Detoxification and Elimination of α-pinene in North American Porcupines *(Erethizon dorsatum)* at the Alaska Zoo

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Abstract and Specific Aims

North American porcupines *(Erethizon dorsatum)* are known to consume high concentrations of chemical toxins, such as terpenes, which are produced by plants. These compounds are abundant in conifer needles and cambium which is commonly consumed by porcupines. Previous studies have found that eucalyptus feeding herbivores such as the koala, great glider, and ringtail possum have efficient oxidative pathways used to eliminate and detoxify terpenes (Boyle, 1999 & Pass, 2001). However, no studies have been performed on porcupines to see if they too have these efficient oxidative pathways for the terpene, α-pinene. In this study, α-pinene will be injected into the food of two porcupines at the Alaska Zoo. Quantitative analysis of the metabolites in the urine will be performed using gas and liquid chromatography. Glucuronic acid will be tested and measured to indicate the presence of additional detoxification pathways. The specific aim of the study is to find out whether increasing the dose of α-pinene will saturate the oxidation. This will determine (1) whether the oxidation pathway is the only pathway, (2) the rate at which the pathway is saturated (its kinetics), and (3) if the porcupine can further conjugate compounds using other pathways (such as conjugation) when the oxidation pathway is saturated. Understanding how porcupines detoxify and eliminate α-pinene will enhance comprehension of porcupine ability to survive on such a nutritionally limited diet. This knowledge can be used to develop further investigations of porcupine detoxification along with other mammals. Future research may be used to develop evidence-based practice and regulation regarding toxins in the environment.

Staff and Administration Contribution

Dr. Don Spalinger will guide me through this project with his expertise in ecological chemistry of plant-herbivore interactions. This research will fulfill the requirements for Dr. Spalinger's herbivore ecology class by studying porcupine digestion. However, this project will provide a more in depth study of terpene detoxifican in porcupines.

Dr. Ann Jache, instructor for the year long capstone project in design, research, analysis, and presentation will oversee the project. Within the capstone class, a more extensive literature review will be performed to obtain a better understanding of ecology and the practical applications of this research.

Alaska Zoo's IACUC chairperson and curator of the animals, Shannon Jensen, has reviewed the project design and will ensure ethical treatment of the porcupines.

Community Involvement

Preliminary research findings will be presented to the zoo staff in December, 2008. After additional analysis, a final report of the project will be given to the zoo staff in April, 2009. An article describing the findings in lay terms will be provided for community education. Information provided to the zoo staff from findings in the study may be important for use as a diet guideline. The zoo will be informed of the levels of α-pinene that the porcupines can tolerate. With this information, the staff can ensure that they will not exceed the α-pinene tolerance of porcupines in their every day diet.

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Plants produce a variety of chemical compounds that are not directly related to the primary metabolism in the plant. Many of these compounds are important in the life history of the plant (such as attractants for pollinators), but many are also used as chemical defenses against herbivores (Gang, 2005). In order for herbivores to eat plants they must be able to detoxify and eliminate these compounds including terpenes. Terpenes are long-chain, volatile carbon compounds that are potentially toxic and highly abundant in plants (Kopsell, 2008). An example of a familiar terpene is turpentine which is commonly used as paint thinner.

Freeland and Janzen (1974) hypothesized that herbivores have mechanisms to detoxify and eliminate chemical compounds, but there is a limit to the amount of chemicals the herbivore can tolerate. Toxins such as terpenes are dose-dependent, but their detoxification in an herbivore can follow several known pathways. As a consequence, herbivores tend to consume a variety of plants with different toxins to avoid toxicity of any one toxin. Freeland and Janzen's study provided insight into the idea that there are detoxifying and eliminating pathways of chemical compounds. These results motivated Boyle, et al. (1999) to compare the pathways between specialist herbivores (consuming one to two plant types) and generalist herbivores (consuming a wide variety of plants).

Boyle, et al. (1999) studied the saturation point of detoxification and excretory pathways of the terpene, p-cymene, in generalist and specialist herbivores. Detoxification pathways of p-cymene metabolites in a generalist herbivore (brushtail possums) were compared with pathways of specialist herbivores (great gliders and ringtail possums). These specialist herbivores consume p-cymene consistently in their specialized eucalyptus diets. The metabolites of generalist herbivores were found to be conjugated (combining of compounds) with glucuronic acid, which aids in making the p-cymene soluble and therefore more readily excreted. The metabolites in specialist herbivores were found to have higher levels of oxidation, which is a process of bonding oxygen to the toxin causing it to be more soluble and more readily excreted. No conjugation was found in the specialist herbivores. Boyle, et al. concluded that specialist herbivores have developed efficient oxdidative pathways to eliminate terpenes. Since one oxygen atom was bound to the metabolites in specialist herbivores, it can be implied that the pathway was not maximized. The generalist herbivore excreted metabolites with 1-3 oxygens attached, and conjugation to glucuronic acid was observed. These results suggest there was an overcapacity in the oxidation pathway resulting in the use of alternative pathways. It is important for specialized herbivores with nutritionally poor diets to have an efficient pathway to digest chemical compounds in order to expend as little energy on digesting toxins as possible.

Pass, et al. (2001) also performed a study comparing the metabolic pathway of the abundant eucalyptus terpene, 1,8-Cineole, of a specialized eucalyptus herbivore (koalas), a generalist herbivore (brushtail possums), in addition to rats and humans. Consistent with Boyle's, et al. (1999) results, Pass et al. (2001) also found that the oxidative pathway efficiency of herbivores follows a decreasing trend. The specialized eucalyptus

herbivore developed the most efficient oxidative pathway, followed by the generalist herbivore, then the rats, ending with the least efficient pathway which was found in the humans. While studies have been performed on specialized eucalyptus herbivores, no studies have been performed on the ability of mammals, such as porcupines, to detoxify high concentrations of terpenes found in cambium and conifer needles, which are common in the porcupine's diet. A diet incorporating mostly conifer needles and cambium has many chemical compounds and is nutritionally poor in energy. Chemical detoxification and elimination has high energy demands, thus it is important to analyze how porcupines budget their energy in order to live on such a nutritionally poor diet.

Porcupines are considered generalist herbivores because they consume various ground plants and leaves in the summer. However, during winter their diet is restricted to conifer foliage and the phloem and cambium of coniferous and deciduous trees (Roze, 1984). Unlike most herbivores, porcupines consume cambium and conifer needles which have high concentrations of chemical compounds including terpene (Bryant, 1991). In this study, α-pinene, a dominate terpene in spruce (Mardarowicz, 2004), will be injected into bread and fed to two porcupines. Urine will be collected and metabolites of α-pinene will be quantified using gas and liquid chromatography, and will be tested for conjugation using a glucuronic acid kit. These results will compare detoxification pathways of porcupines with the generalist and specialist herbivores that consumed p-cymene terpene.

Methods

Two porcupines from the Alaska Zoo will be kept in individual cages and fed a standard diet for two weeks consisting of 250g of broccoli and 200g of a standard pelleted zoo rations daily. On the seventh day, 1ml of α-pinene will be injected into a piece of bread and fed to the porcupines with their standard diet. On the fourteenth day, 2ml of α-pinene will be injected into the bread and fed to the porcupines with the continued standard diet.

A second trial will occur about two months after the first trial using the same procedure. A two month period between the two trials will allow rest and recuperation for the porcupines. If the saturation point of the oxidation pathway is not reached after 2ml of α-pinene, the dose will be increased in the second trial.

Each time the α–pinene is added to the diet, urine samples will be collected for a 48 hour period (Boyle, 1991). In order to separate the urine from the feces and litter, the cages will have a wire mesh floor. The urine collected will be frozen for preservation until analysis.

Quantitative analysis of the terpene metabolites will be obtained using two methods. First, the urine will be filtered and then the metabolites will be derivatized to prepare the samples for gas chromatography on a Varian CP-3800 gas chromatograph coupled to an ion-trap mass spectrometer. Trimethylsilyl (TMS) derivitization will be completed in order to prepare the samples for gas chromatography. Chromatography will be completed using a VFX-MX 30M capillary column with protocols previously developed in Dr. Spalinger's lab. If necessary, a preparative thin-layer chromatography (pTLC) will be performed to clean up the metabolites (Boyle, 1999).

In addition, an HPLC-MS-MS system will be used to perform liquid chromatography. This recently purchased machine will minimize sample preparation, and will eliminate the derivatization process. This machine will be capable of high resolution and superior definition of metabolite structures. However, the system will not be installed into the ASET lab at the University of Alaska Anchorage (UAA) until February, 2009, therefore the urine will not undergo liquid chromatography until that time.

The urine will also be tested for glucuronic acid. A glucuronic acid kit will be used to break the conjugation of glucuronide from the terpene. Measurements of the amount of glucuronic acid produced will then be recorded.

Gradually adding α-pinene into the food and analyzing collected urine creates a relatively risk free study. However, to ensure ethical treatment of the porcupines, the U.S government's principles for the utilization and care of vertebrate animals will be followed. In addition, the student investigator will complete the University of Alaska Anchorage Institutional Animal Care and Use Committee (IACUC) course.

Anticipated Results:

The α-pinene metabolites in the urine could vary in the number of oxygen atoms attached, signifying the degree of oxidation. One prediction is that the metabolites will be conjugated with glucuronic acid as Boyle, et al. (1999) found. Another possibility is that the compounds may or may not be conjugated, and porcupines may use a different conjugation pathway.

Since porcupines are known to consume high concentrations of terpenes, it is likely that similar results to the specialized herbivores in Boyle, et al. study will occur by having one or more oxygen atoms attached, and no conjugation. However, these porcupines have been raised in the zoo since infancy and have not consumed much terpene in their diets. This may result in a less efficient oxidative pathway causing multiple oxygen atoms and alternative pathways through conjugation. Nonetheless, this study differs from Boyle's research because α-pinene is being used instead of p-cymene and different animals are being tested. This may cause different and unexpected results to occur.

Overall, the important aspects of the study design is to increase the doses of α-pinene and see if oxidation can be saturated. This will determine whether the oxidation pathway is the only pathway used, the rate at which the pathway is saturated (its kinetics), and whether the porcupine can further conjugate compounds using other pathways (such as conjugation) when the oxidation pathway is saturated.

This research builds on the work of Freeland and Janzen (1974), Boyle, et al. (1999), and Pass, et al. (2001), and paves the way for further research on porcupine detoxification along with other mammals. It is further hoped that this basic research about how toxins are processed by mammals contributes to an incipient body of research necessary to produce evidence-based practice and regulation regarding toxins in the environment.

Project Budget:

To complete this study the following projected budget is necessary:

α-pinene 8 doses total~40ml	$40
Brocolli 4 weeks of broccoli~14000g	$100
Pellets 4 weeks of pellets~11200g	funded by zoo
Extractions 30 extractions~($2 ea)	$60
Derivitizing Chemicals	$300
Gas Chromatography 30 to develop methodology~($4 ea) 30 to test~($4 ea)	$480
Liquid Chromatography HPLC mass spec 30 to develop methodology~($4 ea) 30 to test~($4 ea)	$480
Solvents	$60
Tubes	$30
Silica Gel Columns	$100
Glucuronic acid assay kits	$100
Poster	$130
Total	*$1880*

References:

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Project Timeline

Following is a projected timeline for this experiment:

October-November 2008:	Porcupines will be fed standard pellets and broccoli. Terpene will be injected into the diets. Urine will be collected for 48 hours after each terpene injection.
November-December 2008:	The urine will be filtered, the metabolites will be derivitized, and gas chromatography will be performed.
December 2008:	Current findings will be presented to the zoo staff.
January-February 2009:	Porcupines will be fed terpene injected diets. The amount of terpene may be increased from the October/November dose in order to saturate oxidation. The urine will be collected for 48 hours after each terpene injection.
February-March 2009:	Urine from the October/November samples and the January/February samples will be filtered and will undergo liquid chromatography. Glucuronic acid will also be tested and measured.
April 2009:	Findings and diet advice will be given to the zoo along with an article for the zoo's public bulletin.
Mid-April, 2009:	Presentation at the Undergraduate Research Symposium and Bachelor of Liberal Studies (BLS) capstone presentation.
June 15, 2009:	Final written report deadline.

UAA UNIVERSITY of ALASKA ANCHORAGE

College of Arts and Sciences
Department of Biological Sciences

October 22, 2008

Undergraduate Research Evaluation Committee
University of Alaska Anchorage

Dear Committee Members,

I am writing this letter of support for Ms Rachael Lehmkuhl's proposal for undergraduate research on metabolism of plant toxins in the North American Porcupine. In particular, I was informed that she requires a letter of support from the Alaska Zoo to perform the studies we propose. Although there is insufficient time to get such a letter from the zoo to meet the deadlines for this proposal, I can assure the committee that the Zoo has agreed to the research, has approved the research protocol through its IACUC committee (as has UAA's IACUC Committee), and is happy to work with us on the project. In fact, my Biol. 445 (Herbivore Ecology) Class has been doing this research for the past 3 weeks.

The research is of high value, not simply for understanding how porcupines deal with plant toxins, but they are an ideal model animal for potentially understanding detoxification mechanisms in many other herbivores. In addition, the ASET lab at UAA has recently received an NSF grant to obtain a state-of-the-art HPLC-MS-MS system that can efficiently analyze and characterize the metabolites that we anticipate to be produced. Hence, Rachael's project might be one of the first applications to be run on this new machine.

In summary, Rachael has the interest and the drive to succeed in this project, she is supported by both my lab and the Alaska Zoo, and we have state-of-the-art equipment to insure its success. I recommend the proposal highly.

Sincerely,

Dr. Donald E. Spalinger

Associate Professor of Biological Sciences

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