IT TAKES GUTS TO GO GREEN:

IDENTIFYING THE FACTORS THAT LIMIT THE EVOLUTION OF HERBIVORY IN REPTILES

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Abstract

The evolution of herbivory in reptiles is rare and poorly understood. Researchers have speculated why some lineages of lizards have undergone evolutionary shifts from omnivory to herbivory, but no one has studied the mechanistic basis of this form of diet evolution. I will test whether induced shifts in diet (i.e., omnivory to herbivory) adhere to the popular paradigm that form-follows-function in evolution. This hypothesis predicts that major evolutionary changes occur in a predictable pattern: behavior evolving first (e.g., recognizing plants as food), followed by changes in physiology (e.g., gut enzyme activity), and finally morphology (e.g., gut size and proportion). To test this hypothesis on a proximate level, I will compare several physiological and morphological characteristics of the alimentary tracts of omnivorous lizards (*Pogona vitticeps*) raised on one of three diets (carnivore, omnivore, or herbivore). The digestive tract is the focus of this investigation because, unlike other traits associated with specific diets (e.g., teeth), the gut exhibits both flexibility and plasticity in response to changes in diet, thus providing the necessary variation upon which selection could act. I will measure rates of growth, digestive efficiency, and passage rate as whole-organism measures of digestive performance. Gut enzyme levels and nutrient transport rates will provide measures of tissue-level function. Gross and histological measures of gut anatomy will quantify morphological plasticity. My study should elucidate whether physiological and/or morphological characteristics preclude this omnivore from exploiting a strictly herbivorous diet. This is the first study to attempt to elucidate the mechanisms constraining the evolution of herbivory from omnivory, while simultaneously testing the paradigm that major evolutionary shifts evolve in a form-follows-function manner.

Background

Much of the world's organic carbon is sequestered in plant cellulose. This complex carbohydrate offers an abundant source of energy for organisms that can exploit it. Although a diet of plants is common for some vertebrate groups (e.g., mammals), few reptiles have evolved a diet of leafy plant material (herbivory). In fact, less than 1% of extant reptiles eat plants as their primary source of energy and nutrients (Espinoza 2002). The paucity of strictly herbivorous reptiles is a biological enigma that has been the focus of theoretical and empirical study for over 40 years. Early explanations of the scarcity of herbivorous reptiles assumed a lack of specialization. Arguments were made that reptiles lacked some of the fundamental characteristics of more well-known herbivorous vertebrate (e.g., the ability to chew and constant, high body temperatures), and were thus less well suited for exploiting plants (Szarski 1962, Ostrum 1963, Sokol 1967). Subsequent research, however, identified morphological and physiological specializations in herbivorous reptiles and found them to be quite capable of extracting energy and nutrients from plants (Nagy 1977, Bjorndal 1979, Ruppert 1980, Throckmorton 1980, Iverson 1982, Troyer 1984, Karasov and Diamond 1988, Herrel et al. 2004). Nevertheless, with over 40 years of study, the paucity of herbivorous reptiles has yet to be explained.

Although strict herbivory, especially folivory (leaf eating), is exceedingly rare in reptiles, omnivory is more common (Cooper and Vitt 2002, Espinoza 2002). This suggests that there are one or more limitations precluding omnivores from making the evolutionary transition to herbivory (Iverson 1982, Espinoza 2002, Herrel, et al. 2004). At proximate levels, studies suggest that the limits imposed on the gastrointestinal tract by physiology and/or morphology may preclude some reptiles from adopting a strictly herbivorous diet (Ruppert 1980, Espinoza 2002, Herrel et al. 2004). Likewise, comparative studies have shown that the alimentary tracts of

herbivorous reptiles differ significantly from those of nonherbivores in both physiology and morphology (Guard 1980, Dearing 1993, Espinoza 2002, Durtsche 2004). These studies suggest that if a nonherbivorous reptile was to adopt an herbivorous diet (a proximate diet shift), its ability to do so "successfully" would most likely be dependent on its ability to exhibit physiological and morphological plasticity in its gastrointestinal tract. This begs the question Are there unique adaptations of the gastrointestinal tract not found in omnivores that preclude them from evolving strict herbivory (Espinoza 2002)?

Specializations for Herbivory—Although amply available, most plant tissues (especially leaves) are less energy and nutrient rich than animal tissues (Zimmerman and Tracy 1989). Additionally, much of the energy in plant tissues is bound up in inaccessible fiber (i.e., hemicellulose, cellulose, and lignin) or behind cell walls composed of these carbohydrates. Moreover, vertebrates lack the endogenous enzyme cellulase that hydrolyzes the β-1,4 linked residues that form the framework of this fiber (McBee and McBee 1982, Bjorndal et al. 1991, Troyer 1991, Bjorndal 1997, Mackie et al. 2004). Consequently, selection has acted on the alimentary tracts of herbivorous reptiles to increase their ability to exploit this low-quality diet. The guts of herbivorous reptiles have evolved to increase (1) the capacity to hold more plant matter, (2) the absorptive surface area available for the uptake of nutrients, and (3) the space available for the fermentation of fiber by gut endosymbionts (Iverson 1982, Dearing 1993, Stevens and Hume 1995, Espinoza 2002).

Generally, a large gut increases the time that digesta remains in the digestive tract, which tends to promote more thorough digestion (Sibly 1981). Herbivores have relatively larger, more voluminous guts, specifically longer small intestines and more capacious hindguts relative to

nonherbivores (Guard 1980, Iverson 1980, 1982, Bjorndal 1985, Dearing 1993, Stevens and Hume 1995, King 1996, Espinoza 2002, Herrel et al. 2004). A long small intestine increases the uptake of cell solubles released (in part) by mechanical breakdown in the oral cavity and chemical digestion of the "fleshier" non-cellulose portions of plant fiber. However, because most of the energy found in plants is locked within the cell walls, further processing via fermentation in the hindgut must take place before an herbivore can use the potential energy stored within (Bjorndal 1979, Iverson 1982, McBee and McBee 1982, Foley et al. 1987, King 1996, Mackie et al. 2004).

The structure of the large intestine has been documented in a relatively small number of squamate reptiles (primarily herbivores in the lineages Agamidae, Iguanidae, and Scincidae) and some chelonians (Bjorndal 1979, Iverson 1980, Bjorndal 1985, Espinoza 2002, Herrel et al. 2004). These studies have found an overall increase in the size and proportion of the hindgut, which increases the area available for absorption (Iverson 1980, 1982, Mackie et al. 2004). Additionally, some squamate taxa (e.g., iguanids, *Uromastyx*, and *Corucia*; Iverson 1980, 1982) possess transverse folds and valves in their colons that are presumed to slow the passage rates of food and retain cellulolytic gut symbionts (Iverson 1980, 1982, Bjorndal 1997, Mackie et al. 2004). Many herbivorous reptiles also possess a cecum, a blind pouch at the proximal hindgut, which increases the area of the fermentation chamber for the processing of plant fiber, which is accomplished by cellulolytic gut microbes. The products of fermentation are volatile fatty acids (VFAs), which are passively transported across the lumen of the hindgut. These energy-rich molecules (e.g., acetic, butyric, and propionic acid) can provide a substantial portion of an herbivores daily metabolic requirements (McBee and McBee 1982, Foley et al. 1987, Bjorndal and Bolton 1990).

The vertebrate digestive tract exhibits considerable plasticity and flexibility (sensu Piersma and Drent 2003) in morphology and physiology when presented with changes in diet or subjected to cycles of feeding and fasting (Levey and Karasov 1989, Sabot et al. 1998, Secor and Diamond 1998, 2000, Lepczyk et al. 1999, Levey et al. 1999, Starck and Beese 2001, Overgaard et al. 2002, Gugliemo and Williams 2003, Pierce and McWilliams 2004). This ability to adjust to novel diets or changes in food availability may aid individuals in proximate shifts to new diets (e.g., herbivory) by increasing gut capacity, increasing the time a bolus spends in the digestive tract, increasing enzyme activity levels, and increasing nutrient transport rates (Bjorndal 1997, Secor and Diamond 1998, 2000, Witmer and Martínez del Rio 2001). Although remarkably plastic as well as flexible in its ability to adjust to new foods, the question still remains: Could gut function and/or structure limit the evolution of herbivory in some lineages? To determine which of these changes are critical to adopting or precluding herbivory at ultimate scales, a better understanding of how changes in diet affect gut form and function at proximate scales is needed. Given this knowledge we can begin to trace the steps of diet evolution: Which trait(s) must evolve prior to eating primarily plants? Which traits are caused by a shift to herbivory? and Which changes evolve concurrently with a switch to herbivory?

How does Herbivory Evolve?—To understand how diet specialization evolves (i.e., herbivory), it is beneficial to examine how major evolutionary shifts generally occur. One paradigm suggests that major evolutionary shifts, such as changes in diet (i.e., omnivory ⇶ herbivory), evolve in a specific sequence: first behavior, followed by physiology, and finally morphology (reviewed by Huey et al. 2003). The ability of individuals and lineages to adopt and subsequently evolve an herbivorous diet may be limited by any of these three factors.

Behavior is widely considered to be a major evolutionary driving force (Mayr 1960, 1970, 1974, Wyles et al. 1983, West-Eberhard 1989, 2003, Wcislo 1989, Emerson and Koehl 1990, de Queiroz and Wimberger 1993, McPeek 1995, Huey et al. 2003). This is thought to be true for several reasons. First, behavior is extremely labile, so a shift in behavior can introduce new selective pressures on physiology, morphology, and ecology (Mayr, 1960, 1970, 1974, but see Huey et al. 2003). Although behavior can introduce an organism to novel environments and foods, the ability to use these novelties is dependent on the organism's ability to thrive in or subsist on those novelties.

If we could observe a shift from omnivory to herbivory over evolutionary time, we would expect to find specific changes in the digestive biology of individuals undergoing the shift. For example, if an individual or lineage acquires a behavioral tendency or a mutation that increases its dietary niche breadth (e.g., insectivores that start eating plant matter), if advantageous, selection should favor concomitant adaptations, or plastic responses (reaction norms), that enhance procuring, digesting, and assimilating this new food resource. Naturally, such an increase in diet breadth would be accompanied by a number of new physiological challenges (e.g., detoxifying secondary compounds, processing complex carbohydrates like cellulose, etc.). Consequently, to accommodate a shift to eating plants from insectivory adjustments in relative enzyme activity levels and rates of nutrient transport in the gut would be expected (Karasov and Diamond 1988, Levey et al. 1999, Witmer and Martínez del Rio 2001).

These shifts in diet are likely to elicit changes in the gross morphology of the digestive tract as well. In a shift from readily digested animal tissue to that of fibrous plant matter, one might expect to see changes in gut morphology to increase the surface area available for nutrient uptake as well as increase the time digesta remains in the digestive tract (Sibly 1981, Sabot et al.

1998). These changes are most likely to be in size and shape, specifically a lengthening of the small intestine, and if endosymbionts are present, changes adding to the overall volume of the large intestine. As a population of lizards "progresses" (*sensu* Dawkins 1986) from omnivory to herbivory, the changes made to their digestive biology are likely to be "trading off" with each other, each acting, in turn, to "pull" the other along as the differential benefit of each trait is realized and mutations/adaptations improving them arise in the population.

Study System.—Although, it is difficult to study the evolution of herbivory directly, inferences can be drawn from living generalist species that have the potential for evolving this form of ecological specialization (e.g., Sabot et al. 1998, Starck 1999). Given the goals of this study, one such species is the omnivorous lizard *Pogona vitticeps* (bearded dragon). A member of the Old World family Agamidae (several members of which are herbivorous), this species is endemic to Australia and reaches a body size (snout-vent length) of 25 cm. This species undergoes an ontogenetic diet shift, consuming primarily insects as juveniles and gradually increasing proportion of plant matter in their diet as they mature to a point that they are consuming primarily plants as adults (Cogger 2000). This shift from carnivory to omnivory, but not strict herbivory, suggests that one or more qualities of the digestive tract may be precluding this species from evolving a strictly herbivorous diet.

Hypotheses and Predictions

I will test the hypothesis that the evolution of an herbivorous diet has not occurred more frequently in reptiles because of constraints on the gastrointestinal system. Additionally, I will test the paradigm that major evolutionary shifts evolve in a form-follows function sequence such

that behavior evolves first, followed by physiology, and finally by morphology (see Tables 1–4 for specific predictions).

Table 1. Predicted outcomes for digestive performance. Although carnivores are predicted to have higher digestive efficiencies than omnivores, their growth rates should be lower because the carnivore diet lacks some nutrients found in the omnivore diet. Ranks are relatively highest (1) to lowest (3).

	Relative Performance		
Digestive Performance	**Herbivore**	**Carnivore**	**Omnivore**
Digestive efficiency	3	1	2
Transit Rate	3	1	2
Growth Rate	3	2	1

Table 2. Predicted outcomes for enzyme activities. Enzyme activity should reflect substrate availability. However, sugar-catalyzing enzymes are expected to become saturated reflecting a ceiling on upregulation. Ranks are relatively highest (1) to lowest (3).

	Relative Enzyme Activity		
Enzyme	**Herbivore**	**Carnivore**	**Omnivore**
Alkaline Phosphotase	1	3	2
Aminopeptidase	3	1	2
Pepsin	3	1	2
Trypsin	3	1	2
Maltase	1	3	2
Amylase	1	3	2
Isomaltase	1	3	2
Lipase	3	1	2

Table 3. Predictions for rates of nutrient transport. Because protein is typically a limiting nutrient, its uptake should be actively modulated to maximize absorption. Glucose is a passively and actively absorbed nutrient, and is not expected to exhibit changes in modulation.

Relative Nutrient Transport Rates

Nutrient	Herbivore	Carnivore	Omnivore
L-proline	1	3	2
D-glucose	N/S	N/S	N/S

Table 4. Predictions for morphology. Surface area available for absorption and retention will be estimated from measures of gross morphology (two-dimensional area of for-, mid-, and hindgut), and histology of the small intestine. Herbivores are expected to have the largest

Relative Nominal Surface Area

Gut Morphology	Herbivore	Carnivore	Omnivore
Histology	1	3	2
Gross	1	3	2

To determine which digestive components of a lizard's biology are precluding them from making a complete shift to herbivory, I will raise groups of lizards on one of three different diets (omnivore, carnivore, herbivore). Thus, I am "forcing" a behavioral shift to these diets. Making this shift at an early stage in ontogeny (before 50 g) should allow sufficient time for diet to have a development effect (evidenced as norms of reactions) on the digestive characteristics I will measure.

A behavioral shift to a novel resource (plant matter) should only persist if there is a benefit to that organism's fitness. This can be measured in several ways. For this study fitness will be assessed as time (days) to reach sexual maturity. This will be measured as growth trajectories until the lizards reach a body size corresponding to sexual maturity (23 cm), and digestive efficiencies, which provides a whole-organism measure of digestive performance. If lizards exhibit sufficient plasticity in their digestion to efficiently exploit their respective diets, I

expect to find no difference in growth rates or digestive efficiencies. A difference in digestive performance, however, would indicate an inability to exhibit sufficient plasticity in some aspect of a lizard's digestive biology (e.g., morphology and/or physiology), given lizards will be fed enough to maintain growth.. Additionally, because digestive efficiencies will measure how well food was assimilated on a given diet, I expect differences in growth rate to track differences in digestive efficiencies.

Assuming differences in performance are the result of constraints on digestion I will perform additional experiments to identify which aspects of a lizard's digestive biology constrain the exploitation of a particular diet. To do this I will examine several aspects of digestive physiology and morphology. The physiological aspects of digestion include digestive efficiency and passage rate, and rates of nutrient transport and enzyme activity. The morphological variables include measurements of the gross morphology of the alimentary tract, and histology of the small intestine.

Should these traits exhibit reaction norms that facilitate the exploitation of the assigned diet, I expect to see dramatic differences reflecting the types of adaptations typically associated with specific diets (e.g., simple, short alimentary tracts in carnivores vs. long, complex digestive tracts in herbivores). Although differences are generally expected between treatments, certain traits are likely to exhibit more plasticity than others.

I expect that the physiological aspects of the digestive system will exhibit more plasticity than the morphological. Thus, constraints on the amount of plasticity exhibited by morphology will result in reduced performance in those lizards assigned an herbivorous diet. This lack in morphological plasticity is predicted to be in gut length and proportion, which should have a direct affect on passage rates, and ability to retain large volumes of plant matter for long periods

of time, which will subsequently affect digestive efficiencies and growth rates. Such a finding would indicate that the digestive tracts of *P. vitticeps* lack the morphological plasticity to permit proximate diet switches to herbivory. Consequently, this reduced ability to digest plant matter may result in either a loss of body mass or a reduced rate of growth (when compared to those maintained on a carnivore or omnivore diet), thus establishing a selective regime that would favor omnivory. If this species exhibits both the physiological and morphological capacity to proximately shift to an herbivorous diet, then the lack of an evolutionary shift to strict herbivory in this taxon may be explained by some digestive or ecological advantage provided by an omnivorous diet (e.g., Bjorndal 1991). Alternatively, we may be witnessing a gradual and perhaps lengthy evolutionary shift in diet to strict herbivory.

Materials and Methods

Experimental Animals and Husbandry.—Captive-bred juvenile bearded dragons (N = 36) were purchased from several breeders, toe clipped for permanent identification, and measured for body length and mass.

During feeding trials, lizards will be housed individually in ventilated plastic containers (30.0 x 16.5 x 9.0 cm) lined with plastic grating. Containers will be kept in environmentally controlled chambers (CMP 4030 Equipped Chamber, Conviron® Winnipeg, Manitoba, Canada) with a 14 L:10 D photoperiod temperature programmed for 30 °C scotophase and 35 °C photophase (± 1 °C). Containers will be randomly assigned to a new position in one of two chambers each day to ameliorate chamber effects.

Experimental Diets.—Lizards will be randomly assigned to one of three diet treatments (N = 12 per treatment): (1) hydrated ground cricket (carnivorous), (2) hydrated ground rabbit chow (herbivorous), and (3) a 50/50 mix of both (omnivorous). The carnivore diet will consist of adult crickets (*Acheta domesticus*), killed by freezing (ca. –17 °C) and dried to constant mass in a drying oven (105–110 °C). Crickets were ground in a coffee mill and passed through a 1 x 1-mm screen. The herbivore treatment consists of ground (as for the crickets) rabbit chow (Diamond Pacific Products, Perris, CA). According to the manufacturer, this diet consists of 16% protein, 3% fat, and not more than 21% crude fiber. The omnivore diet consists of a 50:50 mix (by mass) of the carnivore and herbivore diets. The diets are hydrated with distilled water (1:2 by mass) immediately before feeding. As in nature, lizards will receive all water from their diet. Calcium powder (phosphorus free) is sprinkled on the food weekly to supplement mineral requirements. During the experiments, lizards will be allowed to feed ad libitum and provided enough food to maintain body mass ($\pm$ 10%). To determine the amount of food ingested, the food provided to each lizard will be weighed prior to and following feeding and a correction factor applied to determine the dry mass.

Digestive Efficiency and Passage Rate—I will estimate digestive efficiency to obtain a whole-organism estimate of gut function. During feeding trials, feces will be collected daily. Apparent digestive efficiency will be calculated using the equation:

$$DE = (\text{dry mass of food ingested} - \text{dry mass of feces}/(\text{dry mass of food ingested}).$$

This method provides an estimate of digestive efficiency because some gut tissues (e.g., epithelial lining) may be eliminated with feces, resulting in an underestimation of true digestive efficiency (Van Soest 1982).

Passage rate will be measured to determine the effects of diet on retention time. This will be measured using undigestible markers (plastic flagging tape), cut finely and mixed into each lizard's respective diet (Grajal and Parra 1995). Data collection will begin with first appearance of markers in feces and end upon collection of 95–100% of tape. Passage rate will be recorded as percent recovered over time in days or hours (depending on how often they defecate). The mean of these data will be recorded as mean retention time (MRT) and used for statistical analysis. Data recorded from these trials will be analyzed via ANOVA ($\alpha = 0.05$). These data will also be used in a linear regression with gut length (see Gross Morphology below) to determine the effects of gut length on digestive efficiency and passage rate.

Gut Enzyme Activity.— I will measure enzyme activity levels to determine whether lizards exhibit plastic responses to processing the differing availabilities of substrates in their diets. Eight physiologically relevant gut enzymes (alkaline phosphatase, aminopeptidase, amylase, isomaltase, lipase, maltase, pepsin, and trypsin) will be measured using established (Vonk and Western 1984), and recently developed methods (German et al. 2004). These methods may require slight modification for use in *P. vitticeps.*

Alkaline phosphatase catalyzes the hydrolysis of phosphate esters to produce inorganic phosphate. In this reaction, alkaline phosphatase cleaves a phosphate ion from the substrate p-nitrophenyl phosphate yielding a di-, p-nitrophenol, and inorganic phosphate. Phosphate produced by this reaction facilitates active transport mechanisms involving kinase.

Aminopeptidase and trypsin are amino-specific proteases that cleave proteins into monopeptides that are absorbed by the brush-border membrane. Aminopeptidase cleaves before

alanine and leucine residues. Trypsin cleaves before arginine and lysine unless followed by proline. High activities for these enzymes would indicate substrate preference for dietary protein.

Lipase is a carboxylic esterase, activated by bile salts, that cleaves ester bonds in the presence of H_2O producing an alcohol and a carboxylic acid anion. High activities for this enzyme would indicate a substrate preference for dietary fats.

Amylase, isomaltase, and maltase are carbohydrases that reduce carbohydrates to glucose and other short-chain sugar molecules. Amylase attacks the α-1-4 linkages of linear chained carbohydrates to produce oligosaccharides of varying structures (depending on the branching pattern of the original polysaccharide) and glucose. Isomaltase cleaves the 1-6 linkages of the disaccharide isomaltose (produced by amylase) to produce two glucose molecules. Maltase attacks a maltose (also produced my amylase) at the α-1-4 linkages of its carbon structure to produce two additional glucose molecules. High activities of these enzymes would indicate a substrate preference for dietary carbohydrates.

Each enzyme requires its own assay that I will not described here. However, I will discuss the choice of gut sections, basic procedure, measuring techniques, and statistical analysis. I will estimate gut performance on the three regions of the small intestine (anterior-, mid-, and posterior-third) and the proximate region of the large intestine (anterior third distal to the cecum [should one be present]). Each section will be homogenized and centrifuged. Supernatants will be collected and stored in small aliquots at –80 °C until just before use in spectrophotometric assays of activities of the eight digestive enzymes. All assays will be carried out at 15 °C using a microplate spectrophotometer. Each reaction will be read against a blank appropriate for each assay, and all reactions will be run at saturating substrate concentrations and for periods

sufficient to measure maximum activity levels. Measurements will be expressed as 1 μmol product liberated min^{-1} per g of gut tissue (*U*).

Rates of Nutrient Transport.—I will use the everted-sleeve method (Karasov and Diamond 1983) to determine the rates at which nutrients (D-glucose and L-proline) produced by digestion are transported across the brush-border membrane of the small intestine. This method measures both carrier-mediated (via an amino acid transporter) and passive transport of amino acids, passive uptake of L-glucose and carrier-mediated uptake of D-glucose (via sodium glucose transporter-1 [SGLT-1]). Data recorded from these experiments will determine whether lizards fed specific diets can match the uptake rates of diet specialists (e.g., carnivore or herbivore) reported in the literature (e.g., Karasov and Diamond 1988, Secor 2000, Andres et al. unpublished data). These trials will commence upon the conclusion of the digestive efficiency/passage rate trials, and will be conducted in the lab of Dr. Stephen Secor (University of Alabama).

Generally, eight lizards will be selected at random from each dietary treatment. These lizards will be euthenized, either via an overdose of sodium pentobarbital or the severing of the spinal cord. This will immediately be followed by the excision of the small intestine, which is then weighed, flushed with ice-cold Ringer's, everted, divided into equal-length thirds, and cut into 1-cm sleeves. Sleeves then undergo a preparatory phase where they are mounted on glass rods and pre-incubated in Ringer's solution at 30 °C for 5 min. Sleeves are then removed from this solution and incubated for 2 min in Ringer's solution containing four radiolabeled nutrients (D-glucose, L-proline, [^{14}C]polyethylene glycol [PEG], and L-[^{3}H]glucose; see below). Both PEG and L-[^{3}H]glucose are used to correct for the amount of radiolabeled nutrient that adheres to the intestine. L-[^{3}H]glucose has an additional function that corrects for D-glucose transported

via passive diffusion. Tissues are then removed and dried overnight at 70 °C in a drying oven. The dry mass of these tissues is taken prior to solubilization (0.1 ml distilled H_2O and 1 ml TS-1 tissue solubilizer). This solution is incubated overnight at 55 °C. Finally, 10 ml of Econofluor is added upon cooling. Radioactivity of solubolized samples is then measured via a Beckman LS 235 liquid scintillation spectrometer.

Data will be analyzed using analysis of variance (ANOVA) to test for positional effects (proximal, mid, and distal small intestine, and large intestine) and diet treatments on nutrient transport rates.

Gross Morphology and Histology.—I will measure both gross morphology and histology of the alimentary tract to measure diet-induced morphological changes (Sabot et al. 1998, Herrel et al. 2004). Guts will be removed from the body cavity, blotted dry, and the mesentery will be removed. Gut regions (fore-, mid-, and hindgut) will be measured for length (cm), mass (g), and two-dimensional surface area will be traced onto paper and digitally photographed. Foregut will be measured from the terminus of the esophagus to the pyloric sphincter, midgut from pyloric sphincter to the ileo-cecal junction, and hindgut from the ileo-cecal junction to the caeco-colonic junction. All measurements will be of the natural (unstretched) length.

After measurements are taken, the hindgut of each specimen will be opened mid-ventrally and inspected for the presence of valves or other structures that could function to slow down food passage. Additionally, the presence of potentially commensal organisms such as nematodes will be recorded. These data will be qualitative.

Histology of the same gut regions (fore-, mid-, and hindgut) will be compared to obtain an estimate of the nominal surface area available for digestion. Preparation will follow (Bode et

al. 1982, Goodland et al. 1991). Small sections (1–2 cm) of each gut region will be removed and preserved in 10% neutral formalin. Tissues are then processed in an automatic tissue processor and embedded in paraffin wax. Paraffin sections (3 µm) will be cut on a microtome and stained with haematoxylin and eosin for histological examination. Samples are then attached to a piece of x-ray film, villi upwards, left for 1 min to adhere, and then immersed in Clark's fixative (75% ethyl alcohol, 25% glacial acetic acid). After 3–24 h at room temperature, the samples are transferred to 75% ethyl alcohol. Samples are then stained in bulk by Feulgen reaction, and examined under a dissecting microscope. Fifteen villi will be measured per sample for height and width. Data will be analyzed using analysis of variance (ANOVA) to test for positional effects (proximal, mid, and distal small intestine, and large intestine) and diet treatments on nutrient transport rates.

Limiting Factors.—Dramatic differences among diets are expected between physiological and morphological variables measured. However, should one of the two factors not prove to be an obviously limiting factor, additional analysis will be conducted to define the potentially most limiting variable(s). A quantitative approach for this analysis has not been identified.

Proposed Timeline

Spring 2005: Conduct digestive efficiency trials

Summer 2005:

- Conduct nutrient absorption and enzyme activity level trials
- Start analyzing data

Fall 2005:

- Finish analyzing data
- Complete first draft of thesis

Spring 2006: Defend thesis

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