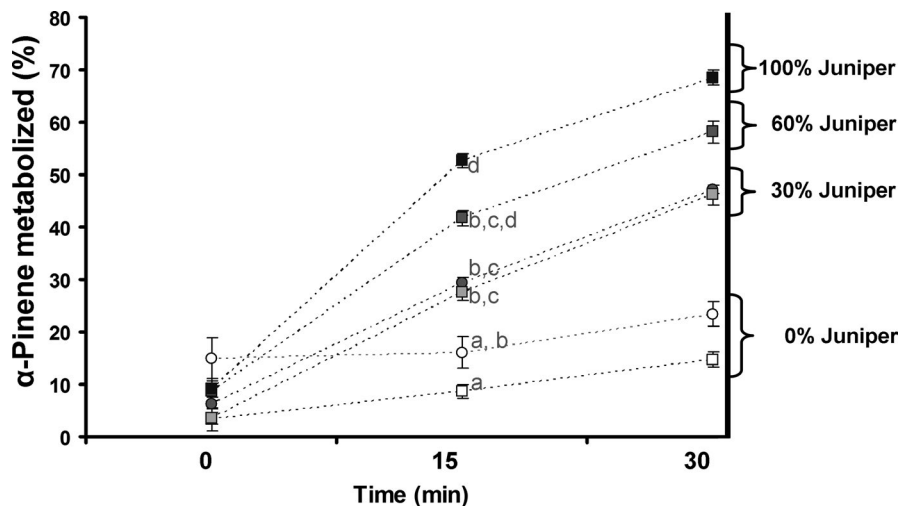
**FIGURE 2** Rate of substrate (α-pinene) metabolism by liver microsomes by diet type for the generalist and specialist species. The percentage of α-pinene metabolized as a function of reaction (incubation) time for specialists (squares) and generalist (circles) induced with increasing levels of dietary juniper as indicated on the right *y*-axis. The shading of the symbols represents increasing juniper in the diet, (from top to bottom: 100%, 60%, 30% and 0% juniper). Each point represents the mean of four individuals run in duplicate. Different letters at 15 min point indicate significant differences (Bonferroni post-hoc tests; ANOVA reported in the results)
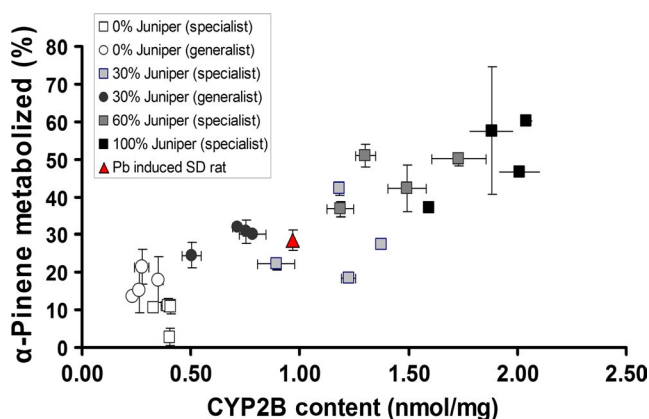


**FIGURE 3** α-pinene metabolism as a function of CYP2B concentration in liver microsomes for the generalist and specialist species. Substrate (α-pinene) metabolism at the 15 min time point plotted against CYP2B concentration across juniper induction treatments and species. Each point represents the average of the technical replicates for an individual microsomal sample i.e., animal. The generalist is indicated by circles, the specialist by squares and the phenobarbital-induced SD laboratory rat by a triangle. Increasing levels of juniper induction are indicated by darker fills [Colour figure can be viewed at wileyonlinelibrary.com]

results below in the context of dietary specialization and other related studies.

## 4.1 | CYP2B concentration and juniper ingestion

In both woodrat species, microsomal CYP2B concentration increased with juniper in the diet suggesting that these enzymes are strongly induced by the ingestion of α-pinene in juniper. Regardless of the level of dietary juniper, the specialist always produced more CYP2B proteins

than the generalist. On the control diet, the specialist produced ~30% more CYP2B than the generalist. When juniper was added to the rabbit chow diet for a resulting 30% juniper diet, this difference increased further, with the specialist producing almost twice as much CYP2B as the generalist. On a 100% juniper diet, the specialist increased CYP2B concentration to more than 4× that of its uninduced state.

The observed differences in CYP2B concentration may result from differences in the feeding behavior of these species. In nature, the specialist feeds on a diet containing a minimum of 85% juniper during most of the year. Lower levels of juniper in the diet, such as those provided in this study, are unnatural. In contrast, levels of dietary juniper are lower for the generalist, and fluctuate from ~15% to 35% of the diet depending on the time of year, with other plant species such as cactus making up a larger proportion of the generalist's diet than juniper (Dial, 1988; Kohl et al., 2014; Orr, Newsome, & Wolf, 2015). Moreover, in an experimental setting, the generalist regulates its food intake to not exceed high levels of juniper ingestion, even if this regulation depresses food intake and reduces body mass. The specialist does not regulate juniper intake and sustains its body mass on diets that are almost entirely juniper (Torregrossa et al., 2011). Because of these differences with respect to juniper ingestion, CYP2B in the specialist may be constitutively expressed at a higher level and may be more responsive to any level of dietary juniper than the generalist. The higher copy number of CYP2B genes in the specialist, (i.e., five more copies than the generalist), may underlie the observed differences in CYP2B concentration between these species on the same level of dietary juniper (Kitanovic et al., 2018). That is, the specialist may always produce more CYP2B enzyme because of its extra gene copies compared with the generalist.

The specialist produced high concentrations of CYP2B enzymes under natural dietary conditions, i.e., 100% juniper. The

concentrations of CYP2B in the specialist on a natural juniper diet were more than 2× higher than measured in the SD rat injected with phenobarbital. This pharmaceutical compound is utilized for its ability to strongly induce CYP2B (Correia, 1996). The high affinity of CYP2B enzymes from rats, woodrats, and humans for terpenes (Wilderman et al., 2014; Wilderman, Shah, Jang, Stout, & Halpert, 2013), coupled with ability of the specialist to strongly induce CYP2B on a juniper diet, is consistent with the hypothesis that these enzymes play a critical role in processing the high levels of terpenes in its juniper based diet.

We are unable to compare CYP2B concentrations of the specialist to those of the generalist on diets with more than 30% juniper because this amount of juniper represents the dietary limit for the generalist. Whether the level of juniper in the diet of the generalist is restricted by a limitation in its production of CYP2B warrants further investigation with approaches other than feeding trials, such as the use of agents such as phenobarbital to explore the upper limit of CYP2B induction in the generalist.

## 4.2 | Limited support for the biochemical trade-offs hypothesis

The metabolism of α-pinene in the microsomal assays was a function of the level of juniper in the diet, as expected by the biochemical trade-off hypothesis. However, the specialist did not metabolize α-pinene more rapidly than the generalist in microsomal assays when both specialist and generalist consumed equivalent rates of juniper (30%). This result is not consistent with the biochemical trade-off hypothesis. This outcome is even more remarkable given that the CYP2B concentration in the specialist was almost 2× that of the generalist. One explanation for this difference is the generalist may have individual CYP2B enzymes that metabolize α-pinene faster than those in specialists. The documented differences in key areas of the CYP2B active site between these two woodrat species likely result in differences in substrate positioning and processing (Kitanovic et al., 2018) and could contribute to differential metabolism of α-pinene.

From an evolutionary perspective, it may be more parsimonious for selection to act on enzyme concentration over quality, as gene duplications particularly in the P450s are common and enable greater enzyme production (Nelson et al., 2004; Perry et al., 2007; Zanger et al., 2007). As mentioned above, the specialist has five more gene copies of CYP2B than the generalist (Kitanovic et al., 2018). Moreover, the evolution of CYP2B in the specialist seems to have been through the conservation of duplicated gene copies, possibly to produce high concentrations of the same CYP2B variant. Alternatively, given that juniper produces many other terpenes (Adams, 1994), the CYP2B variants in the specialist could be honed to metabolize the high quantities of the mixture of terpenes present in juniper, possibly even with higher efficiency than the generalist. It is also conceivable that the CYP2B enzymes in the woodrat specialist oxidize α-pinene more extensively such that the metabolites do

not require subsequent conjugation. Other mammalian specialists (e.g., koala, greater glider, pygmy rabbit) have been documented to oxidize monoterpenes to a greater extent, while using conjugation less than generalists (Boyle et al., 2001; Shipley et al., 2012). For example, the pygmy rabbit (*Brachylagus idahoensis*), a sagebrush specialist, produced metabolites of cineole (a terpene) that were 20% more highly oxidized and far less conjugated relative to those of a generalist counterpart, mountain cottontails (*Sylvilagus nuttallii*, Shipley et al., 2012). We know from previous work that the woodrat generalist, *Neotoma albigula*, relies on conjugation pathways (glucuronic acid and glutathione) to a greater extent than the specialist, *Neotoma stephensi*, in the metabolism of a juniper diet (Haley et al., 2007; Skopec et al., 2007; Sorensen et al., 2005). The specialist relies more heavily on methyl group conjugation, which is energetically less costly than the conjugation pathways used by the generalist (Skopec & Dearing, 2011; Skopec et al., 2007). Distinguishing among these possibilities warrants further exploration.

It is important to recognize that the enzyme metabolism assays were conducted with microsomes. These microsomes contain not only CYP2B, but also other P450 enzymes that could contribute to the observed results. Furthermore, mechanisms other than PSM biotransformation by enzymes in the liver such as efflux transporters also play a role in detoxification at the level of the whole organism (Sorensen & Dearing, 2006).

The rates of α-pinene metabolism by engineered CYP2B35 and CYP2B37 from *N. lepida* were very similar to those by CYP2B1 from the SD rat and to the results (expressed per nmol CYP2B) of the microsomal incubations from the specialist, *N. stephensi* and the generalist, *N. albigula*. These results suggest that CYP2B enzymes metabolize α-pinene at similar rates independent of species. Although not explored, the expression levels of NADPH-cytochrome P450 reductase and cytochrome $b_5$ could also contribute to species-specific differences in total α-pinene metabolism, since differences in the concentration of either protein cofactor relative to the CYP enzyme alters substrate metabolism by the CYP enzyme (Chen, Huo, Halpert, & Wilderman, 2017; Scott et al., 2001; Zhang, Im, & Waskell, 2007; Zhang, Hamdane, Im, & Waskell, 2008).

## 4.3 | Integrating CYP2B concentration with α-pinene metabolism

In the specialist, there was a connection between the level of juniper in the diet, CYP2B concentration and the rate of α-pinene metabolism. CYP2B concentration was positively correlated with the amount of juniper in the diet, with the highest CYP2B concentration documented in the microsomes prepared from the specialist feeding on a natural diet. CYP2B enzymes appear to metabolize α-pinene at similar rates independence of species. For example, we obtained comparable rates of α-pinene metabolism by microsomal samples from the specialist, *N. stephensi* and the generalist, *N. albigula*. These results are suggestive that the liver enzymes may be expected to behave in a comparable manner.

In summary, this study tested aspects of the biochemical trade-off hypothesis and advances our knowledge of the mechanisms in which mammalian herbivores process toxic diets. In some respects, the results challenge the expectations of the biochemical trade-off hypothesis in that we did not find evidence for the specialist having more efficient metabolism of α-pinene, the primary plant secondary compound present in its diet at least at low levels of induction. Enzyme concentration seemed to play a key role in the metabolism of this compound by the specialist, as CYP2B protein was induced to higher levels with increasing quantities of juniper in its diet, which explained much of the variation related to the rate of substrate metabolism. Incorporating measurements of enzyme concentration into future explorations of the biochemical trade-off hypothesis in other animal systems seems warranted.

## AUTHOR CONTRIBUTIONS

T.J.O., J.R.H and M.D.D. designed research; S.K., K.S., M.S., P.R.W. and T.J.O. performed the research; K.S., P.R.W., and J.R.H. contributed reagents or analytical tools; S.K., M.D.D., T.J.O., and P.R.W. analysed data; S.K., M.D.D., T.J.O. and P.R.W. wrote manuscript and addressed reviewers' comments.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study (substrate metabolism rates, P450 and CYP2B concentration) are available in Dryad at (https://doi.org/10.5061/dryad.h18931zft).

## ORCID

*Teri J. Orr* https://orcid.org/0000-0001-6247-5237
*Smiljka Kitanovic* https://orcid.org/0000-0001-9151-6421

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