Intake, apparent digestibility, and digesta passage in leopard tortoises
(*Geochelone pardalis*) fed a complete, extruded feed

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By
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DATE SUBMITTED: December, 2010

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ABSTRACT

Intake, apparent digestibility, and digesta passage in leopard tortoises (*Geochelone pardalis*) fed a complete, extruded feed

The influence of feeding juvenile female leopard tortoises (*Geochelone pardalis*, n=18) a commercially available, complete, extruded feed three (3) or seven days (7) per week on dry matter and digestible energy intake, apparent digestibility of dry matter, organic matter, gross energy and fiber fractions, animal body weight and measurements, digesta transit time, rate of passage, and indigestible fill was evaluated. Both feeding frequencies are commonly practiced with captive tortoises. When fed 7 compared to 3 days per week, dry matter and digestible energy intake was greater. Tortoises gained more g BW, but not when adjusted per kg initial BW. When fed 7 compared to 3 days per week, tortoises grew more in plastron width (PW) and carapace height (CH), but not midline straight carapace length (MSCL), and grew more in calculated shell volume (i.e., a calculated estimate of shell volume using MSCL, PW, and CH), with a higher calculated body condition index (BCI). Providing short fasts (i.e., feeding 3 compared to 7 days per week) may be useful in slowing tortoise growth when animals are provided food *ad libitum*. In general, *ad libitum* feeding, especially of a highly digestible extruded feed, is not recommended for captive juvenile *G. pardalis*, especially when offered food daily. With two data points (detected as outliers) removed due to low fecal output (and resulting unrealistically high apparent digestibility of all nutrients analyzed) of two animals when fed 3 days per week, apparent digestibility of cellulose in tortoises fed 7 (n=18) compared to 3 (n=16) days per week was lower, but no differences were detected in DM, OM,

GE, or any other fiber fractions analyzed. Transit time (TT_1) was shorter and indigestible fill was higher in tortoises ($n=18$) fed 7 compared to 3 days per week, regardless of percent Cr marker recovered. With four animals removed due to $<50\%$ Cr marker recovery, tortoises fed 7 compared to 3 days per week exhibited shorter mean retention time (R_{GIT}), with no differences in digesta transit or indigestible fill. Longer digesta retention when food availability included short periods of fasting may have allowed tortoises to extract more energy from cellulose.

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DEDICATION

This paper is dedicated to my fiancé, Christopher Franske, for believing in me from the beginning, and for keeping me focused. I would also like to dedicate this to my parents, Joanne and Jeff Lickel, for always encouraging me to follow my dreams, wherever they take me – even if that means 3,000 miles from home! Finally, I would like to dedicate this to Flint, the maned wolf that first set me on my path to study comparative nutrition.

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LIST OF ABBREVIATIONS

aDig, %	Apparent digestibility, the fraction of nutrients consumed that are digested and absorbed from the gastrointestinal tract, without taking endogenous nutrient contributions into consideration, calculated as the amount of a nutrient consumed minus the amount of that nutrient lost in the feces, as a percent of the amount of the nutrient consumed
ADF	Acid detergent fiber, composed of cellulose, lignin, cutin and ash
ADL	Acid detergent lignin, composed of lignin, cutin and ash
ADL _{OM}	Acid detergent lignin on an organic matter basis, calculated as ADL-ash
Ash	Inorganic (mineral) content, determined gravimetrically
BCI	Body condition index, calculated in this study as $BCI = BW_g/SV_{cm}^3$ [Loehr et al., 2007]
BW	Body weight
Cell	Cellulose, calculated as sADF-ADL
CH	Carapace height, the height at the greatest point of the third vertebral scute
DE	Digestible energy, the amount of food energy available to the animal after fecal energy loss
DEI	Digestible energy intake
DM	Dry matter, the non-water fraction of a sample
DMB	Dry matter basis
DMI	Dry matter intake
FMR	Field metabolic rate
GE	Gross energy, total potential energy content of a food
HC	Hemicellulose, calculated as NDF - sADF
ME	Metabolizable energy, food energy available after fecal, urinary, and gaseous losses

LIST OF ABBREVIATIONS (continued)

MSCL	Midline straight carapace length, the maximum straight line length between the anterior and posterior edges of the carapace
NDF	Neutral detergent fiber, composed of hemicellulose, cellulose, lignin, cutin and ash
OM	Organic matter, calculated as DM-ash
PW	Plastron width, the width along the plastral suture between the pectoral and abdominal scutes
R _{GIT}	Mean retention time, an estimate of the average time food and digesta are retained in the digestive tract, calculated as $R_{GIT} = \Sigma(n \cdot t) / \Sigma n$ where n is the amount of marker recovered, and t is the time (h) between administration of the marker dose and the end of collection [Udén, 1978; Holleman and White, 1987; Stevens and Hume, 1995]
sADF	Acid detergent fiber determined sequentially after NDF analysis, composed of cellulose, lignin, cutin, and ash
SV	Shell volume, calculated as $SV, \text{cm}^3 = (\pi \cdot MSCL \cdot CH \cdot PW)/6$ [Loehr et al., 2007]
T ₅₀	Time (h) to cumulative recovery of 50% of an indigestible marker dose in feces post dosing [Barboza, 1995]
T _{MAX}	Time (h) to recovery of the maximum marker concentration in a single fecal sample post dosing [Barboza, 1995]
TT ₁	Transit time, or time (h) to first appearance of an indigestible marker in feces post dosing [Holleman and White, 1987; Stevens and Hume, 1995]
V _N	Indigestible fill, an estimate of the volume of the digestive tract for undigested material, based on the product of an estimate of the flow rate (total fecal output per collection) total passage time (R _{GIT}) observed, calculated as $V_N, g = F \cdot R_{GIT}$, where F is the total fecal output [Holleman and White, 1987; Barboza, 1995]

LITERATURE REVIEW

Introduction

Tortoises in captivity are commonly kept on feeding schedules where they are offered food three times per week. This type of feeding schedule has been recommended, but not objectively evaluated, for captive adult tortoises [Boycott and Bourquin, 1988; Boyer and Boyer, 1996]. Daily feeding is recommended for hatchling animals [Boycott and Bourquin, 1988; Boyer and Boyer, 1996]. Feeding frequency may affect intake, digestibility, digesta passage, and growth in captive tortoises. There are two issues related to the influence of feeding frequency in tortoises 1) their adaptations to vast fluctuations in food and water availability and sources in their natural environment, and 2) their continuous foraging strategy when food is available.

Geochelone pardalis

The leopard tortoise (*Geochelone pardalis*) is the second largest tortoise on mainland Africa and fourth largest tortoise in the world [Highfield, 1990; Fife and Fife, 2006]. The species has a broad range from Sudan, south to the Cape Province of South Africa, occupying Sudan, Ethiopia, Somalia, Kenya, Uganda, Angola, Zambia, Natal, Mozambique, Namibia, Botswana, Tanzania and South Africa [Auerbach, 1987; Patterson, 1987; Iverson, 1994; Fife and Fife, 2006]. There are two proposed subspecies: *G. p. pardalis*, that occupies only southern Namibia and the western Cape of South Africa; and *G. p. babcocki* that occupies most of the rest of southern Africa [Auerbach, 1987; Fife and Fife, 2006]. A

separate tortoise species, *G. sulcata*, inhabits Africa's northern regions [Auerbach, 1987].

Geochelone pardalis has a yellow to brown carapace with black spots and striations [Auerbach, 1987]. It is uniquely characterized by the lack of a nuchal scute on the anterior margin of the highly domed carapace [Auerbach, 1987; Patterson, 1987; Bonin et al., 2006]. Carapacial scutes of free-ranging individuals may be smooth or raised, and marginal scutes may be flared. Abnormal or pronounced pyramiding can occur under captive conditions, though the cause has not been specifically defined [Auerbach, 1987; Wiesner and Iben, 2002; Fife and Fife, 2006]. Some of the literature suggests that causes of pyramiding, which itself has not been clearly defined, may include overfeeding, rapid growth rates, high protein diets, calcium or vitamin D deficiency, or an improper calcium:phosphorous ratio in the diet [Stearns, 1989; Ritz et al., 2009]. Some attribute pyramiding to low relative humidity [Wiesner and Iben, 2002; Fife and Fife, 2006]. One source recommended relative humidity in captivity be kept below 40%, though the author made no reference to the range of relative humidity in the species' natural environment [Norton, 2005].

Feeding Ecology

Geochelone pardalis is an herbivorous grazer, consuming mostly grasses and succulents in their native habitat [Auerbach, 1987; Patterson, 1987; Highfield, 1990; 1996; Milton, 1992; Rall and Fairall, 1993; Paull, 1996; Mason et al., 1999; Kabigumila, 2001]. Observations of feeding behavior in northern Tanzania showed that the natural diet was 97.8% plants (47 species from 21

families), most of which were succulent forbs, or non-woody flowering plants [Kabigumila, 2001]. Observations of *G. pardalis* feeding behavior in South Africa concluded that grasses and bulbs were the principal components of the diet, but succulents were preferred [Milton, 1992; Rall and Fairall, 1993; Mason et al., 1999]. Fallen fruits, such as the marula fruit (*Sclerocarya birrea*) in southern Africa, may also be consumed [Auerbach, 1987]. Quantitative information on feeding frequency in nature is lacking.

Opportunistic ingestion of bone, feces and carrion has been observed [Auerbach, 1987; Patterson, 1987; Highfield, 1990; 1996; Bonin, 2006]. This behavior may be partially attributed to presumed increased calcium and phosphorus requirements for shell growth and egg production [Boycott and Bourquin, 1988].

Captive tortoises have been observed rushing 3-4 m to approach and attempt to bite bright colors, but color preference has not been investigated in food selection [Boycott and Bourquin, 1988]. Investigated using extracts of ginger, anise and rose, and a control of water, *G. pardalis* demonstrated no preference for any of the scents over others or the control [Douglass et al., 2009].

Geochelone pardalis is terrestrial and diurnal [Boycott and Bourquin, 1988; Bonin et al., 2006]. They are adaptable and can be found in montane grasslands, arid bushvelds and tropical coastal plains where rainfall can range from <100 mm to 1400 mm per year [Patterson, 1987]. Sympatric *Kinixys spekii* spends about 6.6% of potential daily activity time (time period between the first and last signs of activity in a *K. spekii* study group), and approximately 34% of

actual observed daily activity foraging in the Gokwe District of Zimbabwe, but no activity budget data has been published on *G. pardalis* [Hailey and Coulson, 1999]. This herbivorous grazer likely spends a similarly large portion of each day foraging in nature.

Most tortoise species obtain water from foods or by drinking dew [Harless and Morlock, 1979; Boycott and Bourquin, 1988]. Some use rain as a drinking water source by modifying their posture, directing water down the carapace to their mouth [Harless and Morlock, 1979; Boycott and Bourquin, 1988]. When free water is available, *G. pardalis* will drink by extending the neck and immersing the lower jaw, or often the entire head, closing the jaws and using the throat muscles to force water into the esophagus [Harless and Morlock, 1979, personal observations].

Thermal Biology

Geochelone pardalis is ectothermic [Bjorndal, 1997; Stahl and Donoghue, 2010]. As such, their metabolic rate is approximately 25% that of endothermic mammals of equal body mass, and does not increase with cold ambient temperatures [Stahl and Donoghue, 2010]. Ectotherms depend on external or environmental heat sources to thermoregulate. In a study spanning one year in the Sengwa Wildlife Research Area (Gokwe District, Zimbabwe), the mean field cloacal temperature of *G. pardalis* (n=63, mean BW = 4.0 kg) was $32.6 \pm 3.6^{\circ}\text{C}$, with a range of 25.0-39.4°C [Hailey and Coulson, 1996]. In the same study, *G. pardalis* sightings (e.g., activity) were positively correlated with both daily

minimum and maximum ambient temperatures as well as rainfall, but not with humidity [Hailey and Coulson, 1996].

Tortoises are also heterothermic (i.e., they exhibit wide fluctuations in body temperature) [Stahl and Donoghue, 2010]. They may respond to ambient temperature fluctuations by hiding in vegetation or undergoing periods of dormancy in vacant burrows, though the latter has only been observed in the proposed southern subspecies, *G. p. pardalis* [Highfield, 1990; Bonin et al., 2006]. Excessively low temperatures could cause decreases in metabolic rate, food intake and digestive performance, and excessively high temperatures could cause decreased food intake and weight loss [Stahl and Donoghue, 2010]. Sympatric *K. spekii* exhibited thermoregulatory behaviors including seeking shade and “wetting” for evaporation via urination, eye watering, and salivation when mean body temperatures exceeded 32°C, though salivation did not occur until mean body temperature reached 38.4°C [Hailey and Coulson, 1996].

Age Classes

Chelonian age classes may be described as hatchling, juvenile or adult, though specific information relating age to classification has not been well documented in *G. pardalis*. Relationships between age and size have been described in captive individuals, but there has been no demonstrated correlation between captive and wild growth rates [Boycott and Bourquin, 1988]. One source described hatchling age as 0 – 6 months and adult age as 15 years and older [Highfield, 1990]. Animals between 6 months and 15 years would then be

considered juveniles before sexual maturity and subadults after reaching sexual maturity.

Wild *G. pardalis* generally reach sexual maturity at 12 – 15 years while those in captivity may reach maturity earlier at 5 – 8 years [Highfield, 1990, Paull, 1996]. This may be related to the effects of relatively higher growth rates observed in animals in captivity compared to those under free-ranging conditions [Ritz et al., 2009]. One captive male *G. pardalis* successfully bred at 4 years [Highfield, 1996]. Size may be more indicative of sexual maturity than age in tortoises [Highfield, 1990]. Captive males generally reach maturity at about 5 years and 22.9 cm straight carapace length (SCL) [Paull, 1996]. Captive females generally reach maturity at 6.5 years and 25.4 cm SCL [Paull, 1996]. *Geochelone pardalis* egg production has been observed at 8 kg BW [Highfield, 1990; Walls, 1996].

Growth and Morphometrics

Hatchling *G. pardalis* range from 17 – 35 g BW and 3.8 – 5.0 cm SCL [Auerbach, 1987; Boycott and Bourquin, 1988; Bonin et al., 2006]. The carapace and plastron of a hatchling remain soft for several months, but become increasingly firmer throughout the first year [Boycott and Bourquin, 1988; Boyer and Boyer, 1996]. Total shell (carapace and plastron) mass to BW proportions in captive *Testudo* and *Geochelone* species increases with age (n=87), with proportions of $21.4 \pm 3.7\%$ in hatchlings, and $30.2 \pm 5.8\%$ in juveniles [Koelle et al., 2007]. Juveniles exhibit the fastest growth rate, with a decline at sexual maturity [Boycott and Bourquin, 1988].

Adult *G. pardalis* can reach 10-20 kg BW, with heavy individuals reaching body weights above 40 kg [Auerbach, 1987; Patterson, 1987; Boycott and Bourquin, 1988; Highfield, 1990]. Total observed shell mass to body weight proportions of adult captive *Testudo* and *Geochelone* species in one study was $35.4 \pm 3.3\%$ [Koelle et al., 2007].

Typical SCL of adult *G. pardalis* is between 30 – 45 cm, though SCL as great as 60 – 68 cm has been observed [Harless and Morlock, 1979; Boycott and Bourquin, 1988; Walls, 1996; Ferri, 2002]. *G. pardalis* may reach a carapace height (CH) of 30 cm [Boycott and Bourquin, 1988].

Males often have a more elongate carapace while females have a highly domed, wide carapace [Paull, 1996]. Males generally weigh 66% as much as females of the same length, and develop a longer, thicker tail and slightly concave posterior plastron to facilitate copulation [Auerbach, 1987; Paull, 1996; Walls, 1996]. The anal plastron scutes form a “V” shape in males and a “U” shape in females, perhaps to facilitate female egg laying [Paull, 1996].

Carapacial scute rings develop during tortoise growth. These rings are asymmetrical and do not necessarily grow annually; they are therefore unreliable indicators of age [Miller, 1932]. Primary growth rings are formed by growth of the marginal papillae of hatchling scutes, and subsequent growth occurs from the edges of those areas [Miller, 1932]. The size of the hatchling scute does not change with age [Miller, 1932].

Gastrointestinal Tract

Headgut

Tortoises lack teeth, using their beaks to grasp and crop food with scissor-like movements [Guard, 1980; Boyer and Boyer, 1996]. Hatchling chelonians have an egg tooth, or protrusion on the rhamphotheca (i.e. horny beak), that aids in puncturing the egg shell during hatching, and wears away with age [Boycott and Bourquin, 1988]. Chelonians have wide tongues that do not extend past the lower jaw [Boyer and Boyer, 1996]. Observations of *Gopherus agassizii* anatomy revealed that the tongue and esophagus have a thick cornified mucosal epithelium including numerous mucus glands [Barboza, 1995]. Chelonian salivary glands produce only mucus, and do not produce any digestive enzymes [Guard, 1980; Boyer and Boyer, 1996].

Foregut

Chelonians have a hyoid apparatus attached by muscles to their sternum and lower jaw that facilitates swallowing [Harless and Morlock, 1979]. Force applied by the basihyal bone also aids swallowing [Harless and Morlock, 1979]. The *G. pardalis* stomach is thick-walled and spindle-shaped and can be found near the left lobe of the liver along the ventral left side [Boyer and Boyer, 1996; Taylor et al., 1996]. The chelonian stomach has both gastro-esophageal and pyloric valves, and two types of glandular epithelia, including fundic glands in the rostral portion and pyloric glands in the aboral portion of the stomach [Guard, 1980; Boyer and Boyer, 1996]. The combined area of these regions was 23% of

the total gastrointestinal tract in free-ranging *G. agassizii* (n=7, mean BW=2033 ± 946 g) [Barboza, 1995]. Observed *G. agassizii* stomach pH (n=7) was more acidic than any other segment of the gastrointestinal tract, consistent with secretion of HCl and pepsinogen by fundic glands [Guard, 1980; Barboza, 1995]. The pyloric glands secrete only mucus [Guard, 1980].

Mean complete gastric emptying in captive *G. pardalis* (n=6, 3 ♂♂ at 3-5 kg and 3 ♀♀ at 11-21 kg) occurred at 6.2 h (range = 5-9 h) after oral administration of barium sulfate to the stomach, with contents initially entering the small intestine via the pyloric sphincter at an average of 0.7 h (range = 0.2-1 h) post marker administration [Taylor et al., 1996].

Midgut

The chelonian small intestine is the primary site of nutrient absorption [Boyer and Boyer, 1996]. The *G. pardalis* small intestine is convoluted, with feathery, wavy mucosa that has villi and crypts extending 2 mm [Taylor et al., 1996]. Intestinal constriction and subsequent dilation suggestive of smooth muscle peristalsis was observed more frequently in the small intestine than in the hindgut in *G. pardalis*, suggesting more rapid transit in the midgut compared to the hindgut [Taylor et al., 1996]. In the same study, radiographs showed contents emptying the small intestine completely at an average of 10.8 h (range = 9-15 h) after oral administration of barium sulfate to the stomach [Taylor et al., 1996].

There were more mucus-secreting goblet cells in duodenal compared to ileal epithelia of wild adult *G. agassizii* (n=7) [Barboza, 1995].

Hindgut

Herbivorous reptiles, such as *G. pardalis*, are hindgut fermenters [Bjorndal, 1997]. The primary sites of microbial fermentation are the proximal colon and cecal dilatation, a small expansion of the large intestine that separates the small and large intestines [Boyer and Boyer, 1996; Bjorndal, 1997; Cork et al., 1999]. The cecal dilatation, rather than true cecum, or blind sac, increases surface area for digestion and provides space for symbiotic microorganisms to thrive (Figure 1) [Guard, 1980; Boyer and Boyer, 1996; Taylor et al., 1996].

The *G. pardalis* large intestine is dilated and smooth, extending from the small intestine at the ileocolic valve [Boyer and Boyer, 1996; Taylor et al., 1996]. Numerous mucus-secreting goblet cells were observed in the thin-walled colon, the longest section of the gastrointestinal tract, of free-ranging *G. agassizii* (n=7) [Barboza, 1995]. Observations of volatile fatty acid concentrations found in the hindgut of *Chelonia mydas* suggested the cecum provided an average of 15.2% of the daily energy budget, assuming all organic acid end products were absorbed [Bjorndal, 1979].

Although there has been no direct measure of digesta content mass in *G. pardalis*, up to 13% of body mass in free-ranging *G. agassizii* (n=7, mean BW 2033 ± 946 g) was attributed to digesta contents (moisture = $80 \pm 3\%$), the majority occurring in the hindgut [Barboza, 1995].

In captive *G. pardalis*, barium sulfate was initially observed entering the large intestine 7.0 h (range = 5-8 h) post-administration to the stomach, and did not empty from the large intestine completely until 153.0 h (range = 144-166 h) post-administration [Taylor et al., 1996]. Long retention despite the lack of a true cecum or capacious colon may be a result of anti-peristaltic action in the hindgut, as digesta is forced antigrade into the cecal dilatation for further microbial fermentation. Peristaltic and anti-peristaltic movement aids in retention of digesta in this region, functionally increasing digestive capacity for further microbial fermentation [Bjorndal, 1997].

The large intestine empties into the cloaca where water can be reabsorbed from a bursa [Guard, 1980; Boycott and Bourquin, 1988; Boyer and Boyer, 1996; Taylor et al., 1996]. Desert species of tortoises such as *G. pardalis* are uricotelic; excreting primarily uric acid, the ammonium salt of urea [Miller, 1932; Harless and Morlock, 1979]. This is an adaptation for water conservation, as less toxic uric acid requires less water for dilution than urea before excretion as a white solid [Miller, 1932].

Zoo Nutrition

Emphasis on zoo nutrition has grown in recent years. Before the implementation of nutrition programs, development of zoo diets was based primarily on domestic animal feeding models and word-of-mouth “success” stories. As the field grew, emphasis shifted towards science-based nutrition with scientific research underlying diet formulation and feeding programs. Digestibility and digesta transit studies are two fundamental types of nutrition research that

contribute toward expansion of our knowledge of species nutritional adaptations as well as provide benchmarks on which captive feeding programs may be based.

The goal of this study was to investigate the influence of feeding captive leopard tortoises a commercially available extruded tortoise diet seven versus three days per week. Information from this controlled study of one captive colony may be useful for developing feeding plans for animals of the same species as well as other tortoises and herbivorous reptiles.

The test diet, Mazuri Tortoise Diet (Mazuri[®] No. 5M21), is relatively high in fiber, a nutrient group of general importance to herbivorous reptiles. Tortoises and other herbivorous reptiles in captivity are often fed various diets of commercial produce and hay, but are also fed inappropriate items such as cat or dog food. Recommendations for *G. gigantea* included a diet containing 24.4% neutral detergent fiber (NDF) and 17.6% acid detergent fiber (ADF), on a dry matter basis, to be more consistent with the diets of this species in its natural environment [Edwards, 1991]. The natural diet of *G. gigantea* is similar to that of *G. pardalis*, consisting of mostly grasses [Edwards, 1991]. These recommendations were used in the formulation of the test diet used in this study, and the neutral detergent and acid detergent fiber values listed in the manufacturers analysis of the test diet are 29.3% and 17.5% on a dry matter basis, respectively [Anonymous, 2009].

Knowledge of energy digestibility can be very useful in diet formulation, as one can predict the energy needs of a species using allometric equations to

estimate how much of a particular diet to offer in order to satisfy predicted energy needs. Currently, there is no information on energy digestibility of the test diet. Information from this study can be used to produce a digestible energy (DE) value of the diet for diet formulation.

Digestibility Trials

Digestibility refers to that fraction of nutrients consumed that are digested and absorbed from the gastrointestinal tract. Digestibility may be expressed as true or apparent. Apparent digestibility (aDig), used throughout this study, is calculated as the amount of a nutrient consumed minus the amount of that nutrient lost in the feces, as a percent of the amount of the nutrient consumed. True digestibility uses a similar calculation, but also takes into consideration endogenous nutrient contributions such as sloughed mucosal cells, microbial debris, and enzymes [Van Soest, 1987]. For comparative purposes, a summary of digestibility observations in herbivorous chelonians is provided (Table 1).

Studies of digestibility evaluate nutrient concentrations of test diets as well as concentrations that are absorbable by the study animal. Trials begin with a preliminary period, known as acclimation or adjustment, and follow with collection periods [Schneider and Flatt, 1975]. Preliminary and collection period durations depend on species-specific digestive physiology, digesta transit rate, and nutrient composition and digestibility of test diets [Schneider and Flatt, 1975].

Table 1. Mean ($\pm$ S.D., when available) apparent digestibility (aDig, %) of dry matter (DM), organic matter (OM), gross energy (GE), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and cell wall by herbivorous chelonians.

Diet	n	aDig, %						
		DM	OM	GE	NDF	ADF	ADL	Cell wall ^a
Leopard tortoise (<i>Geochelone pardalis</i>) [Hailey, 1997]								
<i>Brassica</i> , kale	5	--	82.2 ± 1.2	--	--	--	--	--
<i>Lolium</i> , grass	5	--	63.9 ± 4.0	--	--	--	--	--
Desert tortoise (<i>Gopherus agassizii</i>) [Barboza, 1995]								
<i>Schismus barbatus</i>	8	63 ± 4	--	--	65 ± 5	--	--	--
High fiber pellet	4	53 ± 2	--	--	29 ± 7	--	--	--
Herbage	8	69 ± 4	--	--	57 ± 6	--	--	--
Low fiber pellet	2	76 ± 2	--	--	60 ± 14	--	--	--
Desert tortoise (<i>Gopherus agassizii</i>) [Meienberger et al., 1993]								
<i>Erodium cicutarium</i>	19	63.3 ± 5.0	--	68.8 ± 4.5	36.8 ± 9.0	--	--	--
<i>Schismus barbatus</i>	20	49.7 ± 8.4	--	48.3 ± 8.0	59.1 ± 9.1	--	--	--
Red-footed tortoise (<i>Geochelone carbonaria</i>) [Bjorndal, 1989]								
Guava fruit	4	--	36 ± 1.3	32 ± 0.5	--	--	--	7 ± 1.5
Mango fruit	6	--	69 ± 6.1	65 ± 4.6	--	--	--	40 ± 6.3
Lantana foliage	4	--	38 ± 3.5	34 ± 3.1	--	--	--	37 ± 3.3

Table 1 (continued). Mean ($\pm$ S.D., when available) apparent digestibility (aDig, %) of dry matter (DM), organic matter (OM), gross energy (GE), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and cell wall by herbivorous chelonians.

Diet	n	aDig, %						
		DM	OM	GE	NDF	ADF	ADL	Cell wall ^a
Yellow-footed tortoise (<i>Geochelone denticulata</i>) [Bjorndal, 1989]								
Guava fruit	5	--	36 ± 0.8	32 ± 0.7	--	--	--	7 ± 0.5
Mango fruit	5	--	71 ± 3.4	67 ± 4.1	--	--	--	42 ± 4.3
Lantana foliage	5	--	41 ± 5.6	37 ± 4.8	--	--	--	41 ± 2.3
Gopher tortoise (<i>Gopherus polyphemus</i>) [Bjorndal, 1987]								
<i>Aeschynomene americana</i> leaves	7	--	68 ± 2.6	61 ± 3.2		62 ± 0.8	34 ± 3.7	73 ± 1.2
Florida redbelly turtle (<i>Pseudemys nelsoni</i>) [Bjorndal and Bolten, 1993]								
<i>Hydrilla verticillata</i>	5	80 ± 2	81 ± 2	75 ± 1	--	--	--	86 ± 1
<i>Spirodela polyrhiza</i>	6	29 ± 6	30 ± 0.6	25 ± 7	--	--	--	16 ± 2
Speke's hingeback tortoise (<i>Kinixys spekii</i>) [Hailey, 1997]								
Brassica, kale	6	--	76.8 ± 4.4	--	--	--	--	--
Aldabra tortoise (<i>Geochelone gigantea</i>) [Hamilton and Coe, 1982]								
Tortoise turf ^b	32	30.4 ± 12.5	--	--	--	--	--	--

Table 1 (continued). Mean ($\pm$ S.D., when available) apparent digestibility (aDig, %) of dry matter (DM), organic matter (OM), gross energy (GE), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and cell wall by herbivorous chelonians.

Diet	n	aDig, %						
		DM	OM	GE	NDF	ADF	ADL	Cell wall ^a
Galapagos tortoise (<i>Geochelone nigra</i>) [Liesegang et al., 2001]								
Diet mix I ^c	11	--	91 ± 1	--	--	--	--	--
Diet mix II ^c	11	--	93 ± 1	--	--	--	--	--
Diet mix III ^c	11	--	96 ± 2	--	--	--	--	--
Galapagos tortoise (<i>Geochelone nigra</i>) [Hatt et al., 2002]								
Diet mix ^d	11	--	67.5 ± 12.8	--	--	--	--	--

^aCell wall is calculated as ash free NDF [Bjorndal, 1987; Bjorndal, 1989; Bjorndal and Bolten, 1993].

^bTortoise turf includes a mixture of grasses and herbs on Aldabra atoll. The diet also included *Sporobolus virginicus* and *Guettarda speciosa*.

^cDiet mixes I, II, and III included crude cheese, hay, mixed herbs and vegetables, and a mineral supplement.

^dThe diet mix included fresh and dried rye-grass, *Ficus* and *Salix* browse, fennel, celery, parsley, carrots, apples, pears, banana, cottage cheese, and a vitamin/mineral supplement.

Acclimation is a period during which the animal receives the test diet before the collection period, and is typical for studies testing only one diet [Schneider and Flatt, 1975]. For the purposes of this study, all preliminary periods were referred to as acclimation as the test diet did not change. During collection, the experimenter continues to feed the test diet in the same manner as the acclimation period, while also collecting digestive and/or metabolic wastes. Feces are most commonly collected as they represent the undigested portion of the diet and are relatively easy to obtain. Urine and blood may also be collected during a study, provided the right enclosures or equipment are available [Schneider and Flatt, 1975]. Urine may be analyzed to determine what nutrients were metabolized, and blood may be analyzed to observe changes in the nutrient profiles indicative of changes in nutrient absorption.

In previous studies of digestibility and digesta transit in tortoises, preliminary periods of 3-67 days were used [Bjorndal and Bolten, 1993; Barboza, 1995; Liesegang et al., 2001; Tracy et al., 2006; McMaster and Downs, 2008]. In a digestibility study in the green iguana (*Iguana iguana*), an herbivorous lizard, an 8 week acclimation period was used following a 7-10 day adjustment [Baer et al., 1997].

Visible markers such as fluorescent dyes, food particles, thread loops, and plastic beads or strips are often used as a visual signal of the beginning and/or end of fecal collection [Bjorndal, 1987; Bjorndal and Bolten, 1990; Meienberger et al., 1993; Baer et al., 1997; Hailey, 1997; McMaster and Downs, 2008]. Predetermined lengths based on previous data can also be used (Table 2).

Table 2. Predetermined collection period lengths used in studies of digestibility and/or digesta transit in herbivorous chelonians.

Species	Length, d	Source
<i>Geochelone pardalis</i>	5-7	McMaster and Downs, 2008
<i>Gopherus agassizii</i>	10-36	Meienberger et al., 1993; Barboza, 1995
<i>Geochelone nigra</i>	10	Liesegang et al., 2001
<i>Gopherus polyphemus</i>	14	Bjorndal, 1987
<i>Pseudemys nelsoni</i>	28	Bjorndal and Bolten, 1993

Food intake is measured as the difference between dry matter offered and unconsumed (orts). Intake can be expressed in units of weight or energy, and is often normalized per unit of body weight (BW) or metabolic body size (MBS). Metabolic body size is a measure of metabolically active body tissues, measured as $BW_{kg}^{0.75}$, and scales the energy output of an organism [Stevens and Hume, 1995]. Intake parameters are generally normalized using MBS when comparing across species, based on the assumption that intake is a function of metabolic requirements, and using BW when studying only one species, based on the assumption that intake, more specifically gut fill, is linearly related to body size [Van Soest, 1987].

During a digestibility trial, intake should be constant to minimize uncontrolled variation [Schneider and Flatt, 1975]. One method is to establish voluntary intake during the acclimation period, and to restrict that amount by 10 - 40% during the collection period. This method requires specific attention to the animal's physiology and careful observations of health status to determine what level of restriction can be sustained [Schneider and Flatt, 1975]. In a similar digestibility trial with tortoises, a 15% restriction during collection was applied [Bjorndal, 1989].

Nutrients Considered

Dry matter (DM) is the non-water fraction of a food. Expressing nutrient concentration as a function of dry matter is widely accepted in diet formulation as it represents those nutrient concentrations without variable influence of water. It is important, when working on a dry matter basis (DMB), however, to indicate the percent moisture of the diet as-fed, or the diet when prepared for feeding, including its water content.

Ash is a measure of the inorganic (mineral) content of a diet. When ash is subtracted from DM, this represents organic matter (OM).

Gross energy (GE) is the total potential energy content of a feed, but only a portion of it can be used by an animal. Digestible energy (DE) is the amount of energy available to the animal after fecal energy loss, but before urinary or gaseous energy loss. It can be measured by multiplying the gross energy content and gross energy digestibility of a feed or diet. The DE content of a single feed or diet, just as in the digestibility of any nutrient, can vary between species. One of the goals of this study was to measure the DE of Mazuri Tortoise Diet (Mazuri® No. 5M21) for *G. pardalis*. Because DE is a measure of how much energy of a feed or diet a species can utilize, it may be more accurate to use measured DE in diet formulations, rather than GE or calculated metabolizable energy (ME). Metabolizable energy is the energy available from a feed after fecal, urinary, and gaseous losses.

Fiber components, such as neutral detergent fiber (NDF), cell wall, acid detergent fiber (ADF), acid detergent lignin (ADL), acid detergent lignin on an

organic matter basis (ADL_{OM}), hemicellulose and cellulose, are especially important in herbivore diets. Neutral detergent fiber is a collective measurement of the cell wall components: hemicellulose, cellulose, lignin, cutin and ash [Goering and Van Soest, 1970]. Cell wall is ash free NDF [Bjorndal, 1987; Bjorndal, 1989; Bjorndal and Bolten, 1993]. Acid detergent fiber includes cellulose, lignin, cutin and ash [Goering and Van Soest, 1970]. Hemicellulose can be calculated as NDF minus ADF [Goering and Van Soest, 1970]. Acid detergent lignin (ADL) includes lignin, cutin and ash, so cellulose can be calculated as ADF minus ADL, but ADL_{OM} is lignin and cutin on an organic matter basis only [Goering and Van Soest, 1970].

Digesta Transit

Digesta transit cannot be specifically defined for a species [Stevens and Hume, 1995]. It can be influenced not only by specific animal physiology and mass-specific metabolic rate, but also by the water and fiber content of the diet, environmental and body temperature, and feeding frequency [Stevens and Hume, 1995; Boyer and Boyer, 1996].

Indigestible markers are useful for studies of digesta transit, as well as for digestibility studies. Markers should be inert and must not be elements or nutrients of interest in the study [Schneider and Flatt, 1975]. They should be completely indigestible so that they can be recovered fully in the feces. They must be safe for the animal and not cause any physiological changes detrimental to the study [Schneider and Flatt, 1975]. Specifically for digesta transit studies, markers must also be in a stable equilibrium with the fraction they label, and not

migrate from one fraction to another [Udén et al., 1980]. Though it is possible to force feed a marker to an animal unwilling to ingest it, it is preferable to create a method of delivery in which the animal can willingly consume the marker to mimic actual feeding behaviors and timing.

Though markers should not contain (or react with) any nutrients of interest in the study, an exception lies within using chromium mordanted fiber as a marker [Schneider and Flatt, 1975]. Chromium mordants are chromium (Cr) bound to particles of NDF. The addition of Cr renders the cell wall (NDF) insoluble and indigestible [Udén et al., 1980]. The NDF needs to be accounted for when calculating digestibility, but does not act as a “nutrient of interest”.

Measures of digesta transit include transit time (TT_1), mean retention time (R_{GIT}), time to 50% marker recovery (T_{50}), and time to maximum marker concentration (T_{MAX}). Transit time is defined as the time to the first appearance of an indigestible marker in the feces [Stevens and Hume, 1995]. Mean retention time is the average time that it takes for a pulse dose of an indigestible marker to be eliminated in the feces [Stevens and Hume, 1995; Barboza et al., 2009]. Time to 50% recovery is the time at which 50% of the ingested marker is cumulatively recovered. Time to maximum marker concentration is the time to observed recovery of the maximum concentration of a marker in the feces [Barboza 1995]. For comparative purposes, a summary of digesta transit observations in herbivorous tortoises is provided (Table 3).

Table 3. Transit time (TT_1)^a, time to maximum marker concentration (T_{max})^b, and mean retention time (R_{git})^c of digesta measured with multiple markers in herbivorous tortoises.

Diet	n	Marker	TT_1 , d	T_{MAX} , d	R_{GIT} , d
Desert tortoise (<i>Gopherus agassizii</i>) [Barboza, 1995]					
<i>Schismus barbatus</i> , NDF=64.6% ^d	8	Cr ^e	--	14.9 ± 2.4	14.2 ± 1.8
		Yb ^e	--	5.2 ± 1.4	9.6 ± 1.1
		Co ^e	--	4.8 ± 1.8	6.8 ± 0.8
High fiber pellets, 10x3 mm, NDF=48.4% ^d	4	Cr ^e	--	12.7 ± 5.6	14.8 ± 4.8
		Yb ^e	--	9.7 ± 3.7	13.2 ± 3.1
		Co ^e	--	6.9 ± 3.6	7.8 ± 3.8
<i>Sphaeralcea ambigua</i> , NDF=29.1% ^d	8	Cr ₂ O ₃	--	9.6 ± 1.5	9.5 ± 1.3
Low fiber pellets, 10x3 mm, NDF=18.1% ^d	2	Cr ₂ O ₃	--	5.3 ± 0.01	6.7 ± 0.2
Leopard tortoise (<i>Geochelone pardalis</i>) [Taylor et al., 1996]					
Fasted for 5 days and anesthetized	6	BaSO ₄	6 – 6.9	--	--
Leopard tortoise (<i>Geochelone pardalis</i>) [McMaster and Downs, 2008]					
<i>Medicago sativa</i> , 19.36% carbon fiber ^d	9	flowers, stalks	--	--	8.7
<i>Solanum lycopersicum</i> , 9.55% carbon fiber ^d	9	skins, seeds	--	--	2.2 ± 0.3

Table 3 (continued). Transit time (TT₁)^a, time to maximum marker concentration (T_{max})^b, and mean retention time (R_{git})^c of digesta measured with multiple markers in herbivorous tortoises.

Diet	n	Marker	TT ₁ , d	T _{MAX} , d	R _{GIT} , d
Desert tortoise (<i>Xerobates agassizii</i>) [Meienberger et al., 1993]					
<i>Erodium cicutarium</i> , NDF = 25.7% ^d	19	8 plastic	17.8	--	--
<i>Schismus barbatus</i> , NDF = 72.4% ^d	20	strips	21.8	--	--
Red footed tortoise (<i>Geochelone carbonaria</i>) [Guard, 1980]					
2 mm long particulate marker	4	2.2 mm	--	--	11.25
5 mm long particulate marker	4	diam.	--	--	11.9
10 mm long particulate marker	4	tubing	--	--	15.1
Aldabra tortoise (<i>Geochelone gigantea</i>) [Hamilton and Coe, 1982]					
Tortoise turf ^f	44	tissue	6 – 19	--	--
<i>Sporobolus</i> (coastal grass)	12	paper	10 – 22	--	--

Table 3 (continued). Transit time (TT₁)^a, time to maximum marker concentration (T_{max})^b, and mean retention time (R_{git})^c of digesta measured with multiple markers in herbivorous tortoises.

Diet	n	Marker	TT ₁ , d	T _{MAX} , d	R _{GIT} , d
Galapagos tortoise (<i>Geochelone nigra</i>) [Hatt et al., 2002]					
Diet mix ^g	4 ^h	Co	--	--	9
	4 ⁱ	Co	--	--	9
	4 ^h	Cr	--	--	8
	4 ⁱ	Cr	--	--	12
	4 ^h	C ₃₆	--	--	9
	4 ⁱ	C ₃₆	--	--	12

^aTT₁ is the time to first appearance of the marker in the feces [Stevens and Hume, 1995]

^bT_{max} is the time to the maximum marker concentration in the feces [Barboza, 1995]

^cR_{git} is the average time it takes for a pulse dose of an indigestible marker to be eliminated in the feces, or an estimate of how long food and digesta are retained in the digestive tract [Stevens and Hume, 1995; Barboza et al., 2009]

^dNutrient concentrations are on a dry matter basis.

^eCr was mordanted to large particles, Yb to fine particles, and Co to the fluid portion of the diet.

^fTortoise turf included a mixture of grasses and herbs on Aldabra atoll.

^gThe diet mix included fresh and dried rye-grass, *Ficus* and *Salix* browse, fennel, celery, parsley, carrots, apples, pears, banana, cottage cheese, and a vitamin/mineral supplement.

^hSample size includes only juveniles.

ⁱSample size includes only adults.

Though the Cr does not separate from the fiber during digestion, it can be released through acid hydrolysis in 1M H₂SO₄ [Udén et al., 1980]. Because of this, when analyzing feces for fiber fractions, fiber digestibility would be slightly underestimated if dietary fiber contributed by the pulse dose of Cr mordants was not subtracted from intake.

Chromium mordants are formed by the reduction of hexavalent dichromate complexes via ascorbic acid [Udén et al., 1980]. Ascorbic acid forms a complex with Cr that is water soluble, such that mordants suspended in water and ascorbic acid bind any unbound Cr. Rinsing them removes the soluble complexes of ascorbic acid and Cr [Udén et al., 1980].

MATERIALS AND METHODS

Eighteen, 5 yr old female juvenile leopard tortoises (*Geochelone pardalis*) hatched from a single clutch at the Department of Herpetology, Smithsonian National Zoological Park (Washington, DC) and transferred to the Animal Science Department, California Polytechnic State University (San Luis Obispo, CA) were used in this study. Use of the animals in this study was reviewed and approved by the California Polytechnic State University Institutional Animal Care and Use Committee (Protocol # 903).

Animals were fed a 1.3-2.5 cm extruded tortoise diet (Mazuri[®] Tortoise Diet, Mazuri[®] No. 5M21, PMI Feeds, St. Louis, MO), formulated for terrestrial herbivorous tortoises. Feed samples from each bag were taken for chemical analysis. Due to the large size of the extruded particles, weighed quantities of the diet were soaked in water for 30 sec prior to feeding to aid ingestion. Tortoises were fed this diet exclusively for approximately 1 yr prior to this study. The experiment consisted of two periods, each including a 28 d acclimation phase and a 35 d collection phase. Two feeding schedules, 3 (3) and 7 days (7) a week, were assessed between 17 July 2009 and 22 November 2009.

Voluntary intake and digestibility of dry matter (DM), organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (sADF), acid detergent lignin on an organic matter basis (ADL_{OM}), and gross energy (GE) were determined in a Latin square crossover experimental design [Neter et al., 1996]. The design was blocked by tortoise ID and treatment order. Half of the animals were

randomly assigned to receive the three day a week treatment first with the other half first assigned to the seven day a week treatment.

Tortoise weights and morphometrics were measured weekly. Body weight (BW, g) was quantified to 0.1 g using a digital balance (Delta Range® SB32001DR, Mettler-Toledo, Inc., 1900 Polaris Parkway, Columbus, OH 43240).

Measurements included:

- a. Midline straight carapace length (MSCL), the maximum straight line length between the anterior and posterior edges of the carapace, measured to 0.1 cm;
- b. Carapace height (CH), the height at the greatest point of the third vertebral scute, measured to 0.1 cm with a 40-cm metal combination square (Johnson Level & Tool Mfg. Co. Inc., 6333 West Donges Bay Rd., Mequon, WI 53092-4456);
- c. Plastron width (PW), the width along the plastral suture between the pectoral and abdominal scutes measured to 0.01 cm with 150 mm dial calipers (General Tools Mfg. Co. LLC, New York, NY 10013).

Shell volume was calculated as $SV, \text{cm}^3 = (\pi \cdot \text{MSCL} \cdot \text{CH} \cdot \text{PW})/6$ [Loehr et al., 2007] and a body condition index using the formula $\text{BCI} = \text{BW}_g/\text{SV}_{\text{cm}^3}$ [Loehr et al., 2007; Lickel and Edwards, 2009].

Tortoises were individually offered the soaked, complete pelleted diet at 0930 h and orts removed at 1900 h. Orts were dried at 50°C to constant weight to determine feed intake. Those receiving food 3 days a week were fed on Mondays, Wednesdays, and Fridays only. Food found displaced from the bowl

at any time during the day was placed back in the bowl to prevent it from drying to the floor or heat pad. Drinking water was available *ad libitum* throughout the study.

Tortoises were individually housed indoors. There were six, 5.5 x 3 m rectangular enclosures, each divided into three 2.75 x 1 m pens by a 13 cm plastic beam (Figure 2).

Figure 2. Scaled diagram of the tortoise barn at the Swine Unit of California Polytechnic State University, San Luis Obispo, CA.

The southern side of the building was partially exposed to unfiltered, natural sunlight with 89 cm tall, fabric sun screening 130 cm off the floor that covered a portion of the open wall and created a pattern of unfiltered, natural sunlight exposure and shade in each pen. Supplemental fluorescent lighting was provided on a 10L:14D schedule. Each animal was provided a 23.5 x 44 x 29 cm shelter with an 18 cm entrance on the east side.

Minimum and maximum ambient temperatures were recorded twice daily using an indoor/outdoor digital thermometer (Acu-Rite®, Chaney Instrument Company, Lake Geneva, WI). Each enclosure included a 121.5 x 60.5 cm heat pad (Stanfield®, Osborne Industries, Inc, Osborne, KS) maintained between 25 –

35°C (Figure 2). Temperatures of supplemental heat pads and concrete floors were measured daily at 0900 h and 1900 h with a noncontact infrared thermometer (Raytek Mini Temp MT, Raytek Corporation, Santa Cruz, CA).

Each pen was spot cleaned daily with a 4% chlorhexidine diacetate solution (Nolvasan S, Fort Dodge Animal Health, Fort Dodge, IA) and reusable cotton towels. Each pen was thoroughly disinfected every Monday morning at 0700 h by scrubbing with water and antibacterial soap, while the animal was temporarily housed in a 50 gallon stock tank (Rubbermaid® No. 4243, Newell Rubbermaid Inc., Atlanta, GA).

Acclimation – *determining voluntary food intake*

During each 28 d acclimation phase, voluntary food intake was determined. To allow *ad libitum* feeding during acclimation, the diet was initially offered at 200% of calculated daily field metabolic rate (FMR) of herbivorous reptiles, using the following allometric equation:

$$\text{FMR, kJ ME/d} = (0.232)(\text{BW}_g)^{0.813} \text{ [Nagy et al., 1999]}$$

The calculated ME of the diet (12.13 kJ/g), was used in this calculation [Anonymous, 2009]. For example, a 1500 g tortoise, fed 100% FMR, 7 days a week, would receive:

$$\text{FMR, kJ ME/d} = (0.232)(1500 \text{ g})^{0.813} = 88.6 \text{ kJ ME/d}$$

$$88.6 \text{ kJ ME/d} / 12.13 \text{ kJ ME/g} = 7.3 \text{ g test diet/day}$$

and a 1500 g tortoise, fed 100% FMR, 3 days a week, would receive:

$$88.6 \text{ kJ ME/d} \cdot 7 \text{ d} = 620.2 \text{ kJ ME per week}$$

$$620.2 \text{ kJ ME} / 3 \text{ feedings per week} = 206.7 \text{ kJ ME/feeding}$$

$$206.7 \text{ kJ ME} / 12.13 \text{ kJ ME/g test diet} = 17.0 \text{ g test diet/feeding}$$

The extruded diet was weighed in each food dish, a 14 cm diameter plant pot saucer, using an Ohaus Navigator™ (model NOB110). If the exact quantity was not attained without dividing feed pellets, the closest value above the target quantity was recorded. Water was added to the brim of each dish and left for 30 sec. Water was then discarded and the food dish placed in the northwest corner of the pen at 0930 h (Figure 2).

Orts were collected at 1900 h. If ≤ 1 g of daily food remained for an individual, the weekly amount offered was increased by an amount equivalent to an additional 25% FMR. Food quantities offered were only increased on Mondays, Wednesdays and Fridays, such that both treatment groups had equal opportunity for diet increases.

Voluntary DM intakes for individual tortoises were calculated as the difference between food offered and Orts on a dry matter basis.

Collection

Each acclimation phase was immediately followed by a 35 d collection phase. Collection dates for period 1 occurred from 17 August 2009 to 20 September 2009, and collection dates for period 2 occurred from 19 October 2009 to 23 November 2009.

Tortoises were offered 85% of their mean voluntary energy intake (kJ/d·MBS) as established during the preceding acclimation to encourage consistent intake [Schneider and Flatt, 1975; Bjorndal, 1989]. Food was prepared and collected as described during acclimation.

An indigestible marker (see below) was administered orally, by hand-feeding, as a pulse dose on the first day of collection. All feces were collected into resealable plastic bags at 0900 h, 1400 h and 1900 h and frozen at -20°C for later analysis. Fecal samples were dried at 100°C to constant weight in a forced-air drying oven (Stabil-Therm No. ESP-400BC-4, Blue M Electric Company, Blue Island, IL) and ground in a stainless steel laboratory mill through a 2 mm screen (Wiley Mini Mill; Thomas No. 3383L60, Thomas Scientific, Swedesboro, NJ) (Appendix A).

Collection – *indigestible marker preparation*

The test diet was ground in a stainless steel laboratory mill through a 2 mm mesh screen (Wiley Mini Mill; Thomas No. 3383L60) (Appendix A) and macro-portion of NDF extracted [Goering and Van Soest, 1970]. A ratio of 50 g ground test diet per 1 L neutral detergent solution was used to boil the diet for 1 h in a 3 L beaker under a cool water condenser stabilized with a ring stand [Goering and Van Soest, 1970]. Ten (10) mL alpha amylase (Ankom No. FAA) was added to the solution after 5 minutes of rapid boiling. After 1 h, the solution was filtered through a polyethylene filter bag (50 µm pore size), rinsed with deionized water, rinsed with acetone, and air dried in a ventilation hood.

Chromium was mordanted to extracted NDF (Appendix B) [Uden, 1980]. All dry, extracted NDF (114.2 g) was weighed. Chromium, as sodium dichromate dihydrate ($\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$), was added at 14% of NDF dry weight, to 4 volumes of water to completely cover the NDF in a 42 x 31 cm aluminum pan. The mixture was covered with an identical pan and placed in a 100°C drying oven

(Precision Scientific No. 114) for 24 h. The solution was then filtered through a polyethylene filter bag (50 µm pore size) and rinsed thoroughly with deionized water. The Cr-mordanted fiber was suspended in deionized water in a 3 L beaker with 57.1 g (50% of initial fiber weight) L-ascorbic acid ($C_6H_8O_6$) for 1 h. After 1 h, the material was filtered again through a polyethylene filter bag (50 µm pore size) and rinsed thoroughly with deionized water. The Cr-labeled fiber was dried at 65°C to a constant weight in a drying oven (Precision Scientific No. 114) [Udén, 1980].

Each animal was dosed with forty (40) mg Cr in 2.45 g mordanted neutral detergent fiber (16,300 ppm Cr), mixed with 4.9 g Mazuri No. 5M21 (ground through a 2 mm screen) and 14.7 mL H_2O . This mixture was formed into pellets resembling the normal diet by extrusion through a syringe (Monoject 12 mL syringe, 20 X 1-1/2") with the luer-loc tip removed. The pellets were left to air-dry overnight and were fed to tortoises on the first morning of each collection at 0930 before feeding. The weight of the test diet incorporated in the pellets (4.9 g) fed to each tortoise was subtracted from the amount of food offered that day.

Collection – *determining digestibility*

Dry matter (DM) was determined gravimetrically by drying ground subsamples in 50 mm diameter aluminum pans in a forced air oven for 16 h at 105°C (Precision Scientific no. 1054) [Undersander et al., 1993a]. Ash was sequentially determined gravimetrically by combustion at 575°C for 12 h in a muffle furnace (Barnstead/Thermolyne no. F6000) [Undersander et al, 1993b]. Organic matter (OM) was calculated as DM minus ash.

Composite fecal samples for each period, representing each animal-treatment combination, were created using equal amounts of each sample within that combination, when available. Chromium analysis for evaluation of digesta transit was given priority, such that some samples were not represented in the composite if the full sample was needed for Cr analysis.

Gross energy (GE) of feed and composite fecals was determined using a Parr adiabatic calorimeter (Parr Model No. 1261) [Parr Instrument Company, 1994]. Feed and composite fecals were analyzed for neutral detergent fiber (NDF) and sequential acid detergent fiber (sADF) using an Ankom²⁰⁰ fiber analyzer (Ankom No. A200) and Ankom Technology Methods 6 and 5, respectively [Ankom Technology, 2006]. Acid detergent lignin (ADL) of feed and composite samples was determined using the Ankom Technology method for determining acid detergent lignin in beakers [Ankom Technology, 2005]. Apparent digestibilities (aDig, %) of nutrients, including DM, OM, GE and fiber (NDF, sADF, ADL_{OM}), were calculated as [Schneider and Flatt, 1975]:

$$\text{aDig, \%} = [(\text{nutrient intake} - \text{fecal nutrient output}) / (\text{nutrient intake})] \cdot 100$$

Collection – *determining digesta transit*

Ground fecal sub samples were prepared for Cr analysis by acid digestion in 125 mL Erlenmeyer flasks [Arthur, 1970]. Ten (10) mL 70% nitric acid (HNO₃; CAS # 7697-37-2) was added to flasks in a ventilated hood overnight. Flasks were placed on a steam table in a chemical fume hood designated for hot nitric acid for initial digestion. When the samples were completely dry, they were assessed for color. When dark orange or brown was present, indicating the

presence of organic material, another 10 mL aliquot of nitric acid was added. This process was repeated until there were no further signs of organic material.

Dried samples that were yellow, white, or green in color, with no signs of organic material, received 5 mL of 69-72% perchloric acid (HClO_4 ; CAS # 7601-90-3) and the flask was transferred to a hot plate in a perchloric acid hood. Flasks were heated until all traces of green (i.e., indication of Cr in the wrong oxidation state in chromic oxide, Cr_2O_3 , which could not be read on the AAS) became clear, yellow, or orange/red in color (i.e., indication that Cr is present as dichromate, $\text{Cr}_2\text{O}_7^{-2}$). Digestion continued if there were any black specks (i.e., organic material) on the sides of the flasks as well.

After acid digestion, flasks were rinsed with 10 mL of 0.25% calcium phosphate solution ($\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6 + 10 \text{ mL HClO}_4$), scraped with a rubber policeman into 125 mL volumetric flasks, and further rinsed with double deionized water [Arthur, 1970]. They were then brought up to volume in the 125 mL volumetric flask with double deionized water.

Samples were initially analyzed by flame atomic absorption spectrometry (Perkin Elmer AAnalyst 300) using the continuous graphics function to determine absorption. Subsequent dilutions were made using a 0.025% calcium phosphate solution to bring samples >5 ppm Cr down into the 1-5 ppm range. Final AAS readings were obtained using a standard curve including standards of 0.25, 0.50, 0.75, 1.0, 2.0, 3.0, 4.0, and 5.0 ppm [Arthur, 1970].

Chromium concentrations of subsamples were then applied to fecal sample weights to determine absolute Cr concentrations in each sample. Cr

content in the diet was multiplied by the total intake amount during each collection to determine the approximate amount of Cr contributed via diet intake. This amount was divided by 35 to represent daily Cr intake and was subtracted from individual fecal Cr content.

Transit time (TT_1) was determined as the first appearance (h) of Cr in the feces post dosing [Holleman and White, 1987; Stevens and Hume, 1995].

Time to 50% marker recovery (T_{50}) was calculated as the time (h) to cumulative recovery of 50% of the marker dose (20 mg). Time (h) to recovery of the maximum marker concentration, T_{MAX} (mg Cr/g fecal DM), was calculated as the time (h) to recovery of the maximum marker concentration in a single sample [Barboza, 1995].

Mean retention time (R_{GIT}) was calculated as the average time (h) to recover the Cr mordants, using the following formula:

$$R_{GIT} = \Sigma(n \cdot t) / \Sigma n \text{ [Udén, 1978; Holleman and White, 1987]}$$

where n is the amount of Cr recovered in mg, and t is the time (h) between administration of the 40 mg dose and the end of the collection period (840 h).

Indigestible fill (V_N) is an estimate of the volume of the digestive tract based on the product of an estimate of the flow rate (total fecal output per 35 d collection) and R_{GIT} , an estimate of the total passage time observed [Holleman and White, 1987]. The calculation is $V_N = F \cdot R_{GIT}$, where F is the total fecal output during the 35 d collection [Holleman and White, 1987].

Statistical Analyses

Analysis of variance (ANOVA) using the general linear model (GLM) function in Minitab 16.1.1 (Minitab Inc., State College, PA) was used to compare the dependent variables between the two feeding frequencies. Categorical variables were created to indicate the pattern in which the treatments were received, and the order position of each response variable. Pattern, order position, and treatment were indicated in the model, and pattern was also nested within tortoise ID [Neter et al., 1996] to account for variation that may have occurred due to which pattern the treatments were in as well as variation inherent in individual tortoise physiology.

The exact model specified was “Pattern OrderPosition Treatment TortoiseID(Pattern)” [Neter et al., 1996]. Tortoise ID was also indicated as a random factor because these 18 tortoises represent only a small sample of the entire population of captive leopard tortoises. Differences were interpreted as statistically significant at $P < 0.05$.

Relationships between periods 1 and 2 in ambient and enclosure temperatures were analyzed using the general regression function in Minitab 16.1.1. To account for temporal correlations in daily temperature data, lags of one and two days were included in the model, along with period. An attained Durbin-Watson coefficient > 1.4 indicated that autocorrelation was no longer present. Differences were interpreted as statistically significant at $P < 0.05$.

Predictions

The following predictions were tested:

1. Daily dry matter intake (DMI), and digestible energy intake (DEI), would be greater on the 7 compared to 3 day per week feeding schedule.
2. Indigestible fill, V_N , would be greater on the 7 compared to 3 day per week feeding schedule. The gastrointestinal tract would be stimulated by exposure to food on a more consistent basis and may expand its capacity for undigested portions of food through distention of gastrointestinal tract walls [Van Soest, 1987].
3. Body weight gain would be greater on the 7 compared to 3 day per week feeding schedule. If tortoises have greater intake on the 7 day per week feeding schedule, then I would expect to find greater body weight gain as well.
4. Transit time (TT_1), would be faster on the 7 compared to 3 day per week feeding schedule. Digesta would move more rapidly through the digestive tract on the 7 day per week feeding schedule. Seven day per week feeding may stimulate the digestive system on a more consistent basis and metabolism may be increased.
5. Mean retention time (R_{GIT}) would be shorter on the 7 compared to 3 day per week feeding schedule. This was also hypothesized because the 7 day per week feeding schedule may stimulate the digestive system on a more consistent basis and metabolism may be increased.

6. Fiber digestibility would be lower on the 7 compared to 3 day per week feeding schedule. Fiber must be retained in the chelonian hindgut longer than readily available nutrients to be thoroughly digested by microbial symbionts. If digesta transit is faster and digesta retention is shorter, then I would expect that decreasing the amount of time fiber remains in the gastrointestinal tract would decrease its digestibility.
7. Dry matter and GE digestibility would not differ between the 7 and 3 day per week feeding schedules. I hypothesized that apparent digestibility would not be different between feeding schedules because these are readily available nutrients that should not be affected by longer retention.

RESULTS

Test Diet

The test diet was analyzed for DM, OM, ash, fiber fractions, GE, and Cr content (Table 4). Test diet ingredients, listed by the manufacturer in the following order, include ground soybean hulls, ground corn, ground oats, dehulled soybean meal, wheat middlings, cane molasses, dehydrated alfalfa meal, wheat germ, soybean oil, dicalcium phosphate, monocalcium phosphate, brewers dried yeast, calcium carbonate, salt, DL-methionine, choline chloride, menadione dimethylpyrimidinol bisulfite (vitamin K), pyridoxine hydrochloride, d-alpha tocopheryl acetate (natural source vitamin E), cholecalciferol (vitamin D3), biotin, L-lysine, tocopherols (a preservative), calcium pantothenate, vitamin A acetate, riboflavin, folic acid, nicotinic acid, thiamin mononitrate, vitamin B12 supplement, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, and sodium selenite.

Table 4. Selected nutrient analyses of the extruded tortoise diet formulated for terrestrial herbivorous tortoises^a.

Nutrient ^b	Concentration
DM, %	92.6
OM, %	91.9
Ash, %	8.1
NDF, %	29.9
Cell wall (NDF – ash), %	27.4
sADF, %	18.1
ADL _{OM} , %	1.0
Hemicellulose (NDF - sADF), %	11.8
Cellulose (sADF – ADL), %	17.1
GE, kJ/g	18.18
Cr, ppm	2.26

^aAll nutrient concentrations, except dry matter (DM), are on a dry matter basis.

^bAbbreviations: OM = organic matter, NDF = neutral detergent fiber, sADF = sequentially determined acid detergent fiber, ADL_{OM} = acid detergent lignin on an OM basis, GE = gross energy and DE = digestible energy.

Temperature

Average enclosure temperatures and average minimum and maximum ambient temperatures during both collections were determined (Tables 5a and 5b, respectively). Between periods 1 and 2, there were no differences observed in surface temperatures of the concrete floors at 0900 h ($P=0.888$) or 1900 h ($P=0.238$), and no differences in surface temperatures of the supplemental heat pads at 0900 h ($P=0.201$) or 1900 h ($P=0.113$). During collection 1, minimum ambient temperatures were, on average, 1.5°C higher than during collection 2 ($P=0.027$), and collection 1 maximum ambient temperatures were, on average, 2.8°C higher than during collection 2 ($P=0.011$).

Table 5a. Mean ($\pm$ S.D.) surface temperatures ($^{\circ}\text{C}$) of concrete enclosure floors and temperature controlled heat pads at 0900 h and 1900 h for each collection.

Period ¹	Enclosure (0900 h)		Enclosure (1900 h)	
	Floor	Heat Pad	Floor	Heat Pad
1	24.0 $\pm$ 3.1	30.3 $\pm$ 3.1	24.8 $\pm$ 2.5	31.8 $\pm$ 4.2
2	24.0 $\pm$ 3.6	31.8 $\pm$ 4.7	23.4 $\pm$ 3.0	29.5 $\pm$ 3.4
<i>P</i>	0.888	0.201	0.238	0.113

¹Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Table 5b. Mean ($\pm$ S.D.) minimum and maximum ambient temperatures ($^{\circ}\text{C}$) for each collection.

Period ¹	Ambient temperature	
	Minimum	Maximum
1	16.2 $\pm$ 2.8	29.8 $\pm$ 4.5
2	12.8 $\pm$ 3.3	24.5 $\pm$ 4.5
<i>P</i>	0.027*	0.011*

¹Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Initial Body Weights (BW)

Mean ($\pm$ S.D.) initial and overall body weights during collection were determined (Table 6). Mean initial BW for all tortoises on day 1 of the first acclimation period was 1486.1 ± 533.5 g and ranged from 654.7 – 2768.7 g. There was no significant difference in initial weights between treatments ($P=0.123$) on day 1 of each collection. Initial body weights were, on average, 326.6 g higher at the beginning of period 2 compared to period 1 ($P<0.001$).

On average, mean overall BW (the mean BW throughout each treatment or period during collection) was 408.9 g higher when tortoises were fed 7 compared to 3 days per week ($P<0.001$). There was no significant difference in mean overall BW between periods ($P=0.129$).

Table 6. Mean ($\pm$ S.D.) initial and overall body weights (BW, g) of *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week.

		BW, g	
	n	Mean Initial	Mean Overall ¹
Treatment ²			
3	18	1795.8 ± 656.0	1728.2 ± 630.8
7	18	1861.6 ± 757.0	2137.1 ± 837.3
<i>P</i>		0.123	<0.001*
Period ³			
1	18	1665.4 ± 602.6	1892.3 ± 702.3
2	18	1992.0 ± 756.6	1973.0 ± 831.1
<i>P</i>		<0.001*	0.129

¹Mean BW (g) was calculated as the mean BW during each collection. Mean overall BW represents the mean across all animals (n=18) for each treatment and period.

²Treatments were 3 days (3) and 7 days (7) per week.

³Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Voluntary Intake

Mean ($\pm$ S.D.) maximum voluntary DMI (Max DMI) and DEI (Max DEI) were determined (Table 7). Tortoises fed 7 compared to 3 days per week consumed, on average, 6.0 g/d ($P<0.001$), or 3.2 g/d·BW_{kg} ($P<0.001$) more. The same relationship was demonstrated for Max DEI with tortoises consuming 89.8 kJ/d ($P<0.001$), or 49.3 kJ/d·BW_{kg} ($P<0.001$) more.

On average, tortoises consumed 4.1 g/d ($P=0.002$), or 61.9 kJ/d ($P=0.002$) more during period 2 than 1. No statistical difference was detected when intake was adjusted per kg BW ($P=0.087$).

Table 7. Maximum ($\pm$ S.D.) voluntary dry matter intake (Max DMI) and digestible energy intake (Max DEI) of *G. pardalis* (n=18) when offered food *ad libitum* 3 or 7 days a week during acclimation.

	n	Max DMI ¹		Max DEI ¹	
		g/d	g/d·kg BW ⁻¹	DE kJ/d	DE kJ/d·kg BW ⁻¹
Treatment ²					
3	18	11.1 ± 5.2	6.4 ± 1.6	167.1 ± 77.9	95.4 ± 24.2
7	18	17.1 ± 8.0	9.6 ± 1.7	256.9 ± 120.2	144.7 ± 25.7
<i>P</i>		<0.001*	<0.001*	<0.001*	<0.001*
Period ³					
1	18	12.1 ± 6.5	7.4 ± 2.4	181.0 ± 97.8	112.0 ± 36.3
2	18	16.2 ± 7.6	8.5 ± 2.2	242.9 ± 114.8	128.1 ± 32.7
<i>P</i>		0.002*	0.087	0.002*	0.087

¹ Body weight (BW) is expressed as the mean BW during the corresponding acclimation.

²Treatments were 3 days (3) and 7 days (7) per week.

³Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Mean Daily Dry Matter Intake (DMI)

Mean ($\pm$ S.D.) daily DMI was determined (Table 8). Animals fed 7 compared to 3 days per week ate, on average, 4.45 g/d more ($P<0.001$). When adjusted per kg BW, animals consumed 1.97 g/d more when fed 7 compared to 3 days per week ($P<0.001$).

Animals consumed, on average, 2.61 g/d more during period 2 than 1 ($P=0.009$). When adjusted per kg mean overall BW, there was no significant difference detected in DMI between periods ($P=0.628$).

Mean ($\pm$ S.D.) orts recovered were 0.5 ± 1.5 g and 2.0 ± 3.4 g per feeding during periods 1 and 2, respectively.

Table 8. Mean ($\pm$ S.D.) daily dry matter intake (Mean DMI) of *G. pardalis* when offered 85% of mean weekly voluntary intake distributed over 3 or 7 days a week during collection.

		Mean DMI	
	n	g/d	g/kg BW · d ¹
Treatment ²			
3	18	8.75 ± 4.2	4.61 ± 0.8
7	18	13.20 ± 6.5	6.58 ± 1.0
<i>P</i>		<0.001*	<0.001*
Period ³			
1	18	9.67 ± 4.8	5.52 ± 1.5
2	18	12.28 ± 6.6	5.67 ± 1.3
<i>P</i>		0.009*	0.628

¹Body weight (BW) is expressed as the mean BW during the corresponding collection phase.

²Treatments were 3 days (3) and 7 days (7) per week.

³Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*An asterisk indicates statistical significance at $P<0.05$.

Growth

BW Gain

Mean ($\pm$ S.D.) total BW gain and average daily gain (ADG) were determined (Table 9). On average, tortoises gained 99.5 g total, or 2.9 g/d, more when fed 7 compared to 3 days a week ($P=0.002$). There was no difference in total BW gain (g) between periods ($P=0.213$). When BW gain was adjusted per kg initial BW (obtained on the first day of each collection phase), there was no difference between treatments ($P=0.341$), but tortoises gained, on average, 32.3 g total, or 0.9 g/d, more per kg initial BW during period 2 than 1 ($P=0.023$).

Table 9. Mean ($\pm$ S.D.) total BW gain and average daily gain (ADG) of *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week.

	n	Total BW Gain		ADG	
		g	g/initial BW _{kg}	g/d	g/d·initial BW _{kg}
Treatment ¹					
3	18	183.7 ± 98.8	117.4 ± 56.3	5.2 ± 2.8	3.3 ± 1.6
7	18	283.2 ± 165.1	130.0 ± 26.8	8.1 ± 4.7	3.7 ± 0.8
<i>P</i>		0.002*	0.341	0.002*	0.341
Period ²					
1	18	216.3 ± 145.1	107.5 ± 29.2	6.2 ± 4.1	3.1 ± 0.8
2	18	250.5 ± 143.5	139.8 ± 50.7	7.1 ± 4.1	4.0 ± 1.4
<i>P</i>		0.213	0.023*	0.213	0.023*

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Measurements (MSCL, PW, CH, SV)

Midline Straight Carapace Length (MSCL)

Mean ($\pm$ S.D.) MSCL growth during collection was determined (Table 10).

Midline straight carapace length growth was not different between treatments on an absolute basis (cm, or cm/d) ($P=0.231$), or when adjusted per cm initial MSCL ($P=0.219$). MSCL growth was also not different between periods on an absolute ($P=0.387$) or adjusted ($P=0.891$) basis.

Table 10. Mean ($\pm$ S.D.) total and daily midline straight carapace length (MSCL) growth of *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week.

	n	Total MSCL Growth		Daily MSCL Growth	
		cm	cm/initial cm	cm/d	cm/d·initial cm
Treatment ¹					
3	18	0.96 ± 0.36	0.04 ± 0.01	0.03 ± 0.01	0.001 ± 0.0004
7	18	1.10 ± 0.42	0.05 ± 0.02	0.03 ± 0.01	0.001 ± 0.0005
<i>P</i>		0.231	0.219	0.231	0.219
Period ²					
1	18	0.98 ± 0.45	0.05 ± 0.02	0.03 ± 0.01	0.001 ± 0.0005
2	18	1.07 ± 0.32	0.05 ± 0.01	0.03 ± 0.01	0.001 ± 0.0003
<i>P</i>		0.387	0.891	0.387	0.891

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Plastron Width (PW)

Mean ($\pm$ S.D.) total and PW growth during collection was determined (Table 11). On average, animals grew 0.22 cm, or 0.01 cm/d PW, more when fed 7 compared to 3 days per week ($P=0.007$). When adjusted per cm initial PW, animals grew 0.02 cm, or 0.0004 cm/d more when fed 7 compared to 3 days a week ($P=0.001$).

On average, animals grew 0.17 cm PW, or 0.01 cm/d PW, more during period 2 than 1 ($P=0.019$). When adjusted per cm initial PW, animals grew 0.01 cm, or 0.0003 cm/d more during period 2 than 1 ($P=0.046$).

Table 11. Mean ($\pm$ S.D.) total and daily plastron width (PW) growth of *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week.

		Total PW Growth		Daily PW Growth	
	n	cm	cm/initial cm	cm/d	cm/d·initial cm
Treatment ¹					
3	18	0.49 ± 0.20	0.04 ± 0.01	0.01 ± 0.005	0.0011 ± 0.00037
7	18	0.71 ± 0.26	0.06 ± 0.01	0.02 ± 0.007	0.0016 ± 0.00042
<i>P</i>		0.007*	0.001*	0.007*	0.001*
Period ²					
1	18	0.51 ± 0.21	0.04 ± 0.02	0.01 ± 0.006	0.0012 ± 0.00045
2	18	0.68 ± 0.26	0.05 ± 0.01	0.02 ± 0.007	0.0015 ± 0.00042
<i>P</i>		0.019*	0.046*	0.019*	0.046*

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Carapace Height (CH)

Mean ($\pm$ S.D.) total and daily CH growth during collection was determined (Table 12). On average, animals grew 0.23 cm, or 0.01 cm/d CH, more when fed 7 compared to 3 days per week ($P=0.002$). When adjusted per cm initial CH, animals grew 0.02 cm, or 0.0006 cm/d more when fed 7 compared to 3 days a week ($P=0.005$).

There were no differences detected between periods 1 and 2 in PW growth in cm or cm/d ($P=0.695$). When adjusted per cm initial CH, there were also no differences detected between periods 1 and 2 ($P=0.513$).

Table 12. Mean ($\pm$ S.D.) total and daily carapace height (CH) growth of *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week.

		Total CH Growth		Daily CH Growth	
	n	cm	cm/initial cm	cm/d	cm/d·initial cm
Treatment ¹					
3	18	0.36 ± 0.16	0.03 ± 0.01	0.01 ± 0.005	0.0009 ± 0.00038
7	18	0.59 ± 0.19	0.05 ± 0.02	0.02 ± 0.005	0.0015 ± 0.00048
<i>P</i>		0.002*	0.005*	0.002*	0.005*
Period ²					
1	18	0.47 ± 0.22	0.04 ± 0.02	0.01 ± 0.006	0.0012 ± 0.00058
2	18	0.46 ± 0.19	0.04 ± 0.02	0.01 ± 0.006	0.0011 ± 0.00046
<i>P</i>		0.695	0.513	0.695	0.513

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Shell Volume (SV)

Mean ($\pm$ S.D.) total SV growth during collection was calculated (Table 13). On average, animals grew 93.2 cm³ SV more when fed 7 compared to 3 days per week ($P=0.005$). When adjusted per cm³ initial SV, animals grew 0.04 cm³ more when fed 7 compared to 3 days a week ($P<0.001$). Because SV data adjusted per cm³ initial SV did not fit a normal distribution ($P=0.019$), the data was transformed via natural log to satisfy the assumption of normality necessary to run ANOVA ($P=0.130$). The same general result was attained, with higher SV growth when fed 7 compared to 3 days per week ($P<0.001$).

On average, SV increased 69.1 cm³ more during period 2 than 1 ($P=0.011$). There were no differences detected on raw ($P=0.563$) or natural log transformed ($P=0.513$) data when adjusted per cm³ initial SV.

Table 13. Mean ($\pm$ S.D.) total shell volume (SV) growth of *G. pardalis* during collection when fed at 85% of mean weekly voluntary intake distributed over 3 or 7 days a week¹.

		Total SV Growth		
	n	cm ³	cm ³ /initial cm ³	ln(cm ³ /initial cm ³)
Treatment ²				
3	18	204.6 ± 104.8	0.12 ± 0.24	-2.14 ± 0.23
7	18	297.8 ± 160.6	0.16 ± 0.04	-1.82 ± 0.22
P		0.005*	<0.001*	<0.001*
Period ³				
1	18	214.1 ± 99.9	0.14 ± 0.04	-2.02 ± 0.28
2	18	283.2 ± 167.3	0.14 ± 0.04	-1.96 ± 0.25
P		0.011*	0.563	0.513

¹Shell volume (SV) = $(\pi/6) \cdot (\text{MSCL} \cdot \text{PW} \cdot \text{CH})$

²Treatments were 3 days (3) and 7 days (7) per week.

³Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Mean ($\pm$ S.D.) daily SV growth during collection was calculated (Table 14). Animals grew, on average, 2.66 cm³/d SV more when fed 7 compared to 3 days per week ($P=0.005$). When adjusted per cm³ initial SV, animals grew 0.02 cm³/d more when fed 7 compared to 3 days a week ($P<0.001$). Because SV data adjusted per cm³ initial SV did not fit a normal distribution ($P=0.013$), the data were transformed via natural log to satisfy the assumption of normality necessary to run ANOVA ($P=0.108$). The same result was attained, with higher SV growth when fed 7 compared to 3 days per week ($P<0.001$).

On average, animals grew 1.97 cm³/d SV more during period 2 than 1 ($P=0.011$). There were no differences detected on raw ($P=0.563$) or natural log transformed ($P=0.513$) data when adjusted per initial cm³ SV.

Table 14. Mean ($\pm$ S.D.) daily shell volume (SV) growth of *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week¹.

		Daily SV Growth		
	n	cm ³ /d	cm ³ /d·initial cm ³	ln(cm ³ /d·initial cm ³)
Treatment ²				
3	18	5.85 ± 2.99	0.003 ± 0.0007	-5.70 ± 0.20
7	18	8.51 ± 4.59	0.005 ± 0.0010	-5.38 ± 0.22
<i>P</i>		0.005*	<0.001*	<0.001*
Period ³				
1	18	6.12 ± 2.85	0.004 ± 0.001	-5.58 ± 0.28
2	18	8.09 ± 4.78	0.004 ± 0.001	-5.52 ± 0.25
<i>P</i>		0.011*	0.563	0.513

¹Shell volume (SV) = $(\pi/6) \cdot (\text{MSCL} \cdot \text{PW} \cdot \text{CH})$

²Treatments were 3 days (3) and 7 days (7) per week.

³Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Body Condition Index (BCI)

Mean ($\pm$ S.D.) BCI during collection was calculated (Table 15). Mean BCI was 0.0204 higher when animals were fed 7 compared to 3 days per week ($P=0.005$). There was no difference detected in mean BCI between periods 1 and 2 ($P=0.365$).

Table 15. Mean ($\pm$ S.D.) body condition index (BCI) of *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week.

	Mean BCI
Treatment	
3	1.0957 $\pm$ 0.0448
7	1.1161 $\pm$ 0.0379
<i>P</i>	0.005*
Period	
1	1.1085 $\pm$ 0.0445
2	1.1022 $\pm$ 0.0412
<i>P</i>	0.365

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*Statistical significance at $P<0.05$.

Apparent Digestibility (aDig, %)

Data for 18 animals on treatment 7 and period 1 and for 16 animals on treatment 3 and period 2 are reported. Two animals were removed from analysis of apparent digestibility for uncharacteristically low fecal output on treatment 3 during period 2. One animal (307104) did not defecate during the entire second period when fed 3 days per week, and thus had an observed apparent digestibility of 100%, which is a highly unlikely result. With this animal removed, box plots of mean total fecal output by treatment and by period (Figures 3a and 3b, respectively) showed that one data point from another animal (307100) was

an outlier, with low fecal output and a resulting observed aDig DM of approximately 96.7%. This unrealistic result was also removed, resulting in sample sizes $n=16$ for both treatment 3 and period 2, and sample sizes remaining at $n=18$ for treatment 7 and period 1.

Figure 3a. Boxplot indicating mean total fecal DM output (g DM/kg BW) for animals fed 3 or 7 days per week, with an outlier indicated by an asterisk.

Figure 3b. Boxplot indicating mean total fecal DM output (g DM/kg BW) during periods 1 and 2, with an outlier indicated by an asterisk.

Apparent digestibility of DM, OM and GE during collection was determined (Table 16). Raw data for aDig in all animals (n=18) are presented in Appendix C. There were no differences detected for DM ($P=0.823$), OM ($P=0.687$) or GE ($P=0.980$) between animals fed 3 (n=16) or 7 (n=18) days per week. There were no differences detected for DM ($P=0.108$), OM (0.085) or GE ($P=0.080$) between periods 1 (n=18) or 2 (n=16).

Mean apparent digestibility of GE, excluding the two data points discussed earlier, was 82.2%. When multiplied by the GE concentration of the test diet (Table 4), this gives a digestible energy (DE) value of:

$$\text{DE, kJ/g} = 18.18 \text{ kJ/g} \times 0.822 \text{ aDig GE} = 14.94 \text{ kJ DE/g test diet}$$

Digestible energy concentration of the test diet was applied to DMI intake to calculate digestible energy intake (DEI) (Table 17).

Table 16. Mean ($\pm$ S.D.) apparent digestibility (aDig, %) of dry matter (DM), organic matter (OM), and gross energy (GE) in *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week.

		Apparent Digestibility (aDig), %		
	n	DM	OM ³	GE
Treatment ¹				
3	16	84.4 ± 2.4	85.4 ± 2.2	82.1 ± 2.8
7	18	84.4 ± 2.9	85.2 ± 2.8	82.3 ± 3.2
P		0.823	0.687	0.980
Period ²				
1	18	83.6 ± 2.4	84.4 ± 2.3	81.1 ± 2.8
2	16	85.4 ± 2.5	86.2 ± 2.4	83.4 ± 2.8
P		0.108	0.085	0.080

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

³Organic matter (OM) is DM minus ash.

*Statistical significance at $P<0.05$.

Mean Digestible Energy Intake (DEI)

Mean ($\pm$ S.D.) DEI was determined (Table 17). Animals fed 7 compared to 3 days per week ate, on average, 66.4 kJ DE/d more ($P<0.001$). When adjusted per kg BW, animals consumed 15.2 kJ DE/d more when fed 7 compared to 3 days per week ($P<0.005$). Mean DEI per meal was 75.1 kJ DE/meal higher in animals fed 3 days per week when adjusted per kg BW ($P<0.001$).

Animals consumed, on average, 39.0 kJ DE/d more during period 2 than 1 ($P=0.009$). When adjusted per kg mean overall BW, tortoises consumed, on average, 16.6 kJ DE/d more during period 2 than 1 ($P=0.003$). Mean DEI per meal was also 21.4 kJ DE/meal higher during period 2 when adjusted per kg BW ($P=0.012$).

Table 17. Mean ($\pm$ S.D.) digestible energy intake (Mean DEI) of *G. pardalis* when offered 85% of mean weekly voluntary intake distributed over 3 or 7 days a week during collection.

	n	Mean DEI ²		
		kJ DE/d	kJ DE/d · kg BW ¹	kJ DE/meal · kg BW ¹
Treatment ³				
3	16	130.8 ± 62.6	75.1 ± 17.7	175.3 ± 41.2
7	18	197.2 ± 97.6	90.3 ± 14.8	100.2 ± 11.0
P		<0.001*	0.005*	<0.001*
Period ⁴				
1	18	144.5 ± 71.9	74.4 ± 14.6	127.0 ± 32.0
2	16	183.5 ± 99.1	91.0 ± 17.2	148.4 ± 59.5
P		0.009*	0.003*	0.012*

¹Body weight (BW) is expressed as the mean BW during the corresponding collection phase.

²DE = digestible energy.

³Treatments were 3 days (3) and 7 days (7) per week.

⁴Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

*An asterisk indicates statistical significance at $P<0.05$.

Apparent digestibility of fiber fractions during collection was determined (Table 18). Raw data for aDig in all animals (n=18) are presented in Appendix C. There were no statistical differences detected in aDig NDF ($P=0.302$), aDig cell wall ($P=0.280$), aDig sADF ($P=0.152$), aDig ADL_{OM} ($P=0.973$) or aDig HC ($P=0.827$) between animals fed 3 or 7 days per week. Mean apparent digestibility of cellulose was 3.2% ($P=0.037$) and 4.5% ($P=0.009$) higher, respectively, when animals were fed 3 compared to 7 days per week.

There were no differences detected in mean apparent digestibility of NDF ($P=0.067$), cell wall ($P=0.110$), sADF ($P=0.076$), ADL_{OM} ($P=0.191$), cellulose ($P=0.074$), or HC ($P=0.075$) between periods.

Digesta Transit, Digesta Retention, Indigestible Fill

In the following sections, results are reported for all animals (n=18) (Table 19, Appendix D), and with four animals removed (n=14) (Table 20).

Though all animals were included in Table 19, there is a missing value from the 3 day a week treatment and period 2 results, resulting in a sample size n=17, as tortoise 307104 did not defecate during period 2, and thus had no results to report.

Four animals were removed from the second analysis of digesta transit, digesta retention, and indigestible fill analyses due to Cr marker recovery <50% in one or both periods. Fifty percent marker recovery was chosen as the cutoff based on the parameter T_{50} , in which recovery of 50% of the marker was necessary.

Table 18. Mean ($\pm$ S.D.) apparent digestibility (aDig, %) of fiber fractions, including neutral detergent fiber (NDF), sequentially determined acid detergent fiber (sADF), acid detergent lignin on an organic matter basis (ADL_{OM}), cellulose and hemicellulose (HC) in *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week.

		Apparent Digestibility (aDig), %					
	n	NDF ³	Cell Wall ⁴	sADF ⁶	ADL _{OM}	Cellulose ⁷	HC ⁸
Treatment ¹							
3	16	77.6 ± 3.8	77.4 ± 4.0	81.0 ± 3.6	82.0 ± 2.8	87.1 ± 3.0	72.3 ± 4.5
7	18	76.2 ± 5.1	75.6 ± 5.0	78.5 ± 6.3	82.2 ± 3.2	83.9 ± 6.6	72.4 ± 4.7
P		0.302	0.280	0.152	0.973	0.042*	0.827
Period ²							
1	18	75.4 ± 4.0	75.1 ± 4.0	78.2 ± 4.6	81.3 ± 2.9	84.1 ± 5.0	70.9 ± 4.5
2	16	78.5 ± 4.6	78.0 ± 4.9	81.4 ± 5.6	83.0 ± 2.7	86.9 ± 5.7	74.0 ± 4.1
P		0.067	0.110	0.076	0.191	0.074	0.075

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

³NDF is composed of hemicellulose, cellulose, lignin, cutin, and ash.

⁴Cell wall is ash free NDF, or NDF – ash [Bjorndal, 1987; Bjorndal, 1989; Bjorndal and Bolten, 1993].

⁵sADF is composed of cellulose, lignin, cutin, and ash.

⁶Cellulose = sADF - ADL.

⁷HC = NDF - sADF.

*Statistical significance at $P < 0.05$.

Tortoise 307104 was removed due to its 0% recovery for the 3 day per week treatment during period 2. Tortoise 307100 defecated 2.4% of the marker dose in period 1 and 23.1% in period 2, so she was also removed. Tortoise 307107 defecated 29.0% in period 1 and 92.2% in period 2. Though it appears there was adequate recovery in period 2, it may have been a result of residual marker dose from period 1, rendering those observations unreliable. The same logic was applied when removing tortoise 307106, as she excreted 13.3% of the marker in period 1 and 80.6% in period 2.

All four removed animals were randomly assigned to the same treatment pattern, in which they received the 7 day a week treatment first; however, removal of these data points did not affect orthogonality of contrasts, so the statistical model did not require modification.

Marker Recovery

With all animals considered ($n=18$), total marker recovery was $77.1 \pm 28.3\%$. When four were removed from analysis ($n=14$), marker recovery increased to $87.9 \pm 11.6\%$. The observed Cr content in the diet was used to correct for background Cr in the feces (Table 4) contributed by the diet.

Digesta Transit

With all animals included ($n=18$), on average, TT_1 was 43.9 h faster in animals fed 7 compared to 3 days per week ($P=0.031$) (Table 19). There was no statistical difference detected between periods ($P=0.978$) (Table 19). Time to 50% marker recovery (T_{50}) could not be analyzed for all animals ($n=18$) because 4 animals had <50% marker recovery for either or both periods. Time to

recovery of the maximum marker concentration (T_{MAX}) was not different between treatments or between periods with all animals included ($n=18$) (Table 19). There were no differences detected in any of the three measures of digesta (TT_1 , T_{50} , T_{MAX}) transit between treatments or between periods with 4 animals removed ($n=14$) (Table 20).

Digesta Retention

With all animals included ($n=18$), there were no differences detected in R_{GIT} between treatments ($P=0.090$) or periods ($P=0.225$) (Table 19), though the assumption of normality necessary to run ANOVA was violated ($P=0.008$). Transformations of the data did not meet the assumption of normality.

When the 4 animals with $<50\%$ marker recovery were removed ($n=14$), the assumption of normality was met ($P=0.882$). Mean retention time (R_{GIT}) was, on average, 60.2 h (or 2.5 d) longer when tortoises were fed 3 compared to 7 days per week ($P=0.018$) (Table 20). No difference was detected between periods ($P=0.507$) (Table 20).

Indigestible Fill

With all animals included ($n=18$), V_N was, on average, 3.89 g/kg BW higher when animals were fed 7 compared to 3 days per week ($P=0.014$), and 3.13 g/kg BW higher in period 1 than 2 ($P=0.042$) (Table 19). There were no differences detected between treatments ($P=0.760$) or between periods ($P=0.183$) in V_N when 4 animals were removed ($n=14$) (Table 20).

Table 19. Digesta transit, digesta retention and indigestible fill in *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week.

Treatment ¹	n	Digesta Transit			Digesta Retention	Indigestible Fill
		TT ₁ ³ , h	T ₅₀ ⁴ , h	T _{MAX} ⁵ , h	R _{GIT} ⁶ , h	V _N ⁷ , g/kg BW
3	17	190.50 ± 125.2	--	372.6 ± 179.4	399.36 ± 99.5	11.57 ± 3.7
7	18	146.56 ± 81.2	--	295.4 ± 119.9	364.54 ± 84.0	15.46 ± 3.8
<i>P</i>		0.031*	--	0.150	0.090	0.014*
Period ²						
1	18	173.22 ± 114.8	--	337.0 ± 163.5	399.67 ± 80.4	15.09 ± 4.9
2	17	162.26 ± 98.3	--	328.6 ± 149.2	362.17 ± 102.0	11.96 ± 3.4
<i>P</i>		0.978	--	0.925	0.225	0.042*

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

³Transit time is time to first appearance of the Cr marker after dosing [Stevens and Hume, 1995].

⁴Time to 50% recovery (T₅₀) is the time between marker dosing and cumulative recovery of 50% of the marker.

⁵Time to maximum marker concentration (T_{MAX}) is the time between marker dosing and recovery of the maximum marker concentration attained.

⁶R_{GIT} = mean retention time, or the time for the average marker molecule to be excreted after a pulse dose [Stevens and Hume, 1995].

⁷V_N = indigestible fill. It is an estimate of the volume of the digestive tract based on the product of an estimate of the flow rate (total fecal output per 35 d collection) and the total transit time (mean retention time, R_{GIT}). The calculation is V_N = F·R_{GIT}, where F is the total fecal output per the 35 d collection [Holleman and White, 1987].

*Statistical significance at *P*<0.05.

Table 20. Digesta transit, digesta retention and indigestible fill in *G. pardalis* during collection when fed 85% of mean weekly voluntary intake distributed over 3 or 7 days a week, with marker recovery >50% in both periods.

Treatment ¹	n	Digesta Transit			Digesta Retention	Indigestible Fill
		TT ₁ ³ , h	T ₅₀ ⁴ , h	T _{MAX} ⁵ , h	R _{GIT} ⁶ , h	V _N ⁷ , g/kg BW
3	14	185.8 ± 131.0	446.4 ± 156.9	398.4 ± 183.3	409.7 ± 93.1	12.8 ± 3.1
7	14	143.9 ± 75.6	351.4 ± 95.8	286.7 ± 118.6	349.5 ± 79.0	13.3 ± 1.7
<i>P</i>		0.153	0.137	0.053	0.018*	0.760
Period ²						
1	14	178.2 ± 119.3	433.6 ± 129.7	340.1 ± 174.8	394.6 ± 80.5	12.6 ± 2.7
2	14	151.5 ± 95.9	364.1 ± 138.7	344.9 ± 154.3	364.5 ± 99.6	13.6 ± 2.1
<i>P</i>		0.523	0.386	0.498	0.507	0.183

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (1400 h) to 20 September 2009 (0900 h), and collection 2 spanned from 19 October 2009 (1400 h) to 23 November 2009 (0900 h).

³Transit time is time to first appearance of the Cr marker after dosing [Stevens and Hume, 1995].

⁴Time to 50% recovery (T₅₀) is the time between marker dosing and cumulative recovery of 50% of the marker.

⁵Time to maximum marker concentration (T_{MAX}) is the time between marker dosing and recovery of the maximum marker concentration attained.

⁶R_{GIT} = mean retention time, or the time for the average marker molecule to be excreted after a pulse dose [Stevens and Hume, 1995].

⁷V_N = indigestible fill. It is an estimate of the volume of the digestive tract based on the product of an estimate of the flow rate (total fecal output per 35 d collection) and the total transit time (mean retention time, R_{GIT}). The calculation is V_N = F·R_{GIT}, where F is the total fecal output per the 35 d collection [Holleman and White, 1987].

*Statistical significance at *P*<0.05.

DISCUSSION

Test Diet

Nutrient analysis of the test diet was comparable to the approximate nutrient composition provided by the manufacturer (Table 4). Neutral detergent fiber and sADF concentrations were consistent with those recommended for *G. gigantea* based on the species' natural diet [Edwards, 1991]. Fiber sources, however, were very different as the natural diet of *G. gigantea* consists mostly of grasses, which is made up of larger particles of long stem fiber, whereas the extruded feed contains smaller particles of more easily digested fiber from sources such as ground soybean hulls, ground corn, ground oats, dehulled soybean meal, wheat middlings, and dehydrated alfalfa meal. As a result of these differences, it would be expected that apparent digestibility of the extruded feed would be comparatively higher than that of chelonians' natural diet.

Digestible energy of the test diet was measured at 14.94 kJ DE/g test diet. Metabolizable energy in a ruminant is approximately 82% of DE [Van Soest, 1987]. Using this as a conversion factor, the calculated ME of the diet based on experimentally determined DE would be:

$$\text{ME, kJ/g} = 14.94 \text{ kJ DE/g} \times 0.82 = 12.25 \text{ kJ ME/g test diet}$$

Though *G. pardalis* is a hindgut fermenting animal rather than a ruminant, this ME value calculated from measured DE is very close to the ME value of 12.13 kJ ME/g, calculated using the Atwater system, in which 4 kcal are obtained per g protein, 9 kcal per g fat, and 4 kcal per g carbohydrate (nitrogen free extract, or NFE) [Van Soest, 1987].

Some sources report a slightly different system for herbivorous reptiles, with 3.5, 8.5, and 3.5 kcal ME/g protein, fat, and carbohydrate (neutral detergent soluble carbohydrates, or NDSC, calculated as $\text{NDSC} = 100 - \% \text{ Moisture} - \% \text{ CP} - \% \text{ EE} - \% \text{ Ash} - \% \text{ NDF}$), respectively, as well as an extra 2 kcal ME/g fiber when it is converted to short-chain fatty acids in the hindgut [Donoghue, 1999; Stahl and Donoghue, 2010]. Using the guaranteed analysis of the test diet, this calculation would yield:

$$\text{ME, kJ/g} = (3.5 \cdot 31.2\% \text{ NDSC}) + (8.5 \cdot 5.2\% \text{ Fat}) + (3.5 \cdot 15\% \text{ CP}) + (2 \cdot 29.3\% \text{ NDF}) \cdot 4.184 \text{ kJ/kcal} = 11.07 \text{ kcal ME/g test diet}$$

This calculated ME value of 11.07 kcal ME/g test diet is lower than both the ME value calculated using the tradition Atwater system as well as the ME value calculated from measured DE. If ME is 82% of DE, then DE calculated from this value would be:

$$\text{DE, kJ/g} = 11.07 \text{ kJ ME/g} / 0.82 = 13.50 \text{ kJ DE/g test diet}$$

The DE value calculated this way is greater than that experimentally measured in this study. Validation of this system should be considered.

Temperature

Because the animal facility is an open-air building, differences in ambient temperature may have been influenced by environmental conditions during each period (collection 1: 17 August 2009 to 20 September 2009; collection 2: 19 October 2009 to 23 November 2009). In a study in the natural range of *G. pardalis*, seasonal activity, measured as the number of sightings (n=462) within each month, was highly correlated with minimum ambient temperature [Hailey

and Coulson, 1996]. Ninety-five percent of sightings were between October and April, when mean minimum ambient temperatures were 16.4-20.5°C [Hailey and Coulson, 1996]. Mean minimum ambient temperatures in this study were 16.2°C and 12.8°C in periods 1 and 2, respectively. If a similar correlation between minimum ambient temperature and activity in the species' native range holds true in captivity, activity may have been relatively suppressed, and perhaps more so in period 2 than 1.

This does not take ambient temperature of the space between tortoise shelters and supplemental heat pads into consideration as temperature in this space was not measured, but was presumably higher than ambient temperature of the entire building as heat pads were maintained at surface temperatures between 25-35°C. It is also probable that if tortoise activity was “suppressed” at low minimum ambient temperatures in this study, as in the study of *G. pardalis* in its native habitat, it is likely that animals with “suppressed” activity would have been found on their heat pads, especially under their shelters, and although a formal activity budget was not performed, anecdotally, tortoises were frequently observed in that area at 0900 h (i.e., when ambient temperatures were generally at or very near the daily minimum).

Ideally, in future studies, ambient temperature would be measured continuously in areas specific to tortoise activity. One way of achieving this would be to use temperature data loggers affixed to the tortoises' carapaces that would record ambient temperatures at regular intervals. This would give a more specific characterization of temperature conditions during the trial, and in so

doing also provide the means with which to begin an observational study investigating correlations between environmental temperature and food utilization in captivity. If coupled with cloacal temperature monitoring at daily or weekly intervals, a relationship may also be observed between ambient and cloacal temperature that may be characterized and further used to predict tortoise body temperatures at certain ambient temperatures.

Initial Body Weights (BW)

The increase in initial BW of the juvenile animals from period 1 to 2 is suggestive of growth. Though species-typical growth in *G. pardalis* has not been thoroughly characterized, growth is expected in juvenile animals that are approximately 5 yrs old.

Voluntary Intake

Ad libitum feeding was achieved, as all tortoises had orts at the end of both acclimation periods.

In a review of energetics, *G. agassizii* (mean BW 2.12 kg) had a mean FMR of 42.9 kJ ME/d [Nagy et al., 1999]. If the same BW were applied to the herbivorous reptile FMR prediction equation, created from data generated from eight herbivorous lizards and one herbivorous tortoises,

$$\text{FMR} = (0.232) \cdot \text{BW}_g^{0.813} \text{ kJ ME/d,}$$

an herbivorous reptile of the same size would need 117.43 kJ ME/d; an estimate 2.74 times observed *G. agassizii* FMR [Nagy et al., 1999]. It is possible that *G. agassizii* data were comparatively lower than other herbivorous reptiles used in the development of this equation due to the proportionally lower amount of

metabolically active tissues of the chelonian carapace and plastron, absent in the herbivorous lizards included in the development of the prediction equation [Nagy et al., 1999].

Average observed FMR of *G. agassizii* and *Homopus areolatus* were predicted by the equation:

$$\text{FMR, kJ ME/d} = (0.098) \cdot \text{BW}_g^{0.788} \text{ [Brown et al., 2005].}$$

For comparison, if both the formula for *G. agassizii* and *H. areolatus* and the herbivorous reptile formula were applied to a 1.5 kg leopard tortoise (i.e. approximate mean BW at the beginning of this study) to predict daily energy requirements, the formula for *G. agassizii* and *H. areolatus* would predict a requirement of 40.96 kJ ME/d while the herbivorous reptile formula would predict a requirement of 88.64 kJ ME/d. The herbivorous reptile formula predicts FMR of a 1.5 kg leopard tortoise at 2.16 times the FMR predicted by the formula created using data from tortoises. Using the herbivorous reptile FMR prediction equation may therefore over predict *G. pardalis* energy requirements for maintenance or growth based on the comparison to the formula for *G. agassizii* and *H. areolatus*.

Not only did the data used to create the herbivorous reptile FMR prediction equation include mostly herbivorous lizards with a presumably higher proportion of metabolically active body tissue, data included from tortoises were also only representative of *G. agassizii*. There have been observations of higher (by approximately 33%) mass adjusted FMR ($\text{FMR}/\text{BW}_{\text{kg}}^{0.788}$) in a small species, *H. areolatus*, compared to the larger *G. agassizii* [Brown et al., 2005]. *Geochelone pardalis* is a comparatively larger species than *G. agassizii*, so this

may further suggest that the herbivorous reptile equation provides an over estimate of *G. pardalis* or large herbivorous tortoise metabolic requirements.

Data used to create the herbivorous reptile FMR prediction equation was also obtained from mostly adult and some sub-adult animals, whereas all tortoises involved in this study were juveniles. Although a relatively higher metabolic rate of an endothermic animal may be expected in a growing juvenile compared to an adult, this may not be the case in growing juvenile ectothermic tortoises.

Gopherus agassizii juveniles had an observed FMR approximately 95% that of adults during a season when water was readily available, and approximately 50% that of adults when water was scarce [Nagy et al., 1997; Nagy, 2000]. The authors hypothesized that the lack of change in FMR may have been due to changes in activity patterns between adults and juveniles [Nagy et al., 1997; Nagy, 2000]. Although juveniles likely had a higher growth cost, they may have compensated for energy loss by decreasing their activity levels or remaining in areas with lower ambient temperatures to decrease metabolism when resources were not readily available [Nagy et al., 1997; Nagy, 2000]. Also, observed daily energy expenditure of growing juvenile *G. agassizii* and *H. areolatus* was 71% that of adults in captive outdoor conditions when adjusted for body mass, however juveniles included in that study were exclusively *G. agassizii* and adults exclusively *H. areolatus* [Brown et al., 2005]. The authors justified this comparison as adult *H. areolatus* included were

approximately the same size as juvenile *G. agassizii* in the same study [Brown et al., 2005].

Juvenile tortoises may have the same, or even a lower metabolic rate than adults, which further suggests that the herbivorous reptile FMR prediction equation may over estimate juvenile tortoise metabolic requirements. Animals in this study were initially fed at 200% of predicted herbivorous reptile FMR during acclimation periods, and some animals consumed amounts in excess of 400% predicted herbivorous reptile FMR. Feeding at that level likely would not occur in nature, even during peak seasons when forage is readily available. Tortoises in nature would also be required to travel over distances to obtain food, and may not realistically be able to obtain such a high level of intake due to spatial constraints. Tortoises in this study were not forced to expend nearly as much foraging energy in order to consume their diet, as their diets were offered to them in their enclosures, unlike their wild counterparts.

Based on the idea that the FMR prediction equation for herbivorous reptiles may over predict the energy requirements of an herbivorous chelonian, and on the observation of intakes in excess of 400% predicted herbivorous reptile FMR, it seems that tortoises in this study did not effectively self regulate their feeding when offered food *ad libitum*. This suggests that *ad libitum* feeding of an extruded feed may be inappropriate for juvenile *G. pardalis*, and perhaps for the species. Because adult animals were not included in the study, it is unclear how *ad libitum* feeding would have affected adult *G. pardalis* intake. The colony includes one adult female tortoise that, anecdotally, often consumes less

than 100% predicted herbivorous reptile FMR for her BW. It is possible that adult animals in this situation may more effectively self regulate their feeding compared to juveniles of the species.

In this study, the herbivorous reptile FMR prediction equation provided an objective starting point to model *ad libitum* feeding to characterize voluntary intake, however, it may be impractical to use the formula to predict specific juvenile leopard tortoise energy requirements for diet formulation. Instead, it may be more appropriate to use the formula reported for *G. agassizii* and *H. areolatus* when predicting juvenile *G. pardalis* energy requirements for diet formulation.

Mean Daily DMI and DEI

Mean DMI trends during collection were consistent with those observed for voluntary DMI during acclimation. Consistency in intake results can be seen as validation of the procedure to offer 85% of acclimation intake during collection, though ideally, all animals would have consumed all offered diet and no orts would have remained. Additionally, a period between the acclimation and collection phases wherein animals are offered the restricted amount may be more practical in encouraging intake during collection that is consistent with intake during acclimation to minimize variation between phases.

As animal BW increased throughout the study (i.e. growth), mean daily DMI increased from period 1 to 2 on an absolute basis (g, or g/d). No difference in mean daily DMI was detected when adjusted per mean kg BW, as the amount of food offered was not only a function of voluntary intake during the acclimation

phase, but was also calculated weekly based on increasing BW. As animals grew, more food was offered, but in proportion to the animals' BW.

Growth

BW Gain

Mean BW gain (n=18) through the 18 week study appears linear (Figure 4). When mean BW gain is put in perspective with data obtained since the tortoises' acquisition at Cal Poly in April 2008, mean BW gain (n=18) before and after the trial (with animals fed a fixed percent of predicted herbivorous reptile FMR) appears linear, while growth during the trial appears exponential (Figure 5). If growth was exponential during the study, this may explain why mean BW gain was higher per kg initial BW in period 2 compared to period 1.

Figure 4. Mean weekly BW_{kg} of *G. pardalis* (n=18) throughout acclimation and collection of both periods, totaling 18 weeks.

Figure 5. Mean weekly BW_{kg} of *G. pardalis* (n=18) before, during, and after the trial, with lines indicating the beginning and end of the trial.

Compared to other captive and semi-captive (i.e., maintained mostly on native vegetation but sometimes supplemented with greens) *G. pardalis*, mean BW of this group (n=18), as a function of age, appears within the range of existing growth data [Wilson, 1968; Ritz et al., 2009]. Data from the Cal Poly *G. pardalis* colony appears to reach a comparatively higher growth rate when fed *ad libitum* during this trial, indicated by lines in Figure 6, compared to observed mean growth pre-and post-trial when animals were fed a fixed percent of herbivorous reptile FMR. The higher growth rate observed when fed *ad libitum* during this study resembles that observed in previously observed captive animals (Figure 6). Observed mean growth rate of the Cal Poly colony appears more similar to the semi-captive growth rate when fed a fixed percent of predicted herbivorous tortoise FMR pre-and post-trial (Figure 6), though mean BW of the

Cal Poly colony post-trial was considerably heavier than that of the semi-captive animals reported at similar ages.

Figure 6. Growth curve obtained from the literature including data points from captive (filled circles and triangles) and semi-captive (i.e., maintained in outdoor enclosures with access to native vegetation and occasionally supplemented with greens) (open circles) *G. pardalis* overlaid with data obtained from the Cal Poly *G. pardalis* colony, with lines to indicate the approximate duration of this study [Wilson, 1968; Ritz et al., 2009].

When fed *ad libitum* during the acclimation phases of this trial, and when fed at a level higher than 100% predicted herbivorous reptile FMR during the collection phases of this trial, tortoises gained BW at an accelerated rate compared to when fed a fixed amount equivalent to 100% or less of predicted herbivorous reptile FMR. If the semi-captive growth data illustrated in Figure 6 is used as the closest reference to species-typical growth of *G. pardalis*, the observed accelerated growth during this trial suggests that feeding this diet *ad libitum*, or at a level above 100% predicted herbivorous reptile FMR, may be inappropriate for juvenile *G. pardalis*.

Juvenile tortoises with increased growth rates may reach sexual maturity at a premature age. This may have consequences for those animals, and may interfere with the goal of promoting longevity in this long-lived species in captivity.

Accelerated growth in juvenile tortoises can lead to obesity, potentially to the point where a hinge back tortoise, whose main defense is to retract into its shell and use a hinge to close off its shell cavity, may be too large to actually seal itself off into its shell [personal observations].

Accelerated growth has also been associated with high mortality, renal disease, deformities of the shell and skeletal system such as those caused by nutrition-related metabolic bone disease, and pyramiding [Donoghue, 2006; McArthur and Barrows, 2004]. One source claims that pyramiding and abnormal growth are more likely to occur in animals given high quality foods, and in animals exposed to long winter photoperiods, with no seasonal changes such as brumation (i.e., hibernation) or a seasonally restricted diet resembling conditions of food scarcity [McArthur and Barrows, 2004]. High temperatures, such as those experienced in the summer during period 1 of this study, if experienced in nature, could possibly result in water and food scarcity that may slow or halt growth in juvenile tortoises. Without challenging tortoises with simulated periods of food scarcity, or with seasonal temperature changes that may influence digestibility of GE, as observed in the other arid adapted reptilian herbivores, conditions may have the potential to become conducive to overconsumption and obesity [Harlow et al., 1976].

Managed brumation may be useful in controlling accelerated growth in captivity in a group of animals fed in excess or fed a highly digestible diet, as it may prevent continuous annual growth [Donoghue, 2006; McArthur and Barrows, 2004]. Mimicking seasonal food availability may also offer a solution to continuous accelerated growth, as a group of animals can be managed to undergo periods of growth followed by periods during which growth is slowed or stopped by reducing the amount of a diet offered [McArthur and Barrows, 2004].

Two sources suggest feeding adult tortoises three times a week, but without data for validation, or descriptions of amounts [Boycott and Bourquin, 1988; Boyer and Boyer, 1996]. This may be a valid suggestion in the case of *ad libitum* feeding in juveniles, as it was observed in this study that tortoise growth was slower when juveniles were fed *ad libitum* 3 days per week compared to 7 days per week. However, this may not be the case in adult animals, or animals fed a fixed amount of food per week, as these situations were not addressed in this study. What was observed in this study was likely a function of intake, as animals fed *ad libitum* consumed less on a weekly basis when adjusted per kg BW, and gained less BW (per kg initial BW) when fed 3 compared to 7 days per week.

Measurements (MSCL, PW, CH, SV)

Changes in measurements were analyzed as a secondary growth indicator to challenge the idea that BW gain may be a result of gut fill rather than actual growth. Gut fill was also approximately estimated by calculated indigestible fill (V_N), or the gastrointestinal capacity for undigested material. Shell

volume was calculated as a combination of the three measurements to produce a value that was more resistant to variation in individual measurements (i.e. human error).

All growth measurements, except MSCL, demonstrated significant differences between treatments that were consistent with BW gain results (i.e., more growth was observed when animals were fed 7 compared to 3 days per week). This supports the observation that animals grew more when fed 7 compared to 3 days per week, rather than that they simply gained BW.

Plastron width is the only growth measurement that increased more in period 2 than 1, which was consistent with BW gain results. A significant difference in SV growth was detected between periods on an absolute basis, but not when adjusted per cm³ initial SV. Differences in BW gain between periods may then be viewed as a result of gut fill rather than actual growth if SV growth was not higher during period 2 compared to 1, as tortoises consumed more during period 2, and had higher BW gain results. Differences in BW gain between periods without accompanying changes in SV may be suggestive of a change in body composition, but this could not be measured during this study.

Body Condition Index (BCI)

Overall, mean BCI in this study was similar to values reported in the literature for *Homopus signatus signatus* and *G. agassizii* [Loehr et al., 2007]. The BCI applied in this study has not been evaluated in conjunction with other direct or indirect measurements of body composition (e.g. organ weight proportions, dual energy x-ray absorptiometry, ultrasound). It was developed as

a rapid method of condition assessment in the field, and has not been validated for use in captivity [Loehr et al., 2007; Lickel and Edwards, 2009]. A more appropriate method of developing a body condition index might involve regressing body weight and shell volume rather than using a simple linear equation for the quotient of the two, though this would have been outside the scope of this study.

Although a difference in mean BCI was detected between treatments, the biological significance of that difference is unclear. Mean BCI was not different between periods. This is consistent with the lack of difference in all measurements (except PW) between periods when adjusted per unit of the initial measurement. Shell volume, however, is not a definitive measurement. It is a calculated estimate, and may not be accurate enough to assess small changes in growth observed over just a few months. It may become a more useful tool as data is collected and assessed over years of growth.

Apparent Digestibility (aDig, %)

Inconsistent with reports from other species, aDig cellulose was higher than aDig HC (Appendix C). Hemicellulose digestibility is generally higher than that of cellulose in hindgut fermenting species as the pectins and polymers of cellulose are more resistant to digestion by non-ruminant microflora [Van Soest, 1987; Baer, 1994; National Research Council, 2007]. Future studies investigating relationships between calculated aDig HC and aDig cellulose in herbivorous chelonians might provide interesting results for comparison.

Cell contents are easily digested by endogenous enzymes and rapidly absorbed in the herbivore small intestine, whereas fermentation of cell wall components in the hindgut is a much slower process [Van Soest, 1987; Bjorndal, 1989; Bjorndal, 1997]. Because DM and OM of the test diet include significant levels of cell contents, with approximately 72.6% and 74.8% cell contents, and only 27.4% and 25.2% cell wall, respectively (Table 4), high apparent digestibility of DM, OM, and GE (mean aDig >80%) may be expected. The relatively rapid digestion and absorption of cell contents may have contributed to the lack of difference in apparent digestibility of DM, OM, and GE between treatments.

The relatively slower process of cell wall fermentation may have contributed to the increase in apparent digestibility of cellulose when fed 3 compared to 7 days a week, as it was predicted that feeding 3 compared to 7 days a week would slow digesta transit and increase digesta retention. However, no significant difference was observed in apparent digestibility of NDF, sADF, cell wall, or hemicellulose between treatments.

In a study of American black ducks, *Anas rubripes*, animals were fed 7 days a week, and subsequently fasted either 2 or 4 consecutive days per week [Barboza and Jorde, 2001]. Animals exhibited decreased apparent metabolizability of DM, energy, ADF, and cellulose when fasted, but NDF and hemicellulose metabolizability was not different [Barboza and Jorde, 2001]. In this study, apparent digestibility of cellulose was increased when animals were exposed to shorts fasts (i.e., when fed only 3 days per week), with no other differences between treatments. These differences might be related to the

omnivorous feeding pattern of *A. rubripes* compared to the herbivorous feeding pattern of *G. pardalis* that may allow this species to more efficiently digest structural carbohydrate fractions [Bjorndal and Bolten, 1993]. Another factor is the relatively short retention time in birds, with rapid flow through the digestive tract, compared to the long observed R_{GIT} in *G. pardalis* of 381.1 h [Barboza and Jorde, 2001].

In a previous study of *G. pardalis*, aDig OM of *Brassica* (i.e. kale) and *Lolium* (i.e. grass) were $82.2 \pm 1.2\%$ and $63.9 \pm 4.0\%$, respectively (Table 1) [Hailey, 1997]. Apparent digestibility of OM in this study was $86.0 \pm 3.9\%$, a value very similar to that observed in *G. pardalis* consuming kale, and higher than that observed in *G. pardalis* consuming grass [Hailey, 1997]. This may be related to the ingredients used in the test diet as some of the ingredients (e.g., ground corn, ground oats, cane molasses) are more easily and rapidly digested by the animal and/or the symbiotic microflora of the hindgut, compared to grasses. Although this is a higher fiber diet, it also has relatively easily digestible fiber sources (e.g., soybean hulls, ground corn, ground oats, wheat middlings) compared to grasses.

One author suggested that a “high fiber (hence, low-calorie) diet, all-day access to food (instead of meal feeding), much opportunity to exercise through behavioral enrichment, exposure to unfiltered sunlight, a temperature gradient that includes highest preferred temperatures for that species with proper humidity, and continual access to water with weekly soaking” produces a low risk for pyramiding, presumably encouraging healthy growth [Donoghue, 2006]. This

does not, however, address diet ingredients or digestibility. The extruded feed used in this study, though relatively high in fiber was highly digestible (i.e., aDig DM was approximately 84.4%), and was consumed at intake rates in excess of 400% of predicted herbivorous reptile FMR when fed *ad libitum*, with growth rates visibly (by graph) exceeding those of semi-captive animals fed native vegetation. Using those recommendations for juvenile *G. pardalis* fed this extruded diet may not be appropriate as it is very digestible, even though it has a high fiber content.

Digesta Transit, Digesta Retention, Indigestible Fill

Marker Recovery

Mean Cr marker recovery in this study was $77.1 \pm 28.3\%$, approximately six percentage points lower than the $83 \pm 8\%$ recovered in a similar study in *G. agassizii* fed *Schismus barbatus* grass (NDF = 64.6%, DMB) and a high fiber (NDF = 48.4%, DMB) pellet with a 35-day collection period [Barboza, 1995]. Recovery may have been lower in this study due to the nature of the enclosures. Animals in this study were maintained on concrete floors whereas those in the previous study were maintained in metabolism cages, which were likely more effective in allowing researchers to collect fecal samples in their entirety rather than losing small amounts to the uneven concrete. Diets were also different between the two studies, with different ingredients and nutrient compositions, and temperatures were more variable in this study as animals were maintained in semi-outdoor environments, both of which may have influenced the collection time needed to recover the full marker dose. Longer collection times to recover

more of the dose, especially since one of the animals in this study did not defecate during the entire second collection phase may be advised.

Low Cr recovery in this study may have been largely a function of the rate of defecation of the animals involved. Animals in this study, in general, defecated approximately once a week. Some defecated just after the end of the predetermined collection phases, so those defecations could not be included in analysis. Longer collection phases, or collection phase time periods set to a minimum time frame, but also to a certain number of defecations recovered, rather than on time alone, may improve marker recovery in future studies.

Digesta Transit

Increased intake generally results in shorter TT_1 [Bjorndal, 1989; Bjorndal, 1997]. This was observed when all animals were included ($n=18$), as intake was higher and TT_1 was lower (i.e. shorter) when animals were fed 7 compared to 3 days per week (Table 8, Table 19), however, this was not observed when four animals were removed (Table 20). Animals also exhibited higher intake during period 2 than 1, but no statistical differences were detected in TT_1 (Table 8, Table 19, Table 20).

It should be considered that residual (i.e., carryover) marker dosed in period 1, could underestimate TT_1 in period 2 for that animal when <50% of the marker was recovered in period 1 fecals collected for a single animal.

The four animals removed from TT_1 analysis were fed 3 days per week in period 2 and exhibited a corresponding increase in TT_1 (i.e., TT_1 was slower) compared to being fed 7 days per week in period 1. One animal did not have

corresponding data as she did not defecate during the entire collection phase when fed 3 days per week. However, that would suggest a $TT_1 > 35$ d, which is slower than her observed TT_1 when fed 7 days per week. The carryover effect, it seems, would not have affected transit time in this case then, as that effect should result in faster (shorter) observed TT_1 . Removing these animals from TT_1 analysis may have actually obscure a difference in TT_1 between treatments, though it seems difficult to rationalize removing them from all other measures of digesta transit while they remain in TT_1 analysis. Future studies using longer, or other more appropriate, collection periods as described before may provide more reliable digesta transit results for comparison and validation.

Digesta Retention

Generally, animals fed more frequently have shorter retention times [Warner, 1981]. Though not observed when all animals were included in analysis (Table 19), R_{GIT} was significantly shorter among animals fed 7 compared to 3 days per week when four animals were removed for low marker recovery (Table 20). This may be explained by the nature of the calculation for R_{GIT} , as the four animals removed all exhibited $< 50\%$ Cr marker recovery.

One author explained that low marker recoveries can likely lead to underestimates of R_{GIT} [Barboza, 1995]. Due to the nature of the calculation, it seems that if Cr marker recovery was low and stopped early (or paused at low recovery and did not resume until after the collection phase), R_{GIT} would be underestimated, but if the same small amount of Cr marker was excreted over a longer period of time, estimates of R_{GIT} may not have been as low.

For example, two animals are dosed with 40 mg Cr, and each excrete 10% of the marker (4 mg Cr). Animal one excretes 4 mg on the first day and does not excrete anything else for the next 4 weeks of collection. Animal two excretes 1 mg per week during a 4 week collection phase. Neither animal has excreted the full dose by the end of the 4 week collection phase, and thus R_{GIT} cannot be accurately calculated, but the estimates are as follows:

Animal 1: excretes 4 mg on the first day and nothing more

$$R_{GIT} = [(4 \text{ mg})(1 \text{ hr})]/4 \text{ mg} = 1 \text{ hr}$$

Animal 2: excretes 1 mg per week for 4 weeks

$$R_{GIT} = [(1 \text{ mg})(1 \text{ hr}) + (1 \text{ mg})(169 \text{ h}) + (1 \text{ mg})(337 \text{ h}) + (1 \text{ mg})(506 \text{ h})]/4 \text{ mg} = 253.25 \text{ h}$$

The estimate for animal 1 is much lower due to its pattern of excretion, but because only 10% of the marker was recovered, this estimate cannot be accurate. Also, if animal 1 did not defecate until the fourth week, the estimation would be 672 h, even though the same amount of marker was recovered by that point. This is likely because the formula for R_{GIT} assumes continuous digesta volume and flow rates that were not observed [Blaxter et al, 1956; Holleman and White, 1987]. Continuous flow rates were also not observed in this study. For example, one animal defecated six times during period 2, and defecations were at intervals of 197, 24, 211, 202, and 86 h. Criticism was given even to studies in which herbivores were fed at different times of day, due to variation in the rate of production of feces over 24 h [Blaxter et al., 1956]. The formula might need to be re-evaluated for use in other non-continuously excreting animals. Perhaps

samples could be combined weekly or monthly for each animal, based on previous observations of defecation patterns, and the formula could include those larger intervals of time as t_i , rather than hours or days. This might produce more consistent data among animals, though it would still assume continuous flow averaged over each week or month.

Indigestible Fill

Indigestible fill, V_N , was estimated in this study to address questions about digestive capacity. Because the calculation is based on R_{GIT} and fecal output, inconsistent excretions and low Cr marker recoveries may have also misrepresented estimates of V_N as well [Barboza, 1995].

With all animals included ($n=18$), V_N was higher in animals fed 7 compared to 3 days per week, consistent with higher intake and growth observed when fed 7 compared to 3 days per week. It has been previously described that gut capacity is isometric with body size in mammalian herbivores [Demment and Van Soest, 1985].

Indigestible fill during period 1 was higher than during period 2, however, inconsistent with higher observed intake during period 2. When the four animals with <50% Cr marker recovery were removed, no differences were detected. This might be an indication that spurious results were being observed due to inaccurate estimates of R_{GIT} and, subsequently, inaccurate estimates of V_N due to low Cr recovery.

CONCLUSIONS

In general, tortoises fed 7 compared to 3 days per week in this study exhibited higher intake, faster growth, shorter digesta transit and retention times, and lower digestibility of cellulose, but exhibited no changes in digestibility of readily available nutrients (DM, OM, GE) or other fiber components (NDF, sADF, ADL_{OM}, HC). Digesta was retained for shorter periods when food was available every day (i.e., when animals were fed 7 days per week), and was retained longer when food availability included short periods of fasting (i.e., when animals were fed 3 days per week). This strategy may allow a tortoise undergoing a fasting period to extract more energy from its food as digesta retention likely had the largest effect on digestibility of cellulose. Digestion of cellulose by microbial fermentation in the tortoise hindgut is a slow process, and as digesta was retained longer, more complete fermentation may have been permitted, providing more energy to the host animal.

Feeding juvenile *G. pardalis* a highly digestible, extruded diet *ad libitum*, 7 days per week is not recommended. All animals in this study consumed amounts of at least 200% of predicted herbivorous reptile FMR, with some consuming amounts in excess of 400%, and mean growth of the colony was accelerated compared to when mean growth of the colony observed when held at restricted amounts (i.e., 50% and 100% of predicted FMR) pre- and post-trial.

Providing short fasts for tortoises may be useful in slowing growth as animals fed *ad libitum* 3 days per week in this study generally consumed less and grew at a less accelerated rate compared to animals fed 7 days per week.

Another practical way to encourage healthy growth in herbivorous tortoises might include mimicking seasonal food availability to prevent continuous annual growth.

In addition, the FMR prediction equation for herbivorous reptiles is likely an over prediction for herbivorous chelonian FMR. The FMR prediction equation reported for *Gopherus agassizii* and *Homopus areolatus* may be more practical for use in herbivorous chelonian diet formulation [Brown et al., 2005]. Future energetics studies to develop a prediction equation for *G. pardalis*, as well as to evaluate existing herbivorous chelonian FMR prediction equations would be helpful in guiding future herbivorous chelonian diet formulations.

There is also a need for the development of a more appropriate BCI for captive chelonians, such as one involving regression of body weight and shell volume. The BCI used in this study is very useful for researchers assessing initial status of an animal in the field, but may have limitations for application to captive animals due to the nature of the calculation. However, though the simple equation for BCI may be limited by its linear nature, trends in individual measurements were generally consistent with BCI trends. A more complex BCI calculation may need further development, but the current BCI used in this study may still be useful in investigating trends for comparison. Validation of the BCI used in this study could also be done through formal body composition studies to determine its value.

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APPENDICES

Appendix A. Standard operating procedure for grinding dry feed and fecal samples ≤ 50 g in preparation for laboratory procedures.

Animal Science Department
Policies and Procedures

Title	Grinding dry samples (≤ 50 g)
Owner	Mark S. Edwards, Ph.D.
Approval	Mark S. Edwards, Ph.D., Associate Professor
Revision Level	1.0
Revision Date	19-May-2010
Scope	Sub-sampling or sample reduction of an unprocessed sample in the laboratory is frequently the single largest source of variation during the analysis procedure and should be avoided whenever possible. This method is applicable for conversion of pelleted feeds, forages and feces to powder for laboratory analysis.
Principle	The cutting mill is used to obtain a homogeneous sample particle size. Laboratory samples can be passed through 2 mm sieves to reduce to a particle size appropriate for further sub-sampling and analysis.
References	National Forage Testing Association Method 1.1 www.foragetesting.org
Equipment	Cutting mill (Stainless Steel Wiley Mini Mill; Thomas No. 3383L60) Delivery tube with Monel metal sieve top, size 10 mesh, 2 mm openings (Thomas No. 3383N05) Receiver, stainless steel, for threaded adapter (Thomas No. 3383N40) Adapter (plastic screw cap), threaded, size 58 Glass jar, screw cap, 4 oz. (Thomas No. 6180H19) Hopper with cover, stainless steel, 2.5 in. diameter (Thomas No. 3383M15) Front plate, glass (Thomas No. 3383N55) Plunger, wooden (Thomas No. 3383M20) Wet/Dry vacuum, 4.5 gallon (Ridgid WD45500) Micro-kit accessories for wet/dry vacuum (Emerson Tool Co. VT1215) Cleaning instruments (forceps, dental pick, baby tooth brush) Delicate task wiper (Kimtech Science Kimwipes)

Reagents

Isopropyl alcohol, 70% v/v (CAS No. 67-63-0)

Acetone (CAS No. 67-64-1)

Safety

1. IMPORTANT. Thomas Stainless Steel Wiley Mini Mill cutting mill **is not** equipped with a safety shut off switch to prevent operation of the blades with the cutting chamber glass cover off. The cutting mill should be switched off **and** physically disconnected from the electrical source before opening taking off the cutting chamber glass cover.
2. Wearing of hearing protection when operating the cutting mill and wet/dry vacuum is recommended. The associated noise level is on the threshold of being hazardous.
3. The Thomas Stainless Steel Wiley Mini Mill should only be operated in a ventilated hood, and precautions should be taken to avoid inhaling dust when handling samples.
4. Wear safety glasses to protect eyes from material that may be expelled from grinder and cleaning reagents.
5. Do not insert fingers or objects into the grinding mill.
6. Observe all safety and operating instructions supplied by the manufacturer of the grinding equipment.
7. Use standard precautions when working with electrical equipment.
8. Make sure that all electrical equipment is properly grounded and installed and maintained by qualified electricians.

Procedure

1. Inspect the mill for cleanliness.
2. Slide the swing-out platform so it is sitting below the cutting chamber.
3. Attach the delivery tube with the appropriate size Monel metal sieve top (mesh size 10, 2 mm) to the screw cap glass jar with the square receiver sitting between the cap and the jar.
4. Slide into the slot in front of the mill and raise the tube until it hits the stop pin located on the back plate of the grinding chamber.
5. Secure by raising the sliding side-arm support until its groove engages the side arm on the side of the delivery tube, and tightening the knurled nut.
6. Turn the rotor shaft by hand counterclockwise to check for sieve clearance and adjust height of sieve if necessary.
7. Place the glass plate over face of the cutting chamber and secure with the adjustable rod clamp.
8. Insert the hopper into the upper milling chamber

- opening.
9. Attach the line cord to power source and press cord switch to start operation.
 10. Run empty for 5-10 seconds.
 11. Feed sample into the hopper slowly enough so that the mill does not slow down or become jammed. The wooden plunger may be used to force materials that do not pass freely into the chamber.
 12. Allow the mill to run for at least 60 seconds after the last portion has been added to ensure that the entire sample has passed through.
 13. Turn off power. Disconnect cutting mill from electrical supply.
 14. Disassemble the mill in the opposite order of assembly.
 15. Clean the entire mill using Kimwipes, brush, compressed air, and shop vacuum. Wipe thoroughly with Kimwipes to remove fine particles. Using a pair of forceps with a Kimwipe wrapped around it works best to dislodge particles caught behind the knives.
 16. Fecal samples only. A 2-step rinse with fecal samples is required: alcohol to sanitize, acetone for final rinse and drying. Use conservative amounts of both compounds for this process.
 17. All other samples. A 1-step rinse with acetone for all other samples.
 18. Compressed air can be used to facilitate drying.
 19. Do not proceed with processing the next sample until the cutting mill chamber and knives are visibly dry.

Comments

- If mill becomes overheated, power will automatically shut off. Turn off, disconnect from power, and cautiously remove the glass plate to remove sample from grinding compartment. Allow mill to cool prior to continuing use. We generally put lab paper underneath to catch and save sample material.
- The knives need periodic sharpening. Proper alignment of the knives on re-installation is critical to ensure efficient grinding. Distance between stationary and rotating blades should not exceed the thickness of a piece of paper.
- Dust will accumulate in the motor housing compartment and should to be vacuumed out periodically.
- Please report any irregularities so that any maintenance issues can be addressed.

Appendix B: Standard operating procedure for preparing chromium mordants for digesta transit studies.

Animal Science Department
Policies and Procedures

Title	Chromium mordant of plant cell wall
Owner	Mark S. Edwards, Ph.D., Associate Professor
Approval	Mark S. Edwards, Ph.D., Associate Professor
Revision Level	2.0
Revision Date	05-Aug-2009
Scope:	TBD
Principle:	TBD
References:	Uden P, Colucci PE, Van Soest PJ. 1980. Investigation of chromium, cerium and cobalt as markers in digesta: rate of passage studies. Journal of the Science of Food and Agriculture 31:625-632.
Equipment:	Digital scale (3100 g x 0.1 g) Beaker, 3000 mL Hot plate Polyethylene filter, 50 µm Aluminum roasting pan (42.2 cm x 30.2 cm x 6.7 cm) Forced-draft oven
Reagents:	Sodium dichromate dihydrate ($\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$) CAS 7789-12-0 L-Ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) CAS 50-81-7 Water (pretreated via reverse osmosis filtration) (H_2O)
Procedure:	<ul style="list-style-type: none">• Prepare and dry plant cell wall (NDF) from material selected• Weigh plant cell wall fraction; record the initial weight <ol style="list-style-type: none">1. Prepare 4 volumes of a sodium dichromate dihydrate ($\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$) solution containing an amount of chromium equivalent to 14% of the initial weight of ND fiber used

a. Example:

i. $\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O} = 34.9\% \text{ Cr}$
1. $103.992 / 298.00 = 34.9\%$

ii. To label 100 g of fiber

iii. $100 \text{ g fiber} \times 0.14 = 14 \text{ g Cr}$

iv. $14.0 \text{ g} / 0.349 = 40.11 \text{ g Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$

2. Place plant cell wall to be mordanted into the aluminum
3. Add 4 volumes of sodium dichromate dihydrate solution into the aluminum pan; confirm there is adequate solution to cover the entire amount of plant fiber
4. Cover the pan with aluminum foil
5. Bake mixture in a 100 °C oven for 24 h
6. Pour solution through the polyethylene filter
7. Wash the plant fiber thoroughly with purified water
8. In a large beaker, suspend the fiber in water
9. Add ascorbic acid equivalent to 50% of the initial weight of the plant cell wall (the liquid should taste slightly sour)
10. Let the fiber soak for at least 1 h
11. Fiber should turn green in color at this stage.
12. Pour solution through the polyethylene filter
13. Rinse the fiber with purified water until free of soluble (green) material.
14. Transfer remaining material from filter to aluminum roasting pan
15. Dry to a constant weight in a 65 °C forced-draft oven
16. Analyze a sample of the resulting material to confirm Cr content before use.

**Revision
history:**

1. Use of brass sieve discontinued due to potential contamination of sample with lead from brass and solder (50% Pb, 50% Sn); replaced with polyethylene filter bag, 50 µm pore size.
2. Final rinse changed from tap water to purified water

Appendix C. Apparent digestibilities (aDig, %) of dry matter (DM), organic matter (OM), gross energy (GE), neutral detergent fiber (NDF), sequentially determined acid detergent fiber (sADF), acid detergent lignin on an organic matter basis (ADL_{OM}), cellulose (Cell), hemicellulose (HC), and cell wall of *Geochelone pardalis* (n=18) fed at 85% of weekly voluntary intake level distributed over 3 or 7 days.

100

Tortoise	Treatment ¹	Period ²	aDig, %								
			DM	OM ³	GE	NDF ⁴	Cell Wall ⁵	sADF ⁶	ADL _{OM} ⁷	Cell ⁸	HC ⁹
307103	1	7	86.4	87.1	84.4	80.1	79.7	83.5	85.2	88.5	74.7
	2	3	83.1	84.0	80.9	75.4	75.3	78.6	80.1	84.4	70.4
307112	1	7	84.0	85.1	82.0	75.7	79.3	79.8	80.9	86.8	69.1
	2	3	83.2	84.5	81.5	74.4	73.7	78.3	80.0	85.8	68.3
307101	1	3	84.8	85.4	81.9	77.2	76.7	80.9	82.7	87.4	71.3
	2	7	86.7	87.3	84.8	80.3	79.6	83.8	83.8	89.4	74.7
307100	1	3	84.7	85.0	82.2	76.8	75.9	77.1	82.1	81.3	76.3
	2	7	96.7	96.8	96.1	94.6	94.5	95.3	95.7	96.7	93.5
307105	1	7	86.4	87.1	84.3	79.3	78.7	81.3	83.6	86.3	75.9
	2	3	89.2	89.8	87.4	84.2	84.1	86.4	87.1	91.0	80.8
307111	1	3	85.0	85.9	82.5	78.4	78.3	81.4	82.1	88.1	73.6
	2	7	88.2	88.6	86.5	82.7	82.0	85.3	86.4	89.7	78.5
307099	1	7	83.9	84.4	81.2	74.0	72.7	74.7	82.4	78.8	72.8
	2	3	80.8	81.1	78.7	65.6	63.8	63.0	79.0	66.7	69.4
307107	1	3	79.8	80.2	77.1	65.7	74.3	64.8	76.9	68.7	66.8
	2	7	84.5	85.2	81.6	79.0	78.5	82.0	81.4	87.3	74.2
307097	1	7	89.1	89.7	87.7	83.9	83.4	86.1	88.1	90.0	80.6
	2	3	81.4	82.7	78.4	74.9	75.0	78.4	78.8	85.9	69.3
307113	1	7	79.7	81.0	76.6	68.9	68.4	73.0	76.3	82.6	62.3
	2	3	88.6	89.1	86.9	82.2	81.6	84.7	87.2	89.1	78.2

Appendix C (continued). Apparent digestibilities (aDig, %) of dry matter (DM), organic matter (OM), gross energy (GE), neutral detergent fiber (NDF), sequentially determined acid detergent fiber (sADF), acid detergent lignin on an organic matter basis (ADL_{OM}), cellulose (Cell), hemicellulose (HC), and cell wall of *Geochelone pardalis* (n=18) fed at 85% of weekly voluntary intake level distributed over 3 or 7 days.

Tortoise	Treatment ¹	Period ²	aDig, %								
			DM	OM ³	GE	NDF ⁴	Cell Wall ⁵	sADF ⁶	ADL _{OM} ⁷	Cell ⁸	HC ⁹
307110	1	7	83.8	84.9	82.1	75.4	75.0	79.8	81.9	86.6	68.3
	2	3	83.4	84.7	81.7	74.9	74.4	79.9	81.3	87.1	66.9
307094	1	7	84.9	85.3	82.7	76.0	74.8	75.9	83.9	79.6	76.0
	2	3	85.3	86.3	83.0	80.8	80.3	83.5	83.3	88.9	76.7
307109	1	3	82.2	83.3	78.4	75.7	75.4	79.6	78.9	86.9	69.1
	2	7	87.3	88.0	85.6	80.3	79.7	83.6	85.2	89.1	74.8
307106	1	3	81.0	82.2	78.3	73.1	66.7	76.8	79.0	83.5	67.3
	2	7	86.9	88.3	85.5	82.8	82.8	85.6	84.5	90.9	78.3
307104	1	3	83.5	84.2	80.8	73.4	72.5	76.2	81.5	82.3	69.0
	2	7	100	100	100	100	100	100	100	100	100
307114	1	3	81.6	82.5	78.7	73.4	72.8	76.4	78.7	83.1	68.4
	2	7	85.0	86.0	83.3	78.3	77.7	81.5	82.3	87.3	73.1
307098	1	3	82.2	83.3	79.7	75.8	73.7	80.3	79.3	87.1	68.7
	2	7	85.2	86.1	83.5	79.1	78.4	82.1	83.0	87.2	74.6
307102	1	7	82.0	83.1	79.1	74.6	74.0	79.4	79.7	86.1	66.8
	2	3	86.9	87.9	85.4	81.1	80.6	84.9	84.7	90.0	75.1

Appendix C (continued). Apparent digestibilities (aDig, %) of dry matter (DM), organic matter (OM), gross energy (GE), neutral detergent fiber (NDF), sequentially determined acid detergent fiber (sADF), acid detergent lignin on an organic matter basis (ADL_{OM}), cellulose (Cell), hemicellulose (HC), and cell wall of *Geochelone pardalis* (n=18) fed at 85% of weekly voluntary intake level distributed over 3 or 7 days.

Tortoise	Treatment ¹	Period ²	aDig, %								
			DM	OM ³	GE	NDF ⁴	Cell Wall ⁵	sADF ⁶	ADL _{OM} ⁷	Cell ⁸	HC ⁹
Mean	-	3	86.0±5.0	86.8±4.7	83.8±5.9	79.8±7.3	79.6±7.5	82.8±6.4	83.7±5.8	88.4±4.6	75.0±9.0
	-	7	84.4±2.9	85.2±2.8	82.3±3.2	76.2±5.1	75.6±5.0	78.5±6.3	82.2±3.2	83.9±6.6	72.4±4.7
	1	-	83.6±2.4	84.4±2.3	81.1±2.8	75.4±4.0	75.1±4.0	78.2±4.6	81.3±2.9	84.1±5.0	70.9±4.5
	2	-	86.8±4.9	87.6±4.6	85.1±5.5	80.6±7.5	80.1±7.8	83.2±7.5	84.7±5.5	88.1±6.5	76.5±8.4
Mean	All		85.2±4.1	86.0±3.9	83.1±4.7	78.0±6.5	77.6±6.6	80.7±6.6	83.0±4.7	86.1±6.1	73.7±7.2

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (14:00) to 20 September 2009 (09:00), and collection 2 spanned from 19 October 2009 (14:00) to 23 November 2009 (09:00).

³OM = DM – ash.

⁴NDF is composed of hemicellulose, cellulose, lignin, cutin, and ash.

⁵Cell wall is composed of NDF ash free, or NDF – ash [Bjorndal, 1987; Bjorndal, 1989; Bjorndal and Bolten, 1993]

⁶sADF is composed of cellulose, lignin, cutin, and ash.

⁷ADL_{OM} is acid detergent lignin on an organic matter basis, or ADL – ash.

⁸Cell = sADF - ADL.

⁹HC = NDF - sADF.

Appendix D. Marker recovery, digesta transit including transit time (TT), time to 50% marker recovery (T_{50}) and time to maximum marker concentration (T_{MAX}), digesta retention (R_{GIT}) and indigestible fill (V_N) in *G. pardalis* (n=18) fed 85% of weekly voluntary intake distributed over 3 or 7 days.

Tortoise	Trt	Pd	Marker Recovery, %	Digesta Transit, h			Digesta Retention, h	Indigestible Fill (V_N) ⁷	g/BW _{kg}
				TT ³	T_{50} ⁴	T_{MAX} ⁵			
307103	1	7	95.1	167.5	316.5	167.5	304.1	13.2	7.8
	2	3	93.8	119.5	316.5	316.5	285.7	32.0	14.3
307112	1	7	103.0	167.5	503.5	503.5	387.1	13.0	10.9
	2	3	98.5	172.5	340.5	172.5	335.0	21.6	14.1
307101	1	3	83.2	167.5	503.5	316.5	449.5	19.0	11.4
	2	7	89.6	172.5	340.5	340.5	434.1	27.2	12.9
307100	1	3	2.1	124.5	--	--	439.3	55.4	20.6
	2	7	23.4	124.5	--	--	202.2	3.7	1.7
307105	1	7	74.5	167.5	383.5	316.5	361.0	17.0	10.5
	2	3	69.1	297.5	820.5	820.5	570.0	15.2	10.6
307111	1	3	87.7	124.5	580.5	580.5	424.7	14.3	11.8
	2	7	81.7	81.5	316.5	316.5	368.4	15.5	10.9
307099	1	7	81.6	167.5	503.5	167.5	429.4	54.4	17.0
	2	3	85.5	95.5	273.5	172.5	243.1	58.2	13.7
307107	1	3	28.7	57.5	--	--	291.0	37.5	14.8
	2	7	92.5	172.5	--	--	385.4	30.5	14.6
307097	1	7	77.7	167.5	580.5	9.8	471.2	26.9	14.6
	2	3	96.7	95.5	340.5	340.5	493.9	27.6	17.7
307113	1	7	50.3	580.5	580.5	580.5	580.9	42.2	17.7
	2	3	77.7	359.5	460.5	460.5	477.0	50.8	16.4
307110	1	7	89.5	124.5	551.5	551.5	400.7	13.7	12.8
	2	3	92.8	95.5	292.5	292.5	340.1	17.8	13.2
307094	1	7	91.0	119.5	335.5	335.5	389.8	37.9	12.8
	2	3	96.6	287.5	287.5	287.5	359.0	37.8	15.5

Appendix D (continued). Marker recovery, digesta transit including transit time (TT), time to 50% marker recovery (T_{50}) and time to maximum marker concentration (T_{MAX}), digesta retention (R_{GIT}) and indigestible fill (V_N) in *G. pardalis* (n=18) fed 85% of weekly voluntary intake distributed over 3 or 7 days.

Tortoise	Trt	Pd	Marker Recovery, %	Digesta Transit, h			Digesta Retention, h	Indigestible Fill (V_N) ⁷	
				TT ³	T_{50} ⁴	T_{MAX} ⁵		g	g/BW _{kg}
307109	1	3	89.3	124.5	316.5	316.5	389.0	11.6	14.4
	2	7	90.3	76.5	335.5	335.5	381.9	16.1	15.7
307106	1	3	13.1	124.5	--	--	446.0	37.0	16.6
	2	7	80.8	340.5	--	--	467.4	21.7	11.3
307104	1	3	76.3	316.5	676.5	316.5	504.5	37.1	19.0
	2	7	0.0	--	--	--	--	--	--
307114	1	3	106.4	167.5	167.5	167.5	240.8	16.5	9.4
	2	7	98.5	95.5	340.5	340.5	252.9	17.7	12.1
307098	1	3	99.9	76.5	311.5	311.5	372.4	27.4	11.9
	2	7	96.2	47.5	340.5	340.5	240.6	32.5	11.0
307102	1	7	77.9	172.5	436.5	436.5	323.7	11.9	12.9
	2	3	86.2	124.5	292.5	292.5	322.4	10.0	12.6

Appendix D (continued). Marker recovery, digesta transit including transit time (TT), time to 50% marker recovery (T_{50}) and time to maximum marker concentration (T_{MAX}), digesta retention (R_{GIT}) and indigestible fill (V_N) in *G. pardalis* (n=18) fed 85% of weekly voluntary intake distributed over 3 or 7 days.

Tortoise	Trt	Pd	Marker Recovery, %	Digesta Transit, h			Digesta Retention, h	Indigestible Fill (V_N) ⁷	g	g/BW _{kg}
				TT ³	T_{50} ⁴	T_{MAX} ⁵				
Mean	--	3	79.1±27.7	190.5±125.2	436.0±156.9	384.4±183.3	399.5± 99.3	21.9±13.2	12.4±3.9	
	--	7	75.2±29.5	146.6± 81.2	366.9±124.8	276.4±114.5	365.2± 84.7	30.5±14.2	14.1±2.9	
	1	--	73.7±30.3	173.2±114.8	440.5±139.8	321.2±168.6	400.3± 80.9	27.0±14.7	13.7±3.4	
	2	--	80.5±26.6	162.3± 98.3	356.7±138.7	336.0±154.3	362.3±101.9	25.6±14.0	12.8±3.5	
Mean	All		77.1±28.3	167.9±105.7	408.5±143.5	341.6±159.0	381.8± 92.3	26.3±14.2	13.3±3.5	

¹Treatments were 3 days (3) and 7 days (7) per week.

²Each collection lasted 35 d. Collection 1 spanned from 17 August 2009 (14:00) to 20 September 2009 (09:00), and collection 2 spanned from 19 October 2009 (14:00) to 23 November 2009 (09:00).

³TT₁ is the time to first appearance of the diet in the feces [Stevens and Hume, 1995].

⁴T₅₀ is the time to 50% of marker recovery [Barboza, 1995].

⁵T_{max} is the time to the maximum concentration of marker in the feces [Barboza, 1995].

⁶R_{git} is the average time food and digesta are retained in the digestive tract [Stevens and Hume, 1995].

⁷V_N = (R_{git}) · total fecal output [Holleman and White, 1987]