Warren J. Baker Endowment
for Excellence in Project-Based Learning
Robert D. Koob Endowment *for Student Success*

CAL POLY

Proposal Cover Page

Title of Project:
Influences of fiber length on nutrient digestion and transit time in leopard tortoises

Proposal Author: Allison Mullin **Cal Poly Email:** agmullin@calpoly.edu

Student ID: 007309764 **Dept.:** Animal Science

Signature (Optional): Allison J Mullin
Signature provides permission to check financial aid eligibility.

Previous Baker/Koob Endowment funding? (circle one): Yes (No)

Is this request to support a Senior Project or thesis? (circle one): (Yes) No

Team Member(s)	Signature	Cal Poly Email	Department

Anticipated Start Date: Nov 2018 **Anticipated End Date:** December 2018

Total Funds Requested: $ 1050

Faculty Advisor: MARK S. EDWARDS **Department:** ANIMAL SCIENCE

Faculty Advisor email: msedward@calpoly.edu **Telephone:** 805-756-2599

Signature of Faculty Advisor: Mark S. Edwards **Date:** 05 NOV 18

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PROPOSAL NARRATIVE

Project Title: Influences of fiber length on nutrient digestion in leopard tortoises (*Stigmochelys pardalis*)

I. Abstract

Vertebrate animals lack endogenous enzymes to break down insoluble plant fiber, and therefore are dependent on symbiotic gut microbes to utilize this abundant food source. Although chemically higher in fiber, the efficacy of fiber particles in some commercial feeds, physically reduced in size during processing, has not been quantified. A concept developed for dairy cattle, physically effective fiber (peNDF) considers fiber physical characteristics that influence mastication and the biphasic nature of rumen contents. The importance of describing fiber's chemical and physical effects in nonruminant herbivores has also been identified. Tortoises provide unique insights into digestive processes influencing fiber particle length, because unlike mammalian herbivores, they do not reduce food particle size via mastication. We offered 16 leopard tortoises a diet supplemented with Miscanthus grass differing in physical length (1.18 – 4 mm and 8 – 19 mm). Individuals were allocated to treatment based on body weight. Total food intake and fecal output will be quantified. Particulate and solute digesta transit will be measured with single pulse markers. Diet digestibility will be determined indirectly using acid-insoluble ash. Fecal particle size will be quantified via wet sieving. Gut microbiota will be determined via 16S rRNA gene sequencing. Treatment differences will be analyzed by one-way ANOVA. We predict the increasing physical fiber length consumed will result in improved digestibility via increased digesta retention and microbial degradation with no differences in fecal fiber particle size or bacterial taxonomic distribution. Building on previous results, this is a systematic step toward characterizing the peNDF requirements of herbivorous tortoises and other nonruminant herbivores.

II. Introduction

Tortoises provide unique insights into digestive processes that influence fiber digestion, because unlike mammalian herbivores, they do not reduce food particle size via chewing. Under captive conditions, the mean fecal particle size for leopard tortoises (24.18 mm) was at least five times greater than larger, mammalian hindgut fermenters (African elephants, 4.98 mm; domestic horses, 1.12 mm) (Fritz et al., 2010; Clauss et al., 2015). Vegetative plant parts, high in dietary fiber, contribute over 50% of herbivorous animals' annual food intake (Miljutin, 2009).

It has been suggested that nutritionally balanced tortoise feeds be avoided, as little is known about their suitability for these species (McArthur and Barrows, 2004). In response, 'higher fiber' commercial tortoise diets have been introduced. Although higher in fiber concentration, the efficacy of fiber particles in commercial feeds, physically reduced in size during processing, has not been quantified. Previous work shows that extruded diets with

differing fiber types, amounts and sizes have significant effects on digestibility of dry matter, organic matter and energy ($P < 0.0001$) by leopard tortoises (Maxwell et al., In Preparation).

Microbes and vertebrates co-evolved, establishing a persisting symbiotic relationship exemplified in herbivorous animals (Kostic et al, 2013; Ley et al, 2008). Dietary fiber occupies gastrointestinal space and is resistant to digestion by enzymes produced by the host, but may be rapidly or slowly fermented by gastrointestinal microbes (Van Soest, 1994; Hintz et al., 1996). As microbes ferment complex polysaccharides the resulting in short chain fatty acids become available for host absorption. Thus, the colonization of gastrointestinal microbes was essential for evolution towards an herbivorous diet.

"Small" fiber particle sizes have yet to be defined and "longer" fiber may be necessary for non-ruminant herbivores as a means of increasing digesta transit time in the gut, allowing more time for microbial fermentation, digestion, and absorption. Within herbivorous, hindgut-fermenting reptiles, fecal microbiota is dominated by the phylum Firmicutes and Bacteriodetes which are highly cellulolytic groups. Clostridia was identified as approximately 50% of the represented Firmicutes in all free-ranging iguana species and the Aldabra tortoise (Hong et al., 2011), and 97% of the represented Firmicutes in free-ranging gopher tortoises (Yuan et al., 2015). Previously supported research has shown shifts of gastrointestinal microbiota diversity with Clostridia accounting for approximately 20% of represented Firmicutes across isofibrous diets (Modica, 2016).

An objective of this study is to characterize the fecal microbiome of leopard tortoises fed one of three nutritionally complete, herbivorous tortoise diets that varied in only physical particle length. **This work builds on these findings, controlling for variable diet composition and measuring of digesta passage and retention.** We hypothesize that increasing physical fiber length of a consumed forage will result in improved digestibility via increased digesta retention and microbial degradation with no differences in fecal fiber particle size or bacterial taxonomic diversity.

The proposed study utilizes a high level of graduate and undergraduate involvement which will significantly contribute to the future of science-based, comparative animal nutrition through their training, education and participation.

III. Objectives

a. Evaluate the digestibility and suitability of forages as all the diet fed to herbivorous tortoises.
b. Investigate digesta retention time as it relates to microbial fermentation of structural carbohydrates through mordant markers such as Chromium.
c. Interpret changes in bacterial hindgut colonization through 16sRNA sequencing as it relates to diet type

IV. Methodology

Sixteen subadult leopard tortoises (*Stigomchelys pardalis*) will be individually fed one of three Bermudagrass hay diets differing only in physical length. Animal mass and physical measurements are documented weekly. Tortoises will be fed weighed quantities to satisfy 50% of field metabolic rate (FMR, kJ ME/d) (Nagy et al., 1999) based on body weight at the beginning of the study. Water is available ad libitum. The animals are housed individually in an open-air building with regular access to supplemental heat and shade (Higgins and Edwards, 2009). All daily care and maintenance of the tortoises will be performed by students enrolled in

the Reptile Husbandry Enterprise (ASCI-290), a longstanding, year-round 'Learn by Doing' opportunity for students of all skillsets to become involved in industry supported research.

Animals will be gradually transitioned over 14 d during which the standard diet is incrementally replaced with one of three experimental diets. Following will be an acclimation period (42 d) and total collection period (100 d). Each tortoise will be fitted with total fecal collection harnesses that allow for collection of uncontaminated fecal samples. Individual fecal samples will be analyzed via ICP spectrophotometry to determine digesta marker concentrations excreted. Fecal samples will also be searched for acetate bead excretion. Frozen composites will be subsampled for determination of fecal pH, particle size and microbiome. Fecal particle size distribution will be gravimetrically quantified via wet sieving using a vibratory sieve shaker (Restch® AS300 Control). DNA will be isolated from fresh fecal samples using the MO BIO Powersoil DNA Isolation Kit (12888-50). Isolated DNA will be shipped submitted to a commercial lab for analysis of the 16s rRNA gene to determine bacterial species distribution. The remaining composite sample will be analyzed for dry matter, gross energy, TDF, NDF, ADF, acid detergent lignin, crude protein, ash and acid insoluble ash. Digestive efficiencies will be calculated using the AIA marker method (Van Keulen and Young, 1977; Maxwell et al., In Preparation). Students from the Nutrition Research Enterprise (ASCI-490) will contribute to lab analysis. The Nutrition Research Enterprise allows undergraduate students to participate in industry supported lab analysis and nutritional research.

V. Timeline

2018	Winter	Protocol development; IACUC review
	Spring	Undergraduate intern selection; diet preparation
	Summer	Animal trials
	Fall	Animal trials, marker administration
2019	Winter	Data analysis
Spring		Manuscript preparation

I. Final Products and Dissemination

Preliminary, as well as final results will be presented and published in association with: California Animal Nutrition Conference, Comparative Nutrition Society, Animal Science Department Stock Report newsletter (Cal Poly SLO), refereed journals (Journal of Zoo and Wildlife Medicine, Zoo Biology), California Polytechnic State University Animal Science curriculum, American Zoo and Aquarium Association (Nutrition Advisory Group)

II. Budget Justification

The funds requested will contribute directly to sample collection (100 d) for 16 leopard tortoises and pulse marker dosage for these animals.

Consumable supplies necessary for collection and marker administration include:

- *Medium and large nitrile gloves, sample collection (freezer) bags, Velcro tape (for attaching harnesses to carapace), 1600 fluorescent acetate marker beads ($50 for 4500 pieces), and digesta marker preparation and analysis (Chromic oxide reagents, acetic acid, and laboratory analysis for Chromium content).*

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PROPOSAL BUDGET

Student Applicant(s):	Allison G. Mullin
Faculty Advisor:	Mark Edwards, PhD
Project Title:	**Requested Endowment Funding**
Travel *subtotal*	**$**
Travel: In-state	**$**
Travel: Out-of-state	**$**
Travel: International	**$**
Operating Expenses *subtotal*	**$**
Non-computer Supplies & Materials	**$ 900, gloves, acetate beads, mordanting reagents, PPE, sample collection materials**
Computer Supplies & Materials	**$**
Software/Software Licenses	**$**
Printing/Duplication	**$**
Postage/Shipping	**$ 50**
Registration	**$**
Membership Dues & Subscriptions	**$**
Multimedia Services	**$**
Advertising	**$**
Journal Publication Costs	**$**
Contractual Services *subtotal*	**$**
Contracted Services	**$ 100, Cr analysis outside laboratory**
Equipment Rental/Lease Agreements	**$**
Service/Maintenance Agreements	**$**
TOTAL	**$ 1050**

Appendix A.

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05-Nov-2018

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 **Allison Mullin** 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 Allison, 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. Mullin is an exceptional student. She successfully completed our rigorous undergraduate science curriculum with 3.329/4.0 GPA. Additionally, her academic performance was recognized three times on the college Dean's List.

Ms. Mullin'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 work builds on the results of a previous study, controlling for diet variability and quantifying the movement of digesta through the animal's gastrointestinal tract. Dietary fiber is an animal health issue of particular interest, as herbivorous reptiles maintained in managed environments are often fed low fiber diets and/or diets with highly processed fiber particles that may not result in similar fermentation patterns in the hindgut.

As Allison prepared her review of relevant literature, as well as participating in an international Comparative Animal Nutrition symposium, she has come to appreciate 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.

Allison has identified additional funding sources and contributed to those grant applications. The support requested from the **Baker and Koob Endowments** represents a discrete portion of the project related to chemical analysis of markers used to quantify passage of food through the animal's digestive system.

Allison is coordinating all aspects of this project, including producing the test diets, management of animals, coordinating undergraduate labor, and developing novel 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, Allison 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. Mullin 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 Allison would not only greatly benefit from your support, but she would represent the award with a positive attitude and personal responsibility. **Allison has earned my highest recommendation.** If you have any additional questions or would like to discuss Allison further, please contact me at msedward@calpoly.edu or 805.756.2599.

Sincerely,

Mark S. Edwards, Ph.D.
Professor, Comparative Animal Nutrition