



Warren J. Baker Endowment

for Excellence in Project-Based Learning

Robert D. Koob Endowment *for Student Success*

PROPOSAL NARRATIVE

I. Project Title

Factors influencing the hindgut characteristics of the leopard tortoise (*Stigmochelys pardalis*):
Survey of the microbiome

II. Abstract

Anatomical and physiological hindgut adaptations of multiple vertebrate species allow colonization of a diverse ecosystem of symbiotic microorganisms. Microbial enzymatic capabilities allow utilization of dietary components (e.g., cellulose, hemicellulose) otherwise indigestible by the host animal, as well as components that escape endogenous digestive processes in the proximal gastrointestinal tract. Microbial ecosystem demographics and fermentation endproduct production are influenced by the substrates entering the hindgut. Total bacterial counts of 10^{10} /g, as well as protozoa, are measured in reptiles with hindgut fermentation; however, bacterial types and their relative distribution are not clearly described. In conjunction with a broader study examining the influence of fiber particle size on hindgut fermentation characteristics in tortoises, the objective of this proposal is to characterize the gastrointestinal organisms of leopard tortoises offered one of three diets: 1) control diet consisting of only commercially available tortoise food, 2) control diet formula with added cellulose fiber of 2.0 mm length, or 3) control diet formula with added cellulose fiber of 200 μ m length. Microbial DNA will be extracted and sequenced from fecal samples collected from 16 adult female leopard tortoises housed at California Polytechnic State University. Microbial genera analysis and identification will be accomplished by comparing raw sequencing data to published literature and databases. This baseline information will provide a comparative reference for microbiome changes related to dietary management strategies (e.g., varying levels of starch, novel sources of fermentable fiber). These techniques are transferable to other hindgut fermenting reptile species, as well as birds and mammals.

III. Introduction

Fiber is nutritionally defined as an indigestible or slowly digestible feed fraction that occupies space in the digestive tract (Hintz et al., 1997). Despite this seemingly straightforward description, the variability, complexity and unique physiological significance of plant fiber have not only contributed to the difficulty of developing appropriate analytical procedures to measure fiber but also estimating species fiber requirements (NRC, 2003). Hindgut-fermenters rely on microbial digestion in the large intestine to digest otherwise indigestible fiber components that are inevitable with a plant-based diet. Gastrointestinal (GI) microbes classified as bacteria, protozoa, and fungi inhabiting the hindguts of animals consuming high fiber diets aid in fiber digestion by producing digestive enzymes the host animal is incapable of producing itself. Sacculations and mucosal folds in the large intestine allow for greater surface area and contraction to propel larger fibers back toward the cecum for additional microbial fermentation (Sakaguchi, 2003; Stevens and Hume, 1998). This results in long retention time of several hours to several days, increasing the amount of exposure time between fiber particles and microbes (Bjorndal et al., 1990).

Microbiota and microflora reference microbe colonies that inhabit a host niche, while microbiome references the genes of those individual microbes (Hooper et al, 2002). Fiber fermentation by GI microbes relies on symbiosis; the host must maintain a suitable environment for microbes to perform fermentation, while microbes aid in the development of GI tissue and provide the host with energy substrates such as short chain fatty acids (SCFA), which are also a growth substrate for the microbes themselves (Kostic et al., 2013; Leser and Mølbak, 2009). Mammalian microbe densities have been characterized as “relatively low” in the proximal and middle small intestine, but drastically increase in the distal small intestine and colon, indicating microbial contribution to digestion (Hooper et al., 2002). Identification of beneficial microbes across 60 mammalian species varying in digestive strategy (i.e. foregut-fermenting, hindgut-fermenting, carnivores, omnivores), including humans revealed distinct bacterial colonies between foregut and hindgut-fermenters, as well as nonruminant and hindgut-fermenting omnivores (Ley et al., 2008). These results illustrate the relationship of both gastrointestinal tract morphology and diet with microbiome diversity and demographics.

In conjunction with a broader study examining the influence of fiber particle size on hindgut fermentation characteristics in tortoises, the Animal Science and Biology departments propose to collaborate in order to profile the previously undescribed microbial communities of the hindgut of leopard tortoises using 515F primers (Ion Torrent PGM). This baseline will provide further insight into the adaptations of these specialized herbivores, as well as a reference from which changes in the microbiome resulting from various dietary effects may be assessed. These microbiome changes may result in digestibility changes, which could affect nutrient utilization by the animal, compromising health.

IV. Objective(s)

- 1) Identify and compare bacterial community types and densities in the hindgut of captive leopard tortoises fed one of three diet types, exclusively (no added cellulose fiber (control), 2.0 mm length added cellulose fiber, 200 µm length added cellulose fiber).
 - a. Bacterial community identification is only possible through use of DNA sequencing equipment not available on the Cal Poly campus. Funding will make this first objective possible. Without completion of this first objective, further action cannot occur.
 - b. This objective will be co-funded by the East Texas Herpetological Society (please see budget).
- 2) Prepare comparative reference of captive leopard tortoise hindgut bacterial communities based on diet type. This reference may aid in captive herbivorous tortoise diet management, based on hindgut health.
 - a. Funding for the first objective will make the second objective achievable.

V. Methodology

Experimental Design

Microbial DNA will be extracted and sequenced from fecal samples collected from 16 adult female leopard tortoises housed at California Polytechnic State University and stored at -70°C. DNA extraction will be performed using a MO BIO Powersoil® DNA Isolation Kit. This kit is

specifically designed for use with environmental samples including soil and feces. This process requires a 250 mg fecal sample run through the following series of solutions:

- 1) PowerBead Tube containing lysis beads and solution to mechanically disrupt the sample,
- 2) solution C1 performs cell lysis, 3) solution C2 precipitates non-DNA components, 4) solution C3 precipitates non-DNA components, 5) solution C4 binds DNA to the spin filter, 6) solution C5 washes DNA, 7) solution C6 elutes DNA, 8) DNA transferred to silica spin filter to bind DNA, and 9) DNA transferred to final collection tube for send out to laboratory for sequencing (MO BIO Laboratories, 2014). Vortexing, centrifugation, incubation, and supernatant collection and transfer occur between steps.

Methods of Analysis

DNA will be sent to the Mr. DNA® Next Generation Sequencing and Bioinformatics Laboratory in Shallowater, Texas for sequencing. An Ion Torrent™ Personal Genome Machine® (PGM) System using a 515F primer will be used to identify and enumerate bacterial and archaea genera. DNA reads (raw sequencing data) will be returned to investigators for genera analysis and identification to be determined using published literature and computer programs, including GenBank®.

VI. Timeline

2016 Winter DNA extraction

Spring DNA sequencing, Results analysis, Manuscript preparation/dissemination

VII. Final Products and Dissemination

Upon completion of this research, data will be presented and published in association with the following activities:

Events/Industry Meetings

- California Animal Nutrition Conference

Publications

- California Polytechnic State Univ., Animal Science Dept. Stock Report newsletter
- Refereed journals, including: Journal of Zoo and Wildlife Medicine, Zoo Biology

Presentations

- California Polytechnic State University Animal Science curriculum
- American Zoo and Aquarium Association Nutrition Advisory Group

VIII. Budget Justification

MO BIO Powersoil® DNA Isolation (Extraction) Kit: DNA Isolation kit is necessary to extract DNA from 16 fecal samples for laboratory microbial analysis. Solutions and tubes cannot be reused; therefore a kit with the capacity to perform the necessary amount of DNA extractions and allowing for human error is required.

515F Primer PGM Bacterial & Archaea Assay (DNA Sequencing): Microbial analysis of 16 samples following DNA extraction requires equipment and supplies available through a bioinformatics and sequencing service laboratory.

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PROPOSAL BUDGET

Student Applicant(s): Breanna P. Modica	
Faculty Advisor: Dr. Mark S. Edwards	
Project Title: Factors influencing the hindgut characteristics of the leopard tortoise (<i>Stigmochelys pardalis</i>): Survey of the microbiome	Requested Endowment Funding
Travel <i>subtotal</i>	\$
Travel: In-state	\$
Travel: Out-of-state	\$
Travel: International	\$
Operating Expenses <i>subtotal</i>	\$343.00
Non-computer Supplies & Materials	\$263.00 (DNA Isolation Kit)
Computer Supplies & Materials	\$
Software/Software Licenses	\$
Printing/Duplication	\$
Postage/Shipping	\$80.00
Registration	\$
Membership Dues & Subscriptions	\$
Multimedia Services	\$
Advertising	\$
Journal Publication Costs	\$
Contractual Services <i>subtotal</i>	\$710.00
Contracted Services	\$960.00 (DNA Sequencing) - \$250.00 (ETHS Grant) \$710.00
Equipment Rental/Lease Agreements	\$
Service/Maintenance Agreements	\$
TOTAL	\$1053.00

CAL POLY

Animal Science Department
1 Grand Avenue, 010-0147
San Luis Obispo, CA 93407-0255

01-Feb-2015

Michael D. Miller, PhD
Baker and Koob Endowments Coordinator
Executive Vice President for Academic Affairs
California Polytechnic State University
1 Grand Avenue
San Luis Obispo, CA 93407

Dr. Miller:

This recommendation is on behalf of **Breanna Modica** and her proposal submitted for the annual **Baker and Koob Endowments** from **California Polytechnic State University**. I have had the personal privilege of working with Breanna, as her academic advisor, instructor and supervisor, during her undergraduate studies in Animal Science and her graduate committee chair at California Polytechnic State University. Ms. Modica is an exceptional student. A transfer student into our program, she successfully completed our rigorous undergraduate science curriculum with 3.389/4.0 GPA. Additionally, her academic performance was recognized twice on the college Dean's List. Upon completion of the first year of her graduate level courses, she currently maintains a 3.568/4.0 GPA.

Ms. Modica's thesis research is related to the influence of fiber length in leopard tortoises, our research model for grazing herbivorous reptiles with adaptations for postgastric, microbial fermentation. This is an animal health issue of particular interest, as herbivorous reptiles maintained in managed environments are often fed diets lower in fiber than natural diets and/or diets with highly processed fiber particles that may not result in similar fermentation patterns in the hindgut.

As Breanna prepared her review of relevant literature, as well as participating in an international Comparative Animal Nutrition symposium, she began to consider how the microbiome of the gastrointestinal tract may be influenced by dietary substrates differing in fiber length, but not nutrient content. She proactively sought collaborators on-campus across colleges that could advise her in appropriate methodology, as well as data analysis and interpretation. As of this date, all of the necessary samples have been collected and are available for immediate analysis as resources allow.

Breanna identified, applied for and successfully secured partial funding for this phase of her work through a competitive **Herpetological Grant** generously offered by **East Texas Herpetological Society**. The support requested from the **Baker and Koob Endowments** represents the necessary balance to complete sample and data analysis.

Breanna is coordinating all aspects of this project, including formulation and manufacturing of test diets, management of animals, coordinating undergraduate labor, and developing analytical procedures. Quite frankly, without her efforts, our current scholarly work on fiber utilization in herbivorous animals, and the broad undergraduate involvement associated with that work, would not occur. She demonstrates creative problem-solving skills and resourcefulness to accomplish the work required, often with little or no direction from myself.

Simply, Breanna continues to exceed my expectations, not only in the amount of work and impact she has with our students, but the exceptional quality of that work and those interactions. She demonstrates a level of maturity not observed among her peers, which I believe is exemplified by her application for this grant.

In a short time, she has become an important asset to our department and undergraduate students. I am committed to investing in Ms. Modica as a part of her preparation for a career in comparative animal nutrition, and I would strongly advocate her work is worthy of your investment as well.

I am confident Breanna would not only greatly benefit from your support, but she would represent the award with a positive attitude and personal responsibility. **Breanna has earned my highest recommendation.** If you have any additional questions or would like to discuss Breanna further, please contact me at mstedward@calpoly.edu or 805.756.2599.

Sincerely,

A handwritten signature in blue ink that reads "Mark S. Edwards". The signature is written in a cursive style with a large, stylized initial "M".

Mark S. Edwards, Ph.D.
Professor, Comparative Animal Nutrition
Associate Department Head