Effects of Exotic Forage on Mojave Desert Tortoises

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PROJECT SYNOPSIS

In the Mojave Desert, habitat disturbances have promoted the establishment of non-native plants, so that native grasses and forbs are now intermixed with, or have been replaced by invasive, non-native Mediterranean grasses (Beatley 1966; Brooks 1999; Drake et al. 2015). This shift in plant composition has altered food availability for Mojave desert tortoises (Esque 1994). Several studies have documented that changes in native forage can negatively affect the nutrition and health of tortoises (Oftedal and Allen 1996; Nagy et al. 1997; Nagy et al. 1998; Hazard et al. 2009; Hazard et al. 2010; Drake et al. 2016). Furthermore, recent studies on diet of juvenile desert tortoises indicate that when diets are dominated by red brome (*Bromus rubens*), the tortoises respond with less growth, lower immune-competency, and reduced survivorship (Drake et al. 2016; Esque unpublished data). Considering these effects of brome grasses on juvenile tortoise health and that many places in the Mojave Desert are frequently dominated by red brome during desert tortoise feeding seasons, the potential exists for such conditions to seriously impair recovery efforts by the influence of the loss of juvenile tortoises on demographic processes. We hypothesize that the poor health and survivorship of juvenile tortoises was at least partially due to changes in the commensal microbial communities in the gastrointestinal tract (GIT) of the tortoises that may limit nutrient absorption and physiological performance.

To address these questions, we conducted a small experiment with juvenile Mojave desert tortoises (*Gopherus agassizii*; hereafter tortoise) designed to provide data essential for understanding the negative consequences of altered diets by evaluating how potential changes in the gut microbiota of tortoises may influence the growth, health, and survival of tortoises. Juvenile tortoises were housed in out-door predator proof enclosures and fed a diet of either *B. rubens*, commercial tortoise food, or a mixture of native forbs. Juveniles were evaluated for changes in body mass, shell growth, body and clinical condition, and survivorship each month (April - September). Fecal (scat) samples were collected monthly throughout the experiment to document potential changes in microbial composition temporally and among diets using high-throughput sequencing technology. Additionally, blood samples were collected in selected months (April, June, July) and used as part of an on-going effort to identify biomarkers indicative of oxidative status, immune function and physiological stress in tortoises. Samples were used to identify blood analytes, automize laboratory techniques, and explore potential effects of altered diet on animal performance throughout the spring activity season.

Cumulatively, we used this information to explore the relative biological costs/benefits of altered diets, i.e., *B. rubens* when compared to native plant and commercial tortoise food. We explored gut microbes and the host physiological and immune responses to better understand the potential impacts of altered diet, linkages between microbial communities and performance, and to provide new foundations for the evaluation of animal health and improved husbandry conditions for tortoises. We believe this information may inform and enhance the effectiveness of management decisions and support recovery actions for tortoises and similar species.

Our objectives for this project were to:

1. Conduct a dietary experiment to evaluate juvenile tortoise performance (growth, clinical health condition, survival) during periods of activity (April – September).
2. Describe the fecal microbiome (lineages and community diversity) of individuals consuming native, invasive, and commercial plant diets.
3. Synthesize measures of tortoise performance with fecal microbiota diversity.
4. Contribute to ongoing efforts to identify a panel of biomarkers in blood indicative of oxidative status, immune function, and physiological stress for tortoises and compare these responses among diet groups.

1. DIETARY EXPERIMENT

1.1 INTRODUCTION

The general forage preferences and nutritional ecology of Mojave desert tortoises and other *Gopherus* tortoise species have been well studied (reviewed in Esque et al. 2014, Jennings and Berry 2015). In undisturbed systems, the majority of their diet consists of a mix of native annual forbs and grasses, and herbaceous perennial shrubs (Esque 1994, DeFalco 1995, Nagy et al. 1998, Jennings 2002, Van Devender et al. 2002, Tracy et al. 2006, Esque et al. 2014, Jennings and Berry 2015). Of these, spring and summer annual native forbs and grasses are believed to be the most important food sources in most years (Jennings 2002, Esque et al. 2014, Jennings and Berry 2015). Annual forbs encompass a diverse nutritional array, as they have less structural fiber than most perennials, making nutrients, amino acids, and ultimately energy more available. Nevertheless, habitat disturbance and human land use have degraded tortoise habitats throughout the Mojave Desert, creating the opportunity for invading nonnative Mediterranean grasses such as *Bromus* spp. to dominate landscapes and altering diet available for tortoises. Converse to native annual plants, invasive *Bromus* grasses provide a great deal non-digestible fiber (especially as they senesce) thus providing limited nutritional value to tortoises (McArthur et al. 1994; Drake et al. 2016).

Changes in plant communities can have substantial impacts on herbivore populations, as available forage and nutrition are major drivers of animal health and survival. Nutrient-rich food bolsters healthy immune systems and resources needed for growth, resistance to disease, and other key functions, whereas malnutrition often limits growth (Huitu et al. 2003), reduces reproductive output (Henen 1997, 2002, Cook et al. 2004), causes immune deficiencies, and increases susceptibility to infection and other health-related effects (Gregor and Hotamisligil 2011, Schlaudecker et al. 2011, MacIver et al. 2013, Saucillo et al. 2014). Previous research documented dramatic negative effects for juvenile tortoises consuming *Bromus* grass, including reduced growth and immune function, poor clinical health, and increased mortality (Drake et al. 2016; Authors Unpublished work). That work underscored the need to understand the mechanisms behind this decline in tortoises, as it has important management and conservation implications.

Microbial composition within the gastrointestinal tract (GIT) is well documented in vertebrates, as they play vital roles in nutrient absorption and physiological function (Wu and Wu 2012). For tortoises, GIT microbes are essential for breaking down plant biomass and nutrient absorption, and are assumed to play vital roles in metabolism, immune, and physiological functions. We postulated that in addition to nutritional deficiencies and physical damage from *Bromus* spp. (Medica and Eckert 2007; Drake et al. 2016), invasive grasses may dramatically alter commensal microbial communities within the GIT of tortoises. If native habitats were restored and invasive grasses removed, could tortoises consuming native plants bolster their gut flora and improve desired responses (growth, survival) for this imperiled species?

1.2 METHODS

Diet Treatments

We provided three diets that were fed to 30 juvenile tortoises. Each tortoise was assigned to one of three diets: invasive grass, mixture of native forbs, or commercial tortoise food. We used the invasive non-native annual grass - *Bromus rubens*, commercial tortoise diet (Mazuri Diet LS, St.Louis, MO, USA), or a mixture of five annual/short-lived perennial native forbs known in tortoise diets (*Eschscholzia californica* (California poppy), *Gilia capitata* (blue field *Gilia),* *Plantago ovata* (indian wheat), *Oenothera speciosa* (white evening primrose), and *Sphaeralcea ambigua* (desert globemallow); treatments hereafter will be referred to as *Bromus* and forbs diet. *Bromus* grass seed was collected locally, and forb seed was obtained from Comstock Seed (Gardnerville, NV, USA). Forages were grown in 19 L pots filled with a 3:1:1 mix of sand to organic mulch to perlite and maintained in a glasshouse near ambient conditions.

Commercial tortoise diet (Mazuri Diet LS, St. Louis, MO, USA; hereafter referred to as commercial diet) was comprised of ground soybean hulls, ground corn, dehulled soybean meal, ground oats, wheat middlings, cane molasses, dehydrated alfalfa meal, wheat germ, dicalcium phosphate, soybean oil, brewers dried yeast, calcium carbonate, salt, dl-methionine, choline chloride, pyridoxine hydrochloride, d-alpha tocopheryl acetate (form of vitamin E), biotin, cholecalciferol, menadione sodium bisulfite complex (source of vitamin K), calcium pantothenate, vitamin A acetate, folic acid, riboflavin, preserved with mixed tocopherols, rosemary extract, nicotinic acid, vitamin B12 supplement, thiamine mononitrate, citric acid, l-lysine, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, sodium selenite, and cobalt carbonate. This diet is generally comprised of approximately 15% crude protein, 3% crude fat, 18% crude fiber, 12% moisture, and 8% ash (https://www.mazuri.com/mazuri/reptile/tortoise/tortoise-diet-25lb).

Animal Selection

Juvenile tortoises (approximately 1.5 to 4 yr in age) were chosen as the focus of this study because they grow rapidly and were more likely than adults to respond to differences in diet over the span of the research project (Drake et al. 2016). We acquired juveniles from *Tortoise Group*, a local non-profit adoption organization that rehomes displaced or unwanted pet tortoises in Clark County, Nevada. Upon acquisition, juveniles were immediately housed in outdoor pens (2.23 m^2) within a predator proof enclosure. Each pen included an artificial burrow, a native shrub, and a shallow water dish that was filled twice per week. Tortoise cohorts within pens were randomly assigned to one of three diet treatments, providing two replications of each diet. Five tortoises were assigned to each pen using a stratified random design to minimize co-occurrence of related individuals and even distributions of age classes within each pen. Prior to our experiment, all tortoises were fed commercial tortoise diet and watered twice per week for at least eight months, with most individuals housed for 30 months.

Starting on April 10, 2018, forage was supplied *ad libitum* to tortoises by placing one pot of the selected diet into recessed holes within each pen such that plants were flushed with the ground (Figure 1.2.2). Pots were replaced weekly with stock from the greenhouse throughout the experiment. Tortoises receiving commercial diet were fed twice weekly by softening pellets in water prior to feeding. Also, enclosure pens for tortoises receiving the commercial diet were seeded with native annual/perennial plant seed as listed above prior to the experiment. As such, a

few native annual forbs were available as supplemental diet options early in this experiment. All tortoises were watered twice a week throughout the experiment. By early August, annual forage plants had largely senesced and dried out. To ensure food availability for both *Bromus* and forb diet groups, we supplemented them with commercial tortoise food through the end of the experiment.

Soft ticks (*Ornithordorus* sp.) are common ectoparasites of *Gopherus* tortoises (Green 1986; USFWS 2019). Yet, almost no information is available to understand the potential impact ectoparasites have on tortoise health. To reduce potential impacts from ticks, we sprayed the walls within each pen with Bayer Advanced Complete Insect Killer (containing 0.36% Cyfluthrin; Cary, North Carolina, USA) prior to study. We also used Terro Outdoor Liquid Ant Killer and Terro Outdoor Ant Killer Granules (Lancaster, Pennsylvania, USA) for ant control outside the enclosure perimeter. Ant traps were set on May 14th and July 23rd outside enclosure.

Figure 1.2.1. Photographs of captive juvenile Mojave desert tortoises (*Gopherus agassizii*) foraging on their assigned diet of *Bromus rubens* (A) or a mixture of native annual and perennial forb plants (B) on April 26th and June 1, 2018 respectively. Photographs were taken by Jordan Swart.

Animal Growth, Condition, and Survivorship

Tortoises were assessed monthly (April 2 – September 21, 2018) to determine clinical condition, and identify diseases or injury as well as to document growth and survival. Tortoises were assessed for clinical condition by examining the animals' general posture, respiration, face (with specific attention to the eyes, periocular tissue, nares, mouth, tongue, and oral mucosa), skin, and shell for any clinical signs of disease, abnormalities, damage, or discoloration (USFWS 2019). Numerical body condition scores (BCS) were used to assess overall muscle and fat stores with respect to skeletal features of the head and limbs (USFWS 2019). Once classified, a numeric score was assigned to provide a more precise assessment (i.e., score under conditioned 1 - 3; adequate conditioned 4 -6; over conditioned 7 -9). All tortoises were placed into water dishes post assessment to facilitate drinking. Throughout the experiment, all plant debris (specifically *B. rubens* grass seeds including palea, lemma, and awn) found to be impacting the mucosal lining of the eye, tongue, choana, and oral cavity was noted and removed with tweezers. Growth was quantified by measuring the shell plastron length (PL) between the inner notches of the gular and

anal scutes and recorded to the nearest 0.01 mm using digital calipers (Mitutoyo, Aurora, IL). Mass was recorded to the near 1.0 g using digital scales (Mettler Toledo, Columbus, OH).

Growth was analyzed with a series of linear mixed-effects models (R Package lme4 ver 1.1-26) with tortoise individual as random effects to account for repeated measures. Candidate models were compared using Akaike's information criterion corrected for small sample sizes (AICc; Burnham and Anderson 2002) using the package MuMIn (v1.43.17) in R (Team 2020). One tortoise with significant clinical indictors of poor health was removed from the study and returned to husbandry care. Clinical abnormalities (i.e., impacted *Bromus* seeds, pale coloration in the tongue and oral mucosa) and changes in body condition were calculated each month and analyzed with a series of linear mixed-effects models with tortoise individuals as random effects to account for repeated measures and compared using AICc.

Animal survival analysis was completed using a Cox proportional hazard model implemented in R package survival (Therneau and Lumley 2017). The survival curve for tortoises was plotted against diet and sampling periods and compared using log-rank tests and computed 95% intervals for a period of 164 days (Pollock et al. 1989). For the purposes of calculating survivorship, tortoises removed from the experiment because of poor health or individuals presumed to die from heat related stress were considered fatalities.

Tissue Collection

Fecal scat samples were collected from tortoises throughout the experiment to evaluate the microbial community present in each individual. During handling events for health assessments, tortoises were placed in small individual 64 oz disposable poly-coated paper food cups (Choice Paper Company, New York, New York, USA) for approximately 5 – 15 minutes. When available, fresh scat samples were immediately collected each month, placed in sterile cryogenic vial, immediately flash frozen with dry ice, and stored in an ultracold freezer (-70°C) until analyses.

Blood samples (0.05 - 0.20 mL) were collected via subcarapacial venipuncture (Hernandez-Divers et al. 2002) using a 0.96-cm, 26-gauage needle and 1-mL syringe coated in sodium heparin in April, June, and July to evaluate oxidative status, enzymatic antioxidant activity, immune function, and physiological stress parameters. Blood samples in April were immediately flash frozen with dry ice. Blood collected in June and July were immediately placed into lithium heparin tubes (*Becton Dickinson and Company*, *Franklin Lakes*, *NJ*) and kept on wet ice for up to three hours. Plasma (0.05 – 0.12 mL) was separated from samples using centrifugation with a force of 1318x *g*. All tissues were stored in an ultracold freezer (-70C) until analyses.

Scientific Permits and Animal Care Protocols

All activities involving Mojave desert tortoise were permitted by the U.S. Fish and Wildlife Service (Research Permit #TE-030659) and Nevada Department of Wildlife (Scientific Collecting Permit #S39800). Animal care and use protocols were approved by the Western Ecological Research Center - U.S. Geological Survey Animal Care and Use Committee - Review Chair (Document -Mojave Desert Tortoise Research Program).

1.3 RESULTS

Clinical Health Profiles

Overall body conditions (measured by the change in BCS each month) were similar among diet groups ($F_{2,10} = 1.29$, $p = 0.32$) throughout the experiment, as each animal presented with adequate muscle and fat reserves (i.e., BCS 4-6). Surprisingly, tortoise-B6 had limited growth through late summer; however once commercial diet was supplemented starting in August, it presented with excessive fat and muscle (BCS – 7) by September.

Impacted *Bromus* seeds were found embedded within the oral mucosa, tongue, choana, and the palpebral and periocular membranes on occasion in individuals consuming *Bromus* plants starting in June (n = 6; 67%) and July (n = 5; 83%), coinciding with *Bromus* seed production and plants drying out. During oral examinations each month, the membranes of the oral cavity and tongue appeared pale in coloration each month for some of the tortoises eating *Bromus* (30% observations), commercial (20% observations), and forbs (51% observations) diet, possibly related to an overall state of anemia in these individuals. Reddened oral mucosa, often associated with inflammation, were observed in group including *Bromus* (7%), commercial (3%), and forbs (1%) diet. Clinical abnormalities around the cloaca were expected for tortoises consuming *Bromus* plants (Drake et al. 2016). Yet, we observed abnormalities including dried fecal matter and staining from frequent diarrhea around the cloaca were observed in both *Bromus* and forbs diet groups. The majority of cloacal abnormalities were observed in the *Bromus* (n = 5; 50% animals) diet starting in June, whereas 20% (n = 2) of forb animals had similar conditions. Cloacal abnormalities were not observed for individuals foraging on commercial tortoise food.

Ornithodoros ticks were observed on the head, neck, and shell (carapace) for six tortoises in the commercial and forbs diet groups during assessment (Figure 1.3.1). Tortoises consuming commercial diet (C10) in ticks present in April, whereas tortoises in the forb group (F1, F2, F3, F4, F5) had ticks present in May, with repeated observations for F2 in June. We found tick infestations in soil within enclosures for both *Bromus* (Pen 717B, 718B) and forb (Pen 717A) diet groups. Tortoises in tick-infested enclosures were relocated to another pen on July 30th. During burrow excavations in late July, we noted that native desert cockroaches, scorpions, black widows, ticks, and other insect species were present along with organic debris from the *Atriplex* canescens (desert saltbush) shade shrub.

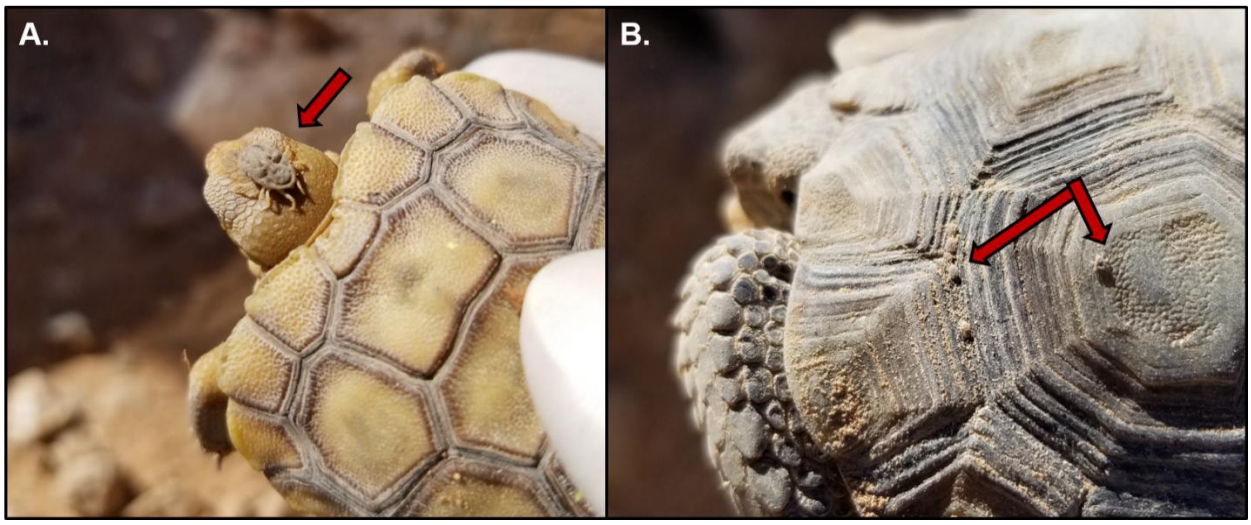

Figure 1.3.1. Photographs of *Ornithordoros* spp. ticks present on the head (A) and carapace (B) of juvenile Mojave desert tortoises (*Gopherus agassizii*) during clinical assessments in May and June 2018. Photographs were taken by Jordan Swart.

Animal Growth and Survival

Growth rates for tortoises varied significantly among diet groups ($F_{2,10} = 203.73$, $p < 0.01$) and among months ($F_{4,85} = 145.36$, $p < 0.01$). Growth rates each month were similar ($F_{1,7} = 0.53$, $p = 0.49$) between *Bromus* (0.82 ± 0.50 mm) and forbs (0.93 ± 0.36 mm) diet groups; however, tortoises foraging on commercial tortoise food grew (3.17 ± 0.44 mm monthly. Growth was greater for animals fed commercial diets each month compared to other groups, with overall cumulative growth more than three times more than other diets groups (Figure 1.3.2).

Diet (LRT = 14.67, df = 2, $p < 0.01$) and month (LRT = 42.43, df = 3, $p < 0.01$) strongly influenced animal survival during this experiment. Survivorship was lowest for animals consuming *Bromus* (*n* = 3; 30%) and higher for animals consuming the forbs (n = 7; 70%) and commercial (n = 10; 100%; Figure 1.3.3) diet, highlighting the importance of the nutrients and dietary fibers found in native plant species and commercial diets. Survival decreased starting in June as temperatures increased and plants senesced and dried. Individuals died that were fed both *Bromus* (*n* = 7, 70.0%) and native forbs (*n* = 3, 30.0%). However, several tortoises consuming *Bromus* diet were either pulled from the experiment in mid-June for poor clinical health (B1), had prior clinical health abnormalities and had not been observed since May (B10), or may have succumb to heat related injury (B9) in late August (Table 1.3.1). Additionally, three dead *Bromus* diet tortoises had had notable hardened urates present during necropsy inspection (Table 1.3.1). Throughout the experiment, some tortoises in both Bromus and forbs diet groups may died from heat related njury and not a direct result of diet (Table 1.3.1).

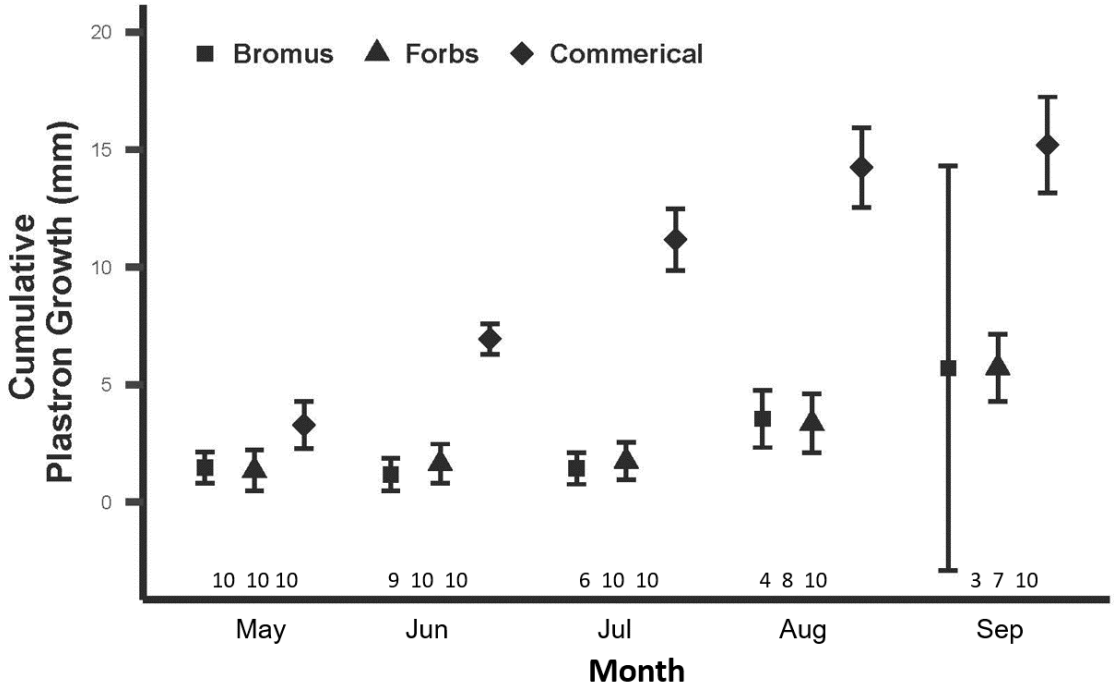

Figure 1.3.2. Mean ± 95% confidence interval for cumulative growth measurements for juvenile Mojave desert tortoises (*Gopherus agassizii*) each month (May–September) foraging on three diets. At the end of the experiment, tortoises foraging on commercial tortoise diet grew significantly more, increasing shell size by 200% compared to tortoises foraging on *Bromus* and native forb diets. The sample size used to calculate growth each month is stated at the bottom of the graph.

Table 1.3.1. Recorded mortality for captive juvenile Mojave desert tortoises (*Gopherus agassizii*) consuming diets of *Bromus rubens* or a mixture of native forb plants. Tortoises were found dead starting in mid-June through September 2018.

Diet	Tortoise ID	Pen	Mortality Date	Notes
Bromus	B1*	717B	6/15/18	Pulled from experiment. Presented with abnormal clinical signs starting on June 1, 2018. Pen was infested with *Ornithordorus* ticks.
	B2	717B	7/26/18	Dessicated in burrow. Pen was Pen was infested with *Ornithordorus* spp. ticks.
	B5	717B	7/26/18	Dessicated in back of burrow. Notable urates present. Pen was infested with *Ornithordorus* ticks.
	B7	718B	8/3/18	Dead on surface; full of urates during necropsy.
	B8	718B	8/3/18	Dead on surface; full of urates during necropsy.
	B9	718B	9/1/2020	Desiccated in burrow. Found upside on 8/14/18; may have succumb to heat related injury.
	B10	718B	7/26/18	Dead in burrow. Animal was not observed since May. Presented with clinical abnormalities such as periocular swelling in May.
Forbs	F3	717A	8/18/18	Likely succumb from heat related injury. Necropsy did not indicate any gross morphological injury.
	F6	718A, 715B	9/7/2018	Found dead in burrow. Was relocated to another pen 715B on 7/30/2018.
	F8	718A	8/24/2018	Found dead upside down.

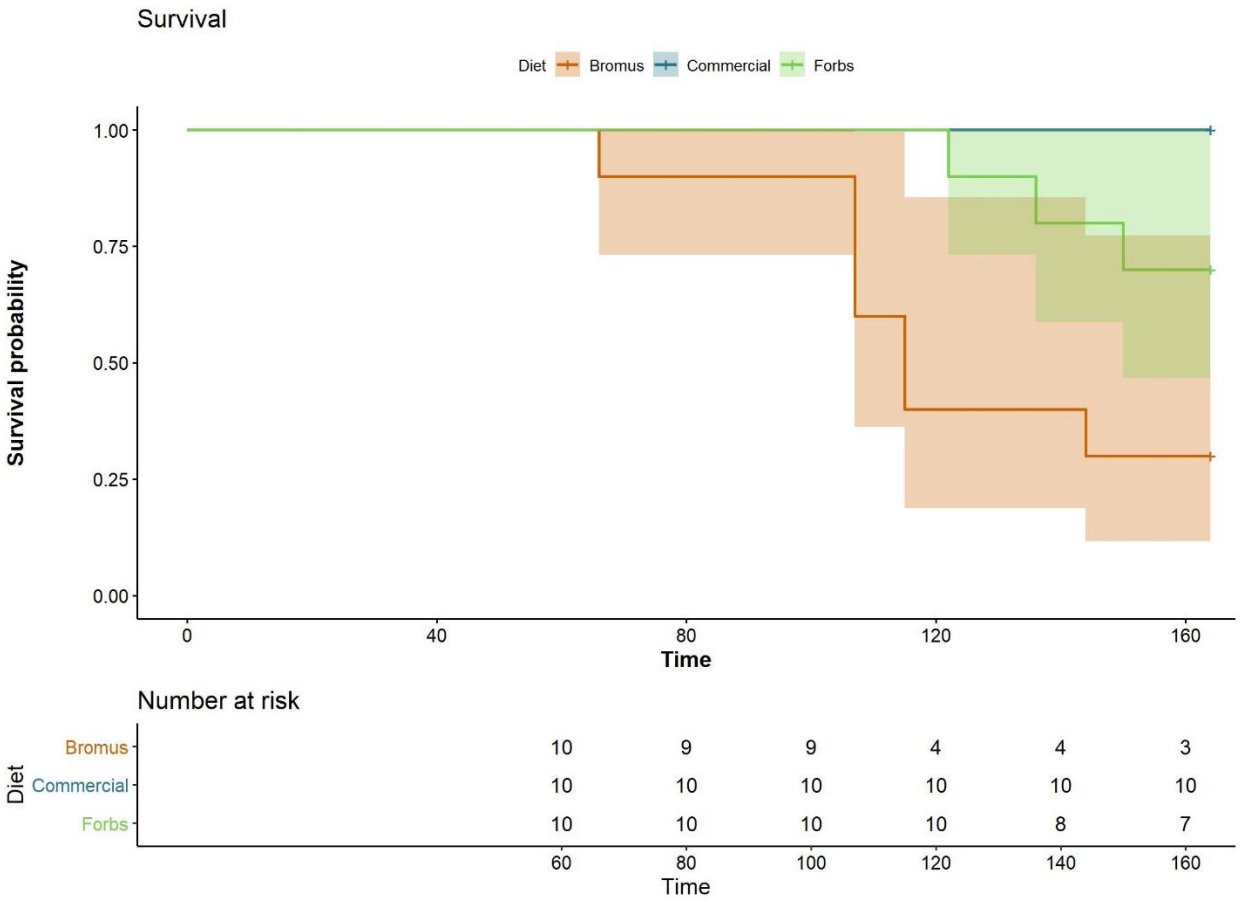

Figure 3.2.3. Survival (survivor) function estimated by the Kaplan-Meier method, including 95% confidence bands. The number of tortoises at risk at different time points is displayed on the graph. All tortoises consuming commercial diet survived. Survival was lower in animals foraging on *Bromus* grass and mortality increased in warmer periods (July-September) for this experiment.

1.4 DISCUSSION

Invasive Mediterranean annual grasses are fully integrated into western North American landscapes, and in the Mojave and Sonoran deserts they have drastically modified the vegetation composition (Beatley 1966, Brooks 1999). Invasive annual grasses such as *B. rubens* occur throughout most of range for Mojave desert tortoises (*G. agassizii*) and are rapidly intermixing with or replacing native plant foods (Esque 1994, Esque et al, 2003), ultimately lowering overall nutritional availability (Oftedal 2002, Oftedal et al. 2002, Tracy et al. 2006). Desert tortoises generally prefer and consume native forb plants that are high in protein, energy content, calcium, magnesium, phosphorus, and potassium and low in fiber (Esque 1994; McArthur et al. 1994; Tracy et al. 2006; Drake et al. 2016). Conversely, *Bromus* grasses contain a great deal of non-digestible fiber (hemicellulose, indigestible cellulose, and lignin representing the fibrous bulk), especially as they senesce, and provide limited nutritional value to tortoises (McArthur et al. 1994; Drake et al. 2016). Prior diet experiments with red brome indicated juvenile tortoises consuming this grass had limited growth, physical injury, reduced immune response activity, and

lower survival than tortoises consuming native diets. We included Mazuri commercial food into this study, as it is commonly used as a forage supplement to tortoises in captive environments. Mazuri commercial diet (content described in methods) is fortified with balanced ratios of protein, vitamins, minerals, and fiber to promote healthy growth and condition in tortoises. However, direct studies have not been conducted to evaluate its dietary impact on the growth and survival of juvenile tortoises, or any work directly with Mojave desert tortoises.

We anticipated that growth and survival rates would be similar among tortoises consuming native forbs and commercial food. However, growth was comparatively reduced for tortoises foraging on native forb plants, and rates were similar to animals consuming *Bromus* grass in our current study and related work (Drake et al. 2016; Esque unpublished data). By the end of the experiment, tortoises foraging on commercial diet increased in body size by ~200% compared to other diets. Although anecdotal, one *Bromus* tortoise switched back to a commercial diet in early August greatly increased its mass and body size after ~5 weeks of foraging on the commercial diet.

Argasid soft ticks, such as *Ornithodoros parkeri* and *O. turicata* are common ectoparasites for tortoises and other desert wildlife (Green 1986). *Ornithodoros* ticks are ubiguitous in low to mid elevations in the Mojave Desert with long life spans (20+ years) and have the ability to survive long periods of starvation (Sonenshine 1991). All stages of *Ornithodoros* ticks are known to parasitize desert tortoises (Green 1986). Although these ticks are commonly associated with tortoises, we were surprised by the frequency and abundance of ticks within our animal enclosures, especially within our forb diet group. Parasitism from ticks and underlying health conditions or disease associated with tortoises in the forb group may have contributed to their limited growth. Each animal was vetted prior to study (quarantined and routinely screened for clinical health conditions and known pathogens such as *Mycoplasma agassizii)*. However, it is possible these tortoises were exposed to novel pathogens or experienced sub-optimal conditions or were provided poor nutrition prior to their receipt, and inclusion in our captive environment. The design of this experiment facilitated two replicate enclosures per diet. One disease individual could have facilitated transmission of pathogens and subsequently health complications could have greatly impacted our findings.

2. COMPARATIVE ANALYSIS OF DIETARY IMPACTS ON FECAL MICROBIOTA

2.1. INTRODUCTION

The gut microbial community of animals is composed of archaea, bacteria, fungi, yeasts, protozoa, viruses, and bacteriophages (Turner 2018), that influence a suite of host characteristics such as physiology, immunity, behavior, and reproduction (Fraune and Bosch 2010; Columbo et al. 2015; Colston and Jackson 2016). Conversely, the host diet and evolutionary history can influence the gut microbiota (Ley et al. 2009; Sanders et al. 2013; Clements et al., 2014). Gut microbes greatly affect digestion, nutrition absorption, maintenance of intestinal mucosal integrity, gut peristalsis, development of immunity and immunomodulation, metabolism of xenobiotics, and disease resistance (Berg 2014; Turner 2018).The symbiotic relationship established between the microbiota and the associated host is particularly relevant within the gastrointestinal tract (Nicholson et al. 2012). Studies on the gut microbiota of different animals have provided a wealth of ecological and evolutionary information showing a strong link with health and diseases (Costa et al. 2012). In addition, the influence of gut microbiota on stress and social behavior has been demonstrated (Cryan and Dinan 2012; Sharon et al. 2016).

Herbivorous reptiles, such as desert tortoises, are hindgut fermenters meaning that they glean some of their important nutrients, including volatile fatty acids, by fermentation processes in the gut within tissues posterior to the small intestine. In that region of the gut, high concentrations of bacteria involved in an endosymbiotic relationship drive the fermentation process to produce short chain fatty acids like acetate, propionate, and butyrate, as well as vitamins and amino acids (Mackie et al. 2004; Stevens and Hume 2004; Hong et al. 2015). Potential changes in diet and consequently GIT microbiota in tortoises could impact their ability to break down plant food, thus reducing nutrient absorption and energy production, and negatively impacting the physiological and metabolic functions of this species.

To date, most studies have focused on the gut microbiota of mammals, especially that of humans, but also of birds, fish, and insects. However, limited research on this topic has been carried out on reptiles (Scheelings et al. 2020) and has focused mainly on carnivorous species (Arriza et al. 2019; Biagi et al. 2019), whereas herbivorous reptiles are still underrepresented.
Since the current study on Mojave desert tortoises was initiated, research on the microbiota of related species including the bolson tortoise (*G. flavomarginatu*s; Garcia-De la Pena et al. 2019), gopher tortoise (*G. polyphemus*; Gilliard 2014), Seychelles giant tortoise (*Aldabrachelys gigantea*; Sandri et al. 2020), Beal's eyed turtle (*Sacalia bealei*; Fong et al. 2020), Krefft's river turtle (*Emydura macquarii krefftii;* Knight et al. 2020), and all marine turtles (Scheeling et al. 2020), have been provided. However, most of these studies focused on microbiota differences between wild and captive populations during a discrete sampling period. Additionally, these studies did not always evaluate the same tissue-microbiota associations, making inference difficult across taxa.

Our study provides the first description of gastrointestinal (fecal) microbial abundance and composition for Mojave desert tortoises. We saw this study as an opportunity to evaluate the

potential for change in microbial composition relative to diet and temporal changes that may occur between months or seasons in this species. We postulated that nutritional deficiencies and physical damage from *Bromus* spp. (Drake et al. 2016, and Medica and Eckert 2007; respectively) would dramatically alter commensal microbial communities within the gastrointestinal tissues of tortoises,

2.2. METHODS

Study Animals and Sample Section

Fecal samples were collected from juvenile tortoises prior to study (April) and monthly afterwards (May – September) throughout the experiment (methods described in Section 1.2). We selected 141 fecal samples from 30 individuals involved in analyses of microbial composition across three diets comprised of: *Bromus*, commercially tortoise food, and native forbs groups.

Microbial Genomic DNA Extraction

Frozen fecal (scat) samples were shipped to Jonah Ventures Laboratory (Boulder, Colorado) and analyzed for whole community genomic DNA. DNA from each sample was extracted using DNeasy kits according to manufacturer protocols (QIAGEN catalog #69504, Germantown, MD). Following extraction, genomic DNA was eluted into 100 µl aliquots and frozen at -20°C. Standard primer sequences (515F and 806R; Caporaso et al. 2011) tagged with adaptor sequences for indexing and Illumina sequencing were used to amplify a portion of the mitochondrial 16S rDNA gene. Reactions were mixed according to standard Promega PCR Master Mix specifications (Promega catalog #M5133, Madison, WI) and amplified using the following conditions: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 45 seconds at 95 °C, 1 minute at 50 °C, and 90 seconds at 72 °C, and a final elongation at 72 °C for 10 minutes. Reactions were then visually inspected on a 2% agarose gel to confirm amplicon size and PCR amplification.

We created 16S libraries by cleaning amplicons using UltraClean 96 PCR Cleanup kits (QIAGEN catalog # 12596-4) following standard manufacturer protocols. Indices were attached by indexing PCR using the Promega Master Mix under the following conditions: initial denaturation of 95 °C for 3 minutes followed by 8 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec. Following visualization of indexed PCR product using gel electrophoresis, we cleaned and normalized each sample using SequalPrep Normalization Plates (Thermofisher catalog #A10510-01, Waltham, MA). Normalized samples were pooled into libraries and these samples were sequenced on an Illumina MiSeq (San Diego, California) at the University of Colorado – Boulder BioFrontiers Sequencing Center using a v2 500-cycle kit (Illumina catalog # MS-102-2003, San Diego, CA).

Microbiome Data Generation and Analysis

We received paired-end sequencing output as demultiplexed FASTQ files, which were used for downstream filtering and analysis in QIIME2 (version 2019.10; Bolyen et al. 2018). We imported reads as a single QIIME2 artifact, and used “dada2” to denoise sequences, filter out chimeras and aberrant sequences, trim paired end reads to 190 nucleotides for R1 and 220 nucleotides for R2, and aligned paired end reads for each sample. We used a SILVA-based 16S

rRNA model (Pruesse et al. 2007) to classify sequence variants for each sample. These classified frequency tables and sequence files were then used for downstream analyses.

Following processing in QIIME2, the "qza_to_phyloseq" function in package "qiime2R" (Bisanz 2018) was used to read in QIIME2 artifacts as a "phyloseq" object using R version 3.6.3 (Team, 2020). In order to reduce complexity of downstream data analyses, we split this dataset into two reduced datasets: one that retains only the first sample taken from each individual, and a second that retains only the final sample taken from each individual. We computed Shannon's alpha diversity metric for each sample using R package "microbiome" (Lahti et al. 2017). We then used function "adonis2" in package "vegan" (Dixon 2003) to: a) compute pairwise Bray-Curtis distances between samples, and b) assess the significance of diet (brome, control, and forb diets), cumulative change in growth metrics (carapace length, mass, and plastron length), and survival status on community composition using a permutation-based ANOVA (PERMANOVA). For diet comparisons, statistical significance was assessed using a PERMANOVA for both the first and final sample datasets; for survival and growth comparisons, PERMANOVAs were only implemented for the final sample dataset. In addition, the effect of maternal identity on both the first and final sample datasets was estimated.

Bray-Curtis distances were used to conduct a principal coordinates analysis (PCoA). In addition to using this PCoA to visualize relationships between samples in multivariate space, we extracted PCoA axis scores and used these scores to calculate the best-supported number of clusters in this dataset using the gap statistic, as implemented in function "clusGap" in package "cluster" (Maechler et al. 2013). We selected the value of *K* that corresponded to a plateau in the gap statistic, and visually inspected the distribution of these clusters in multivariate space.

Finally, we used package "DeSeq2" to identify Amplicon Sequence Variants (ASVs) that were differentially abundant across covariate levels (Love et al. 2014). To investigate if sequence variants differed among diet groups, we ran a DeSeq2 model with all three factor levels for diet treatment (i.e., forbs, brome, and commercial) included; after this overall model, we ran models on pairwise combinations of diet levels as well. Because DeSeq2 performs best with categorical predictor covariates, we computed median cumulative change in mass and plastron length across individuals; and used their median values as thresholds for comparisons of the lower 50% and upper 50% of values for each covariate, and classified samples as either above or below these thresholds (median cumulative change in carapace length = 6.10 mm, median cumulative change in mass = 14.2 g, median cumulative change in plastron length = 5.31 mm). For analyses of survival, we used survival status for each tortoise by the end of the study as the predictor covariate. In each case, we used Wald tests and an adjusted $\alpha = 0.001$ to assess significance.

2.3. RESULTS

Diet Treatments

The complete dataset (141 samples collected from 30 individuals) from our study included 3,085 microbial community lineages across 22 phyla, 38 classes, 73 orders, and 124 families (Figure 2.3.1). In terms of raw abundance, Firmicutes was the most abundant, followed by Bacteroidetes (Figure 2.3.1).

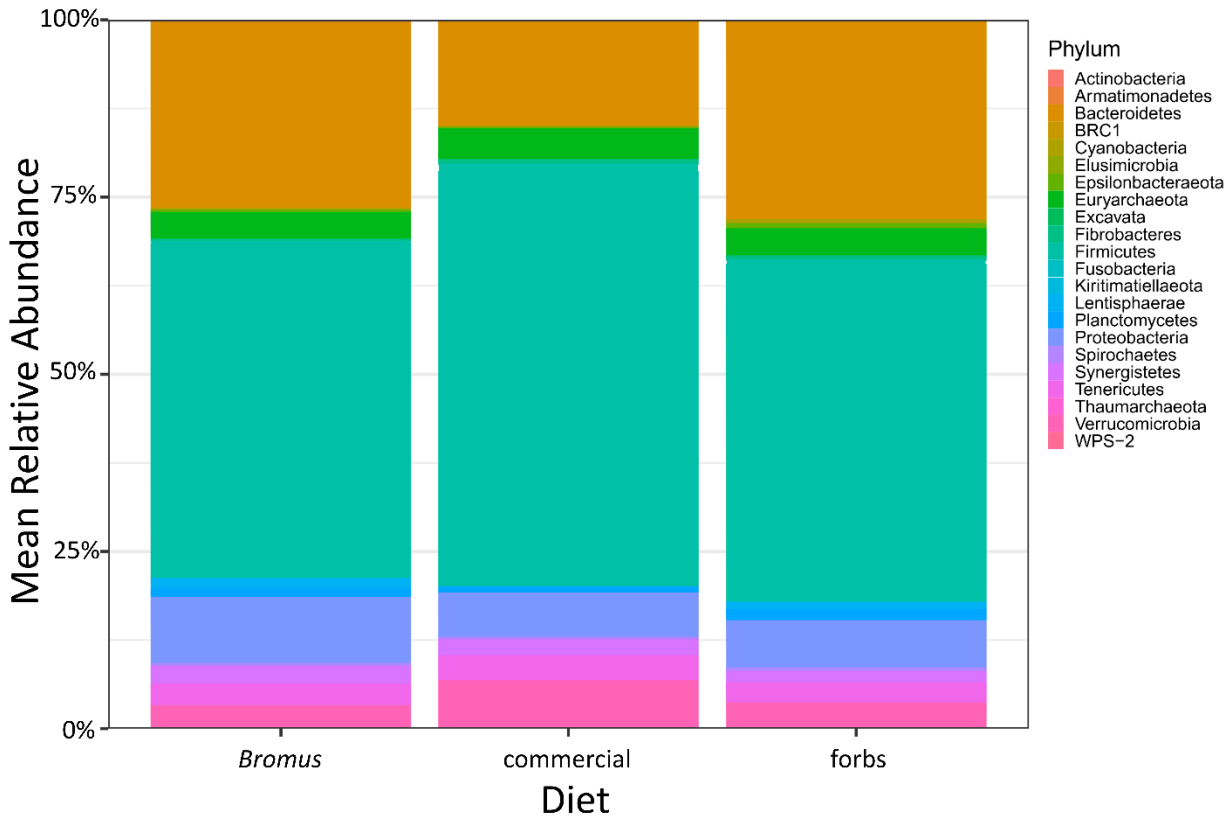

Figure 2.3.1. Mean relative abundance per microbial phylum across diet groups in our study. Patterns of reduced proportions for Firmicutes and increased relative proportions of Bacteroidetes were observed for tortoises consuming *Bromus* and forb diets, compared to animals eating commercial food.

Prior to our study, the microbial diversity (evaluated using PERMANOVA of Bray-Curtis distances) in all individuals regardless of diet were similar ((R^2 = 0.051, F = 1.422, p = 0.181); however, material identity (R^2 = 0.771, F = 2.552, p = 0.001) or likely the enclosure, microenvironment, or diet associated with the maternal female influenced community composition. By September, diet effects (R^2 = 0.107, F = 2.695, p = 0.022) in addition to maternal identity (R^2 = 0.695, F = 2.063, p = 0.008) significantly influenced the microbial composition. Shannon diversity was similar among diets (F = 1.094, p = 0.349; Figure 2.3.2). Although statistically insignificant, microbial community diversity for *Bromus* was slightly lower than that found for commercial and forb diets. For most analyses that follow, we limit analysis and interpretation to the final samples taken for each individual (30 samples total).

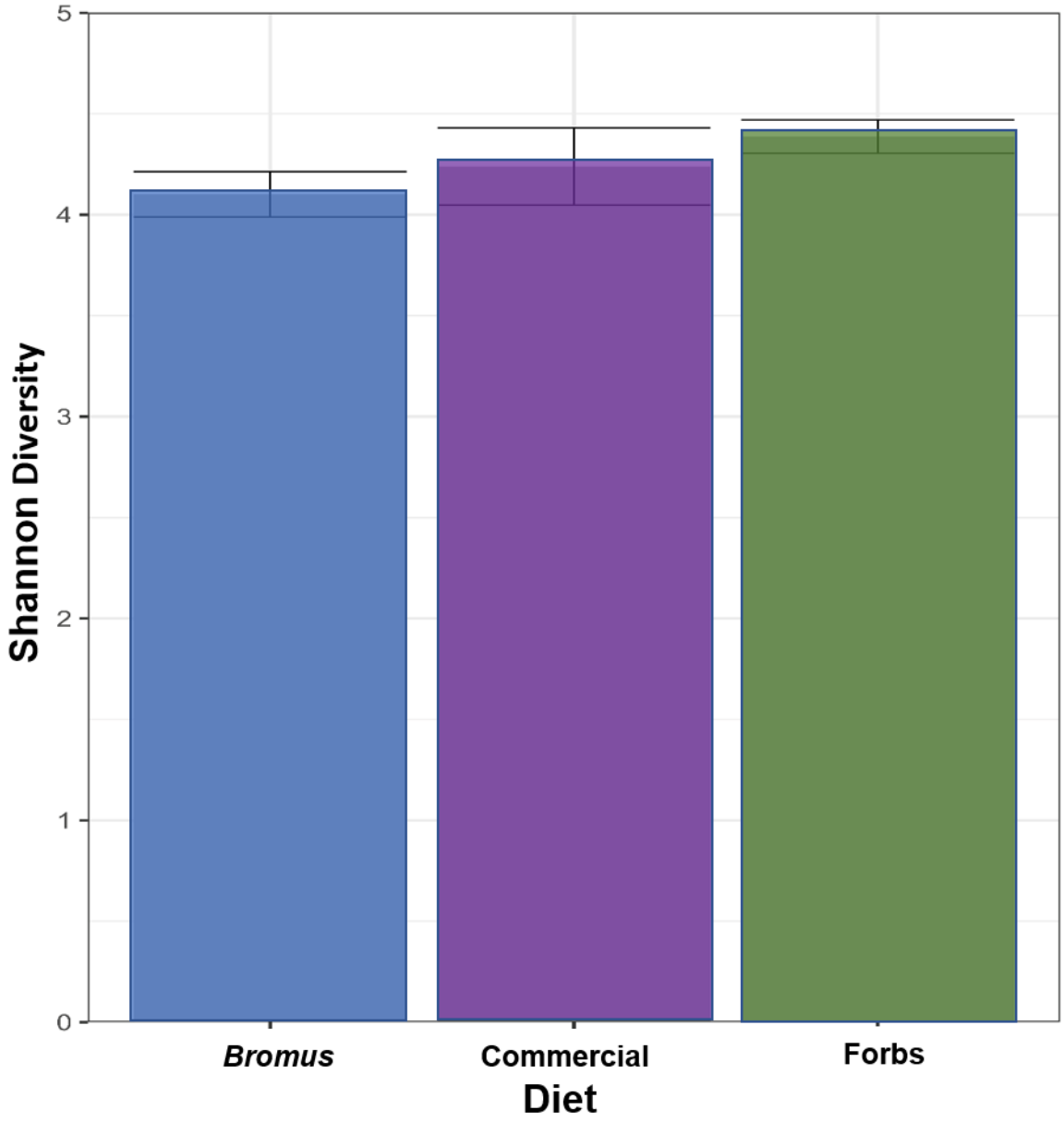

Figure 2.3.2. Mean Shannon diversity for each diet group, presented ±1 Standard Error (SE).

Gap statistic-based clustering of principal coordinate analysis (PCoA) scores for samples collected at the end of the study indicated top support for $K = 3$. Depiction of these clusters along the two most explanatory PCoA axes (explaining 14% and 10% of variation) indicated that tortoises foraging on commercial diet grouped into a single cluster, located centrally along axis 1 and 2. An additional cluster was identified for the *Bromus* and forbs diet samples, with positive axis 1 scores. A final cluster was identified for two samples from the *Bromus* diets (S020081 and S020083) with positive axis 2 scores (Figure 2.3.3). These samples were collected from two tortoises (GS0453 and GS0452, respectively) in early April, and both represented the only samples with sequence data from each tortoise. Thus, the cluster composed of these two samples alone may reflect temporal effects, rather than within dietary group effects.

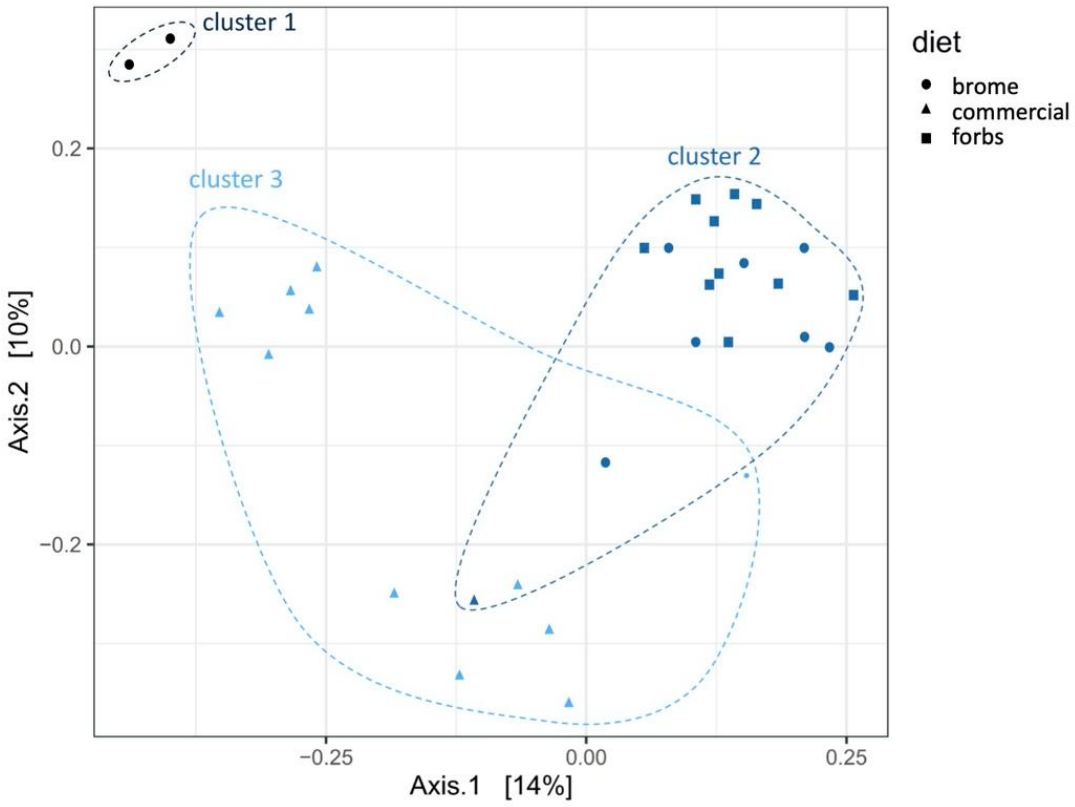

Figure 2.3.3. Principal coordinate analysis (PCoA) based on Bray-Curtis distances between samples. Gap statistic-based clusters ($K = 3$) are presented based on differently colored plots; the coordinate space occupied by each cluster is outlined as well. Point shape is used to denote diet group (circle = brome, triangle = commercial, and square = forbs).

DeSeq2 models identified 33 Amplicon Sequence Variants (ASVs) or microbial lineages that differed significantly across diets, representing nine phyla: Actinobacteria, Bacteriodetes, Cyanobacteria, Epsilonbacteraeota, Euryarchaeota, Firmicutes, Proteobacteria, Synergistetes, and Verrucomicrobia (Table 2.3.1; Figure 2.3.4; Figure 2.3.5). For pairwise comparisons, differing microbial community members were detected as differentially abundant between diets. A total of 18 microbial community members were detected as differentially abundant between *Bromus* and commercial diets, 28 were detected as differentially abundant between commerical and forb diets, and 21 were detected as differentially abundant between *Bromus* and forb diets.

Table 2.3.1. Percent composition of microbial communities that differed significantly for 30 captive juvenile Mojave desert tortoises (*Gopherus agassizii*) consuming diets of either the invasive grass (*Bromus rubens*), commercial tortoise food, or a mixture of native forb plants each month (April – September) during periods of activity in 2018. Deseq2 models identified 33 differentially abundant amplicon sequence variants (ASVs) representing 9 phyla.

Microbial Phyla	Diet	Pre-Experiment Apr	May	Jun	Jul	Aug	Sep
Actinobacteria	*Bromus*	3.3	0.5	0.04	0.0	0.0	0.0
	Commercial	1.9	0.0	0.15	0.0	0.0	0.0
	Forbs	0.0	0.0	0.0	0.0	0.02	0.0
Bacteroidetes	*Bromus*	60.1	75.6	95.6	85.8	59.8	70.0
	Commercial	4.1	8.6	10.5	20.8	20.5	43.6
	Forbs	68.6	63.4	69.6	79.8	72.4	83.3
Cyanobacteria	*Bromus*	0.5	0.07	0.0	0.3	0.0	0
	Commercial	0.0	0.02	0.0	5.6	0.2	0.5
	Forbs	0.4	0.5	1.4	1.5	0.1	1.6
Epsilonbacteraeota	*Bromus*	0.0	8.3	0.06	0.0	0.9	2.8
	Commercial	0.0	0.0	0.0	0.0	2.3	0
	Forbs	0.0	12.2	2.3	0.3	0.4	2.4
Euryarchaeota	*Bromus*	0.1	2.9	0.02	0.0	0.2	6.3
	Commercial	0.0	0.0	0.0	0.0	0	0.3
	Forbs	6.2	0.02	0.85	6.8	1.0	2.5
Firmicutes	*Bromus*	27.3	3.2	2.3	1.4	8.4	2.7
	Commercial	5.6	16.6	16.9	27.4	23.6	25.2
	Forbs	7.9	5.6	3.2	1.9	4.0	2.2
Proteobacteria	*Bromus*	2.9	4.5	1.2	0.7	1.0	0.7
	Commercial	0.0	0.0	0.0	0.0	0.0	0.0
	Forbs	0.0	12.2	8.7	5.9	2.0	1.0
Synergistetes	*Bromus*	4.9	4.3	0.2	5.5	2.8	3.2
	Commercial	0.0	0.0	0.0	0.2	0.0	0.0
	Forbs	0.0	0.09	0.0	0.4	1.3	1.2
Verrucomicrobia	*Bromus*	0.8	0.3	0.5	6.2	26.8	14.3
	Commercial	88.4	74.8	72.4	46	53.3	30.3
	Forbs	16.8	6.0	14.0	3.4	18.6	5.7

Figure 2.3.4. Heatmap showing percent composition of differential microbial communities identified in fecal samples for captive juvenile Mojave desert tortoises (*Gopherus agassizii*) consuming diets of either the invasive grass (*Bromus rubens*), commercial tortoise diet, or a mixture of native forb plants each month (April – September) during periods of activity in 2018 (graphical representation of Table 2.3.1).

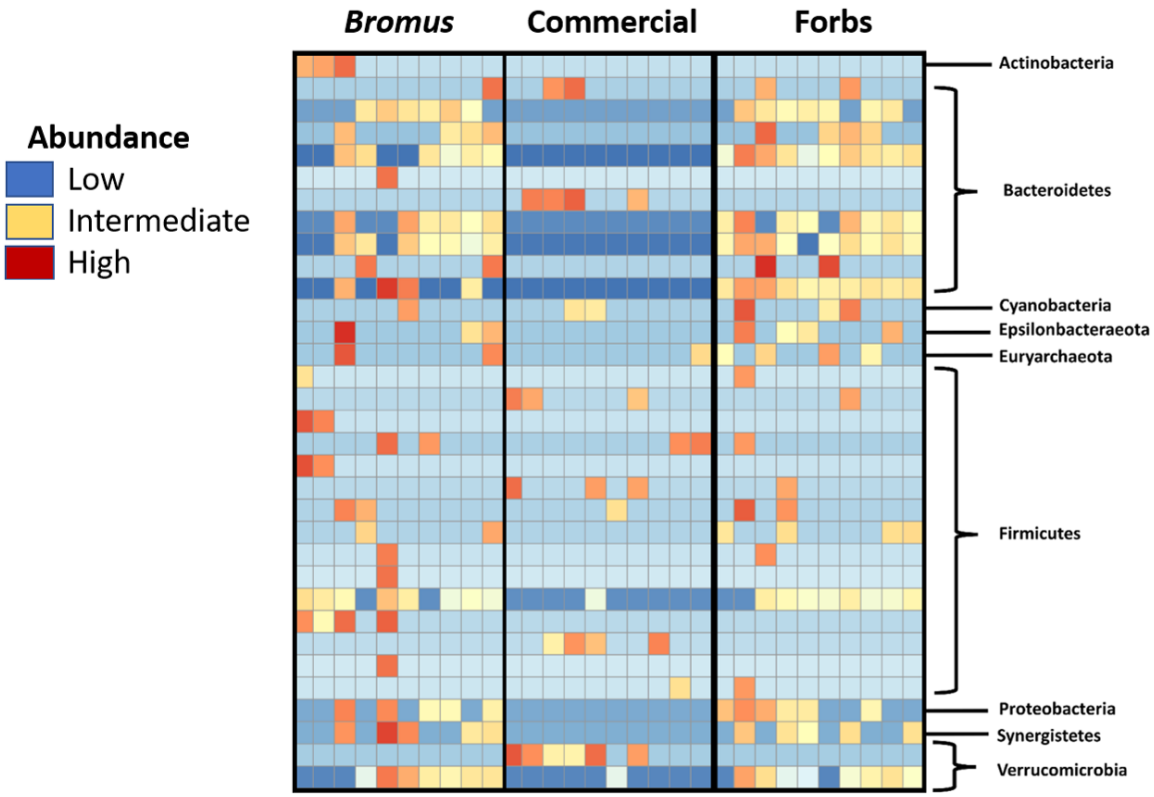

Figure 2.3.5. Heatmap of variance-stabilized abundance estimates for the 33 Amplicon Sequence Variants (ASVs = variant within the microbial community) differentially expressed across diets. Each row corresponds to an ASV and each column corresponds to a sample.

Growth

Cumulative change in plastron length was a significant predicator (R^2 = 0.082, F = 2.503, p = 0.037) of community composition, whereas mass was a marginal predicator (R^2 = 0.064, F = 1.918, p = 0.094) by the end of the study. DeSeq2 models indicated the same 13 ASVs were differentially expressed between individuals that grew less versus individuals that grew more by the end of the study. These ASVs represented five phyla: Bacteroidetes, Euryachaeota, Firmicutes, Lentisphaerae, and Verrucomicrobia. Variants in Lentisphaerae were generally more abundant in individuals that grew less, whereas variants in Euryarchaeota and Verrucomicrobia were more abundant in individuals that grew more (Figure 2.3.6).

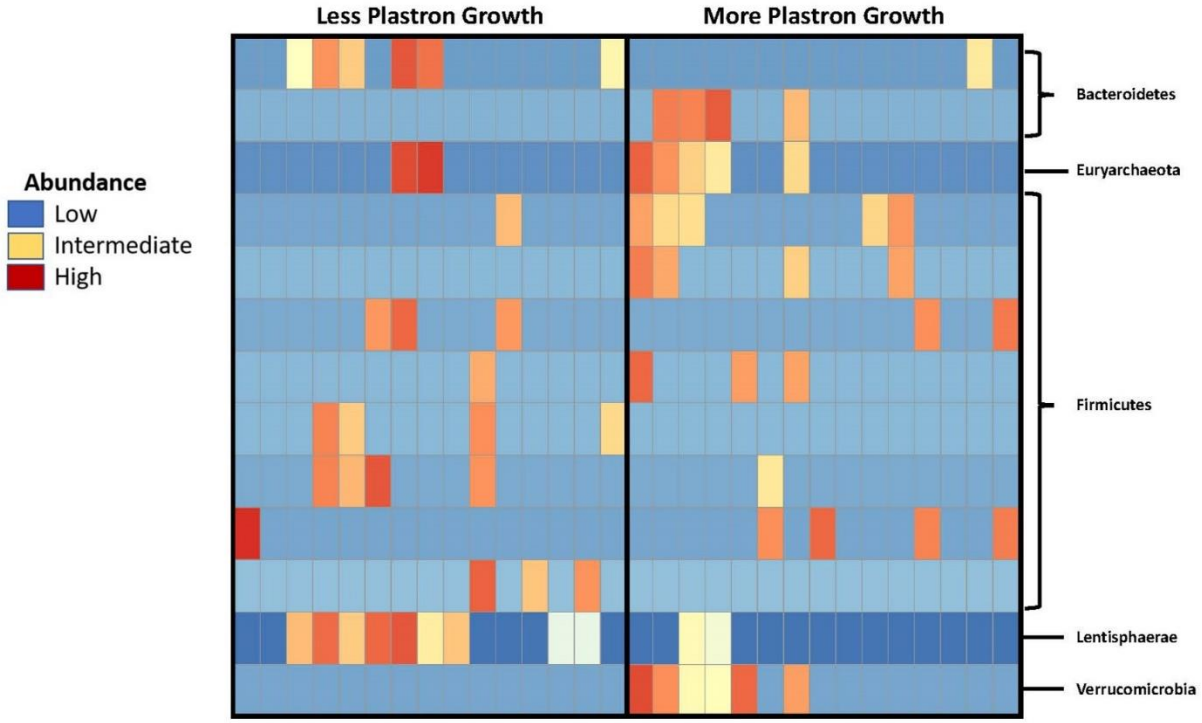

Figure 2.3.6. Heatmap of variance-stabilized abundance estimates for the 16 Amplicon Sequence Variants (ASVs) differentially expressed across diets. Each row corresponds to an ASV and each column corresponds to a sample.

Survivorship

A PERMANOVA of Bray-Curtis distances between fecal samples taken from the last sample available (September for most; earlier for individuals that died) indicated marginally significant associations between microbial community composition and survival (R^2 = 0.074, F = 2.242, p = 0.07). When examining associations between community composition and survival within diet groups for the last samples taken for each tortoise, neither commercial diets (R^2 = 0, F = 0, p = 1; i.e., there was no mortality in this group) nor *Bromus* diets (R^2 = 0.074, F = 0.640, p = 0.623) exhibited significant associations between community composition and survival. There was a marginally significant association detected for results from forb diets (R^2 = 0.216, F = 2.206, p = 0.086). DESeq2 models of differential abundance in response to survival status across diets identified a total of 16 ASVs between tortoises that survived versus those that did not survive (Figure 2.3.8). Within diet groups, 17 ASVs were differentially abundant across survival status within the forbs diet, whereas 21 were differentially abundant within the *Bromus* diet.

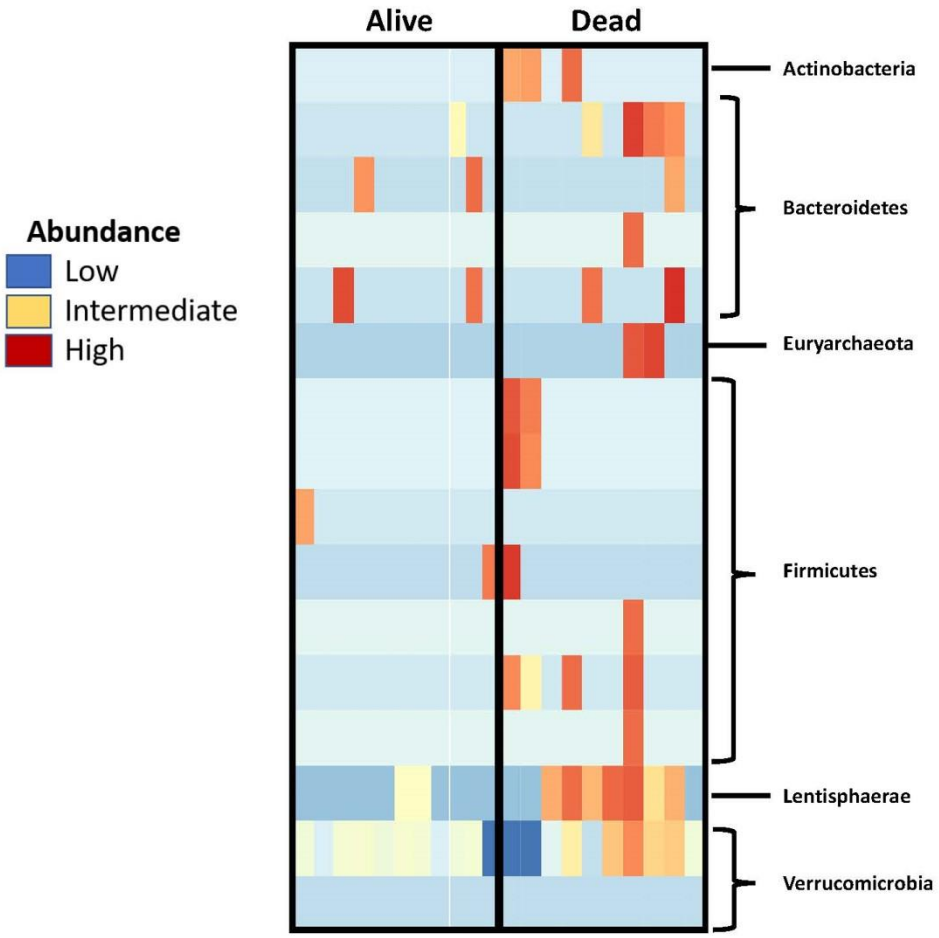

Figure 2.3.8. Heatmap of variance-stabilized abundance estimates for the 16 Amplicon Sequence Variants (ASVs) differentially expressed among alive and deead individuals. Only tortoises consuming Bromus and native forbs diet are included. . Blue cells indicate low abundance, while yellow and orange cells indicate intermediate abundance, and red indicates high abundance. Each row corresponds to an ASV and each column corresponds to the last fecal sample from a tortoise in this experiment.

2.4. DISCUSSION

In this investigation we present the first comprehensive data on the GIT microbial composition for Mojave desert tortoises (*G. agassizii*). Furthermore, this study documented changes in fecal microbiota relative to diet and temporal position in the experiment (month/season) and identified phyla associated with animal growth. We identified 33 microbial lineages (ASVs) representing 9 bacterial phyla including Actinobacteria, Bacteroidetes, Cyanobacteria, Epsilonbacteraeota, Euryarchaeota, Firmicutes, Proteobacteria, Synergistetes, and Verrucomicrobia in fecal samples from *G. agassizii*. Each phylum represents thousands of microbial variants/community organismal groups working interactively with their host to aid digestion, metabolic processes, and immune and physiological functions. In non-model animals, such as tortoises, limited information exists to compare the diversity and abundance for microbiota present within and between species. However, recent related studies have compared wild and captive populations including the Bolson tortoise (*G. flavomarginatu*s; Garcia-De la Pena et al. 2019), gopher tortoise (*G. polyphemus*; Gilliard 2014), and Seychelles giant tortoise (*Aldabrachelys gigantea*; Sandri et al. 2020). We attempted to draw inference from their findings along with similar studies on Beal's eyed turtle (*Sacalia bealei*; Fong et al. 2020) and comparisons among extant marine turtles (Scheeling et al. 2020) when applicable. Most comparative studies documented differences in microbial abundance and diversity in feces between wild and captive populations or age classes (Uenishi et al. 2007; Villers et al. 2008; Xenoulis et al. 2010; Wienemann et al. 2011; Garcia-De la Pena et al. 2019), acknowledging that diet was the likely driver between groups. Unfortunately, most of these studies evaluated a discrete sampling period, potentially missing temporal/seasonal changes that likely occur as plant (food) phenology and availability change.

The dominant phylum of fecal bacteria in our study was Firmicutes, although its abundance varied by diet. Firmicutes play an important role in the metabolism and digestion in the host. For herbivores, Firmicutes assist with fermentation of plant biomass, producing enzymes required to breakdown cellulose and hemicellulose (Sharmin et al. 2013; Qu et al. 2020). Firmicutes are often described within the GIT of herbivorous reptiles (Hong et al. 2015; Garcia de la Pena et al. 2019), as well as a wide variety of mammals (Ley et al. 2008). Bacteriodetes represented the second most abundant phylum in our study, and they are a common gastrointestinal species which produce end-products, promote digestion efficiency, and degradation of simple and complex polysaccharides found in plant cells such as cellulose and hemicellulose (Qu et al. 2020; Yuan et al. 2015; Sandri et al. 2020). Additionally, Bacteriodetes play important roles in preventing bacterial infections in the gut (Hooper et al. 2002). Comparison of the fecal microbial composition in Bolson tortoise (*Gopherus flavomarginatus*) also revealed Firmicutes were the most abundant phylum, although abundance varied for wild and captive animals (93% and 80% respectively). Fibrobacteres was the second most abundant phylum (11%) in captive *G. flavomarginatus* tortoises, however this phlyum was not observed in captive juvenile *G. agassizii* in our study. Cyanobacteria were the second most abundant bacteria in wild *G. flavomarginatus* and also present in *G. agassizii* although less dominant in the community of our juvenile tortoises.

The functions of specific microbiota and their interactions with vertebrate hosts are largely undescribed. However, we believe general information at the phylum or class level can provide

some preliminary insights explaining the composition and abundance of microbiota in relation to tortoise diets and tortoise health in our study. Actinobacteria are known for decomposition and breaking down organic material, but not much is known about their interactions with herbivorous reptiles. A study on the microbiota within Bolson tortoises (*G. flavomarginatus*) found Actinobacteria to contribute only 0.99% of the composition of microbial gut species (Garcia-De la Pena et al. 2019). Bacteroidetes was also identified in our juvenile *G. agassizii* fecal samples, and they are known to produce acids and assist with breaking down plant cells in the digestive tract. In the composition of Aldabra giant tortoise's (*Aldabrachelys gigantea*) microbiome, Bacteroidetes was most prevalent (42%), followed by Firmicutes at 34% (Sandri et al. 2020). Comparisons among wild and captive Bolson tortoises (G. flavomarginatus) also included Firmicutes; however, their microbiota demonstrated that Cyanobacteria was the dominant phyla present among tortoises (Garcia-De la Pena et al. 2019).

We found microbial community composition was a significant predictor of tortoise growth (cumulative change in plastron length) in our study. Generally, communities from Bacteroidetes, Euryachaeota, Firmicutes, Lentisphaerae, and Verrucomicrobia phyla were most associated in differentiating tortoises that grew less or more in our experiment. Variants in Lentisphaerae were generally more abundant in individuals with limited growth, whereas variants in Euryarchaeota and Verrucomicrobia were more abundant in individuals that increased their growth rates throughout the experiment. Microbial community composition was a marginal predicator of animal survivorship; however this compared only included *Bromus* and forbs diet groups.

We conclude this study with more questions than answers. However, this work provided obvious insights into the microbial changes that may occur relative to altered diet from the invasive non-native grass, *B. rubens*. Interestingly, composition among tortoises consuming *Bromus* and native forbs was more similar than animals consuming the commercial diet. This could be an artifact of differing microbes present on natural plants and associated soil, or clinical and physiological differences among individuals and diet groups. As described in the first section of this report, tortoises consuming both forbs and *Bromus* grass performed similarly in growth and clinical condition.

3. UNDERSTANDING PHYSIOLOGICAL DEFENSE SYSTEMS, IMMUNE FUNCTION, AND STRESS IN TORTOISES.

3.1. INTRODUCTION

Animal health plays an important role in the management and conservation of sensitive wildlife (Rodriguez-Jorguera et al. 2016). Physiological biomarkers, when validated and used appropriately, can provide context to the mechanisms underpinning animal health and environmental quality. For conservation efforts, understanding the mechanics of response can provide insight into how animals respond to current and future stressors. Wildlife declines are generally the result of a combination of both human (e.g. overharvest, habitat destruction, or introductions of invasive species, contaminants, and/or disease related agents) and natural (e.g. disease epidemics, drought, flood, climate change) stressors and rarely due to one single factor operating in isolation (Irwin and Irwin 2006; Micheli et al. 2016). The response of an individual or population to environmental stressors often differs according to the cumulative effects of the stressors present in their environment in relation to individual variability of health history, or genetic based resilience factors (Patyk et al. 2015). As a result, understanding the effects of environmental change on animal health requires monitoring and assessment of these complex interactions, ideally using a range of ecological, biomedical and metabolic indicators (Lloyd et al. 2016).Schoeman 2016). Physiological biomarkers, when validated and used appropriately, can provide context and mechanism to describe animal health and environmental quality. Although a number of survey practices (i.e. physical examinations, hematological and biochemical blood panels, serology tests, and pathogen and toxicology screens) can be used to assess wildlife health, these practices have had limited success in accurately tracking health status in free-ranging reptiles (Christopher et al. 2003; Madlinger et al. 2016), thus may require innovation for greater success.

Reptiles present unique challenges in assessing clinical and physiological conditions due to lack of research, challenging logistics of individual capture for repeated assessments, and their metabolic characteristics (Christopher et al. 2003; Allender et al. 2016; Buckley and Huey 2016) that are not well understood at this time (Drake et al. 2017; Drake et al. 2019). As ectotherms, reptiles must partition resources among self-maintenance activities including the immune system and other physiological functions (Zimmerman et al. 2010) by undergoing strong seasonal shifts in behavior, physiology and metabolism (Zimmerman et al. 2010; Vitt 2016). Collectively, such partitioning makes it difficult to accurately measure their physiological stress responses. Age, sex, individual, environmental, and nutritional conditions increase the variability of physiological responses in reptiles (Wright and Cooper 1981; Zapata et al. 1992; Dickinson et al. 2002; Moore and Jessop 2003; Vitt 2016).

Nutritional physiology can provide insight into what components of an animal's diet are required for healthy growth and normal physical development (Tracy et al. 2006). Diets can be examined from the macro-level (e.g. plants, animals, insects) to micronutrients (e.g. copper, zinc, magnesium) in both food and water (Madliger et al. 2016), but specific requirements are not always easy to identify (Madliger et al. 2016) without extensive and invasive experimentation. Despite these drawbacks, standard wildlife diagnostic blood panels (e.g. hematology, biochemistry, serology and cytology) and physical examinations are still routinely used on

reptiles to investigate conditions that may affect blood cells or cause a change in blood cell composition including anemia, inflammation, hematopoietic disorders and parasitemia (Christopher et al. 1999; Christopher 1999; Sheldon et al. 2016; Drake et al 2017). Comprehensive panels often require more blood or plasma than can be safely collected from wild or smaller reptiles, and the interpretation of these data is challenging because of the low number of studies and the lack of reference values for certain species or wild populations (Lloyd et al. 2016). Furthermore, these laboratory tests are not designed for or capable of identifying specific intrinsic or extrinsic stressors impacting animal health.

In the current study, we continued our ongoing efforts to develop a comprehensive panel of blood analytes to evaluate animal stress or physiological dysregulation. We used samples to optimize laboratory procedures and associated chemicals and reactants, thus requiring less blood tissue for future research with this species. We evaluated organic compounds (malondialdehyde-MDA), proteins (interleukin protein (IL -6); alpha-2-macroglobulin (α_2M)), total and targeted proteins (heat shock proteins (HSP70, HSP90)), enzymes (alanine aminotransferase-ALT; aspartate aminotransferase- AST; ALT-AST ratios; lactate dehydrogenase-LDH), and hormones (corticosterone-CORT) in blood. Although several of the selected blood analytes are integrated and play multiple roles in balancing, protecting, and defending physiological mechanisms in vertebrates, we attempted to include each in one of three categories: 1) oxidative stress and antioxidant defenses, 2) immune function, and 3) physiological stress. We predicted that tortoises consuming invasive grass diets (*B. rubens*) would yield higher responses to indicators of oxidative, thermal, immunological, and physiological stress compared to tortoises foraging on nutrient-rich native forb plants or commercial tortoise food.

Oxidative Stress and Antioxidant Defense Systems

Oxidative stress in animals is generally defined as excess production of reactive oxygen species (ROS) relative to antioxidant defense parameters (Shankar and Mehendale 2014). ROS are inescapable by-products of energy metabolism and may cause costly damage to biological structures such as DNA, proteins, and membranes (Bury et al. 2018). Organisms have evolved different means to counter oxidative stress, such as modulation of ROS production, neutralization of produced ROS through free radical scavenging, and the repair or removal of the damaged structures. While normal levels of oxidative stress are guarded by body's self-defense mechanism, overwhelming stress can be detrimental (Shankar and Mehendale 2014). We questioned how tortoises respond to oxidative stress, as they reside in chronically stressful environments with extreme temperature exposure, limited food and water availability, and unique metabolic challenges. Furthermore, we pondered what impact altered diets and their associated nutritional and energy changes have on antioxidant defense systems in tortoises? To explore these questions, we used measures of MDA in plasma as a metric of oxidative stress (Chainy et al. 2016). Authors experience with multiple species (white-tailed antelope ground squirrel -*Ammospermophilus leucurus*, tenrec -*Microgale* spp., and desert pupfish - *Cyprinodon macularius*) indicate that MDA assays provide the most robust means of assessing ROS damage, in those mammals and we sought to clarify their activity in the Mojave desert tortoise.

Immune Function

The vertebrate immune system is a complex network of organs, tissues, circulating cells, and molecules that include both innate and induced mechanisms (Ellis 2012). Tortoises, like most

ecotherms, are known to invest in, and rely on broad innate responses such as non-specific leukocytes, lysozymes, antimicrobial peptides, the complement pathway, and behaviorally induced fever as their primary lines of defense against pathogens and other foreign materials (Rios and Zimmerman 2015; Drake et al. 2019). We used measures of the protein IL-6 and α_2-M as potential markers to capture innate immune responses in tortoises consuming altered diets. IL-6 is a pro-inflammatory cytokine protein that is activated through proteolytic cleavage by the inflammasome during cell damage and subsequent inflammatory signaling (Tanaka et al. 2014; Qing et al. 2020). α_2-M is a large plasma protein typically associated with inhibiting enzymes (proteases), serving as an acute phase protein and contributes to the misfolded protein response during stress (Cater et al. 2019). α_2M concentrations are used clinically to assess various pathologies such as nephrotic and hepatic stress, disease, and cellular dysfunction (Gressner et al. 2007). Tortoises consuming *Bromus* grasses often experience physical injury to their mucosal linings in the mouth, as *Bromus* seeds become impacted and result in infection and inflammation (Medica and Ekert 2007; Drake et al 2016).

Physiological Stress

Physiologic stress from a variety of intrinsic and extrinsic factors results can result in a complex response that involves a variety of endogenous mediators. Several analytes available in plasma such as Heat Shock Proteins (HSP70; HSP90), metabolic enzymes (AST, ALT, AST:ALT Ratio; LDH), and hormones (e.g. corticosterone = CORT) have been used as indicators to evaluate physiological stress. Heat shock proteins are chaperone proteins that assist in proper protein folding during stress, with HSP70 and HSP90 being two of the most widely investigated HSPs and are often associated with thermal and cellular stress (Somero 2020; Gormally and Romero 2020). ALT and AST are intracellular aminotransferases (enzymes) with high expression in liver and muscle tissue. Under various forms of physiological stress and cellular damage, ALT and AST are released into the bloodstream. Lactose DeHydrogenase (LDH) is an intracellular enzyme present in many tissues including blood, liver, kidney, and other organs. Similarly, its elevated presence in plasma is often used as a general indicator for tissue damage. Glucocorticoid hormones [corticosterone = CORT] generated via the Hypothalamic-Pituitary-Adrenal (HPA) axis are also used as indicators of the stress response. Glucocorticoids are a class of steroid hormones released from the adrenal glands during a wide variety of stress stimuli and conditions, including harsh weather, animal manipulation, and disturbances in habitat (Wingfield et al. 1998; Moore & Jessop 2003).

3.2. METHODS

Blood tissue was collected from experimental animals in May, June, and July as previously described (section 1.2.2). However, plasma samples were only available from our June and July sampling events. As such, these samples were used to evaluate blood analytes relative to diet and month when possible. We measured total protein for all samples in June and July. We targeted at least 3 individuals for select proteins and 5 individuals for select enzymes per diet each month for analyses when possible. Tissue collected in April and preserved as whole blood was not used in this experiment.

Protein Concentrations

Prior to analysis, total protein concentrations in each sample were calculated using Bradford protein determination assay (Bradford 1976). This assay follows similar procedures with modified Lowry's determination assay (Lowry et al. 1951; Peterson 1977) yet, requires less sample (~1 µl of plasma per individual in current study) for analysis. Proteins were evaluated using a colorimetric biochemical change and measured using spectrophotometric analysis.

Biomarker for Oxidative Stress and Antioxidant Defense

Malonaldehyde (MDA) – MDA is an organic compound found in packed blood cells that can be used as a metric of lipid peroxidation and oxidative stress (Chainy et al. 2016). We performed a lipid peroxidation assay to quantify malonaldehyde (MDA) levels in plasma based on procedures outlined in Gerard-Monnier et al. (1998). We diluted 5 µl of plasma with 15 µl of sample buffer (20 mM Tris, pH 7.4) and combined this mixture with 10 mM 1-methyl-2-phenylindole in acetonitrile/methanol (3:1). The solution was then added by concentrated methanesulfonic acid containing 34 uM Fe (III) to all tubes. Each tube was vortexed and allowed to incubate in a thermomixer at 45 ^{o}C (300 rpm) for 60 minutes. Samples were centrifuged at 15,000 g at room temperature for 10 minutes. Supernatant was transferred into 96-well plates and absorbance was measured at 585 nm on a Tecan Spark 10M multimode microplate reader (Tecan Austria GmbH, Grödig, Austria).

Biomarkers for Immune Defense

Interleukin 6 protein (IL-6) – IL-6 is a pro-inflammatory cytokine that becomes activated during cellar stress and disease (Qing et al. 2020). We measured IL-6 levels in plasma using a western blot approach. Plasma was subjected to SDS-PAGE western blotting to assess levels of specific proteins. Briefly, 2 µl of plasma of each sample was ran on acrylamide gels. Proteins were then electrophoresed onto PVDF membranes at 400 mA for 3 h at 4 °C. Following transfer, blots were dry-stored at least overnight before overnight incubation in protein blocking buffer (Tris buffered saline with 0.2% tween 20 (TBST) with 5% dry milk w/v) followed by primary antibody (in TBST with 5% dry milk w/v) then secondary antibody (in TBST with 5% bovine serum albumin) incubation. After each antibody incubation, blots were washed for 5 min in TBS followed by two 5 min washes in TBST and a final 5 min wash in TBS. Visualizations for blots were performed on either a Amersham Typhoon Imager using ECL+ (Model #9410, GE Healthcare, Boston, MA, USA) or on a LiCor Odyssey (Lincoln, NE, USA USA). All quantifications were performed in the linear range using either ImageQuant (GE Healthcare, Boston, MA, USA) or LiCor software (Lincoln, NE, USA). Ponceau staining was performed to confirm equal loading of gel lanes. IL-6 proteins were run on 15% acrylamide gels. Blots were incubated with the IL-6 mouse monoclonal antibody at 1:500 (Clinical Proteomics Technologies for Cancer-Development Studies Hybridoma Bank (DSHB) Product #CPTC-IL6-3). Visualization was performed using ECL+ on a Typhoon imager (Model #9410, GE Healthcare, Boston, MA, USA).

Alpha-2-macroglobulin (α_2-M) - We assessed α2M using a western blot (protein immunoblot) approach as described previously to separate and identify targeted proteins (Locke and Tanguay 1996; Ramaglia et al. 2004). α_2-M protein samples were run on 9% acrylamide gels and a rabbit polyclonal antibody (Abclonal Technologies # A1573, Woburn, WA, USA) incubation at a concentration of 1:200. We used a fluorescent secondary antibody (goat-anti-rabbit IRDye

680RD; Licor Biosciences #926-68071, Lincoln, NE, USA) and incubated at a concentration of 1:15,000. Visualization and quantification were performed on a Licor Odyssey Imager and software (LI-COR Biotechnology, Lincoln, NE, USA).

Biomarkers for Physiological Stress

Heat Shock Proteins (HSP-70, HSP-90) – We compared constitutive levels of HSP70 and HSP90 in tortoise plasma using a western blotting as previously described. We evaluated three individuals per diet in both June and July, except for the forbs diet group in July which had two samples available for this assay. Protein samples were run on 8% acrylamide gels. We used rabbit polyclonal antibodies for HSP 70 (Abclonal Technologies #A12948, Woburn, WA, USA) and HSP 90 (Abclonal Technologies #A13501, Woburn, WA, USA) at 1:500 and 1:1000 dilutions respectively. Visualizations for blots were performed using ECL+ on a Typhoon imager. Arbitrary units were used to describe HSPs.

Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) Enzymes - Under certain instances of cell stress and damage, ALT and AST are released into the bloodstream. Increased ALT and AST in the blood are well-documented in various diseases, especially related to the liver and muscles (Cheng et al. 2016; Ahmed et al. 2018; Kleiner et al. 2019). ALT and AST activities in tortoise plasma were measured spectrophotometrically using an ALT and AST detection kits (ALT #A7526-625; AST #A7561-625; Pointe Scientific, Canton, MI, USA) with a Tecan Spark 10M multimode microplate reader (Tecan Austria GmbH, Grödig, Austria). We compared ALT and AST levels for five tortoises per diet group in June and July. Units are expressed in $IU \bullet l^{-1}$.

Lactate Dehydrogenase (LDH) – LDH is an intracellular enzyme involved in metabolism and is found in nearly every cell. Typically, increased presence of LDH in blood indicates cell death and tissue damage, and are known to occur in various cardiac, liver, renal, and skeletal muscle diseases (Chan et al. 2013; Huijgen et al. 1997). We measured LDH activity spectrophotometrically using an LDH activity kit (#L7535-300, Pointe Scientific, Canton, MI, USA) with a Tecan Spark 10M multimode microplate reader (Tecan Austria GmbH, Grödig, Austria). This assay is based on the lactate to pyruvate reaction catalyzed by LDH. The rate of NAD reduction in the reaction results in increased absorbance at 340 nm. Units are expressed in $IU \bullet l^{-1}$.

Corticosterone (CORT) - CORT is a glucocorticoid hormone produced by the adrenal glands in response to stressful stimuli and is commonly used as a stress biomarker in animals (Drake et al. 2012; Gormally and Romero 2020). To assess potential stress related to altered diet, we measured circulating total plasma CORT levels for three tortoises per diet group in June and July. CORT was measured using a corticosterone ELISA assay kit (Cayman Chemical, Ann Arbor, MI, USA; #501320). This assay is based on the competition between sample CORT and CORT tracer. Therefore, the absorbance is inversely correlated to the concentration of CORT in the sample. Absorbance was measured at 412 nm using a Tecan Spark 10M multimode microplate reader (Tecan Austria GmbH, Grödig, Austria). All measurements were within the linear range. Corticosterone concentrations are expressed in $ng \bullet ml^{-1}$.

Statistical Analysis

Blood analytes and ratios were analyzed with a series of linear mixed-effects models (R Package lme4 ver 1.1-23) with tortoise individual as random effects to account for repeated measures. Candidate models were compared using Akaike's information criterion corrected for small sample sizes (AICc; Burnham and Anderson 2002) using the package MuMIn (v1.43.17) in R (Team 2020). Data are presented as mean ± 95% confidence intervals. Statistical significance was assumed when $p < 0.05$.

3.3. RESULTS

Protein Concentrations

Blood plasma proteins are often used as indicators of animal health. We measured total protein concentrations in all available samples and found significant increases in tortoises consuming commercial diets compared to other diet groups (Table 3.3.1; Figure 3.3.1). Overall protein lysate averages were similar for each diet in both June and July (Table 3.3.1).

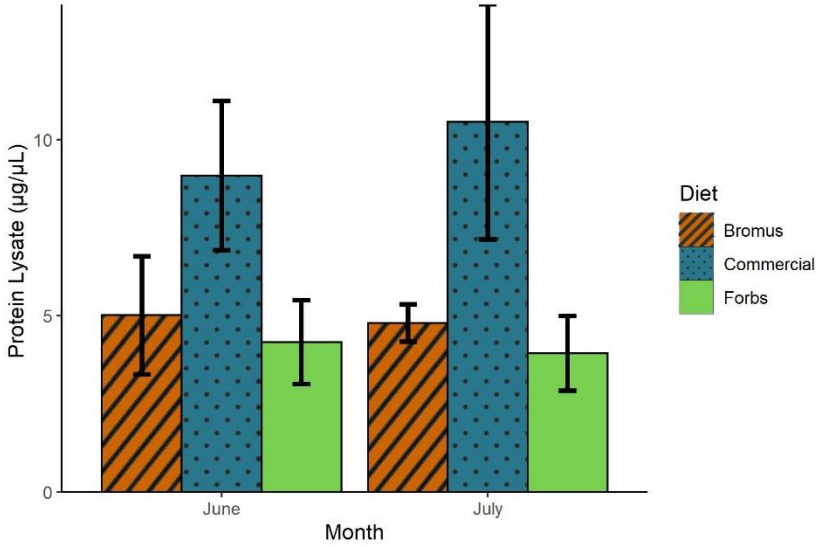

Figure 3.3.1. Mean + 95% confidence intervals for total plasma protein (µg/µL) concentrations in 27 juvenile Mojave desert tortoises (*Gopherus agassizii*) consuming a diet of the invasive grass *Bromus rubens*, commercial tortoise food, or a mixture of native forb plants. Samples were evaluated in June and June 2018. Protein levels were similar among sampling periods, but significantly increased in tortoises consuming commercial diet.

Oxidative Stress

MDA - MDA is a natural biproduct of lipid peroxidation and has been used as a marker for oxidative stress. We measured concentrations of MDA to assess lipid peroxidation/ ROS damage as a function of diet. We found overall [MDA] were similar among diets and between months (Table 3.3.1; Figure 3.3.2). Interestingly, MDA levels increased in July only for tortoises foraging on commercial diet (Figure 3.3.2).

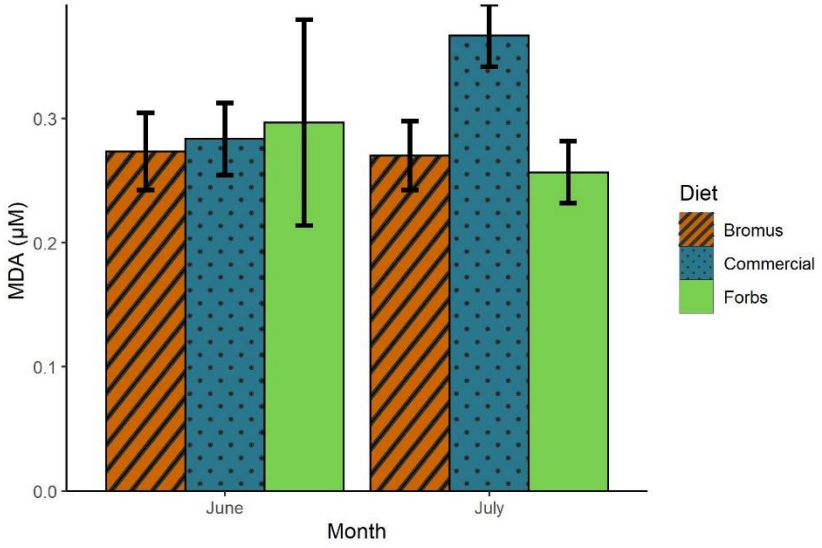

Figure 3.3.2. Mean + 95% confidence intervals for plasma malonaldehyde (MDA) concentrations in juvenile Mojave desert tortoises (*Gopherus agassizii*). [MDA] was evaluated as a potential indicator of oxidative stress for tortoises foraging on diets of *Bromus rubens*, commercial tortoise food, or a mixture of native forb plants and sampling periods. No differences in MDA concentrations were found between diet groups or sampling periods. However, increases in [MDA] were noted in the commercial diet group in July.

Table 3.3.1. Statistical findings for 11 plasma analytes evaluated in 30 juvenile Mojave desert tortoises (*Gopherus agassizii*) relative to diet group (*Bromus*, commercial, and native forbs) and sampling event (months - June and July).

Analyte Type	**Plasma Analyte (unit)**	**Diet**	**Month**
Protein	Total Protein (µg/µL)	$F_{2,24} = 13.25$, $p < 0.01$ **	$F_{1,17} = 0.009$, $p = 0.93$
Organic Compound	[MDA] (µM)	$F_{2,6} = 1.38$, $p = 0.34$	$F_{1,6} = 0.23$, $p = 0.64$
Protein	IL-6 (arbitrary units)	$F_{2,5} = 1.78$, $p = 0.26$	$F_{1,1} = 2.41$, $p = 0.36$
Protein	α_2M (arbitrary units)	$F_{2,5} = 1.78$, $p = 0.26$	$F_{1,6} = 0.31$, $p = 0.60$
Protein	HSP70 (arbitrary units)	$F_{2,6} = 2.65$, $p = 0.15$	$F_{1,5} = 9.48$, $p = 0.03$ **
Protein	HSP90 (arbitrary units)	$F_{2,6} = 1.66$, $p = 0.27$	$F_{1,6} = 0.13$, $p = 0.73$
Enzyme	ALT (IU*1-1)	$F_{2,18} = 1.44$, $p = 0.26$	$F_{1,5} = 4.61$, $p = 0.08$ *
Enzyme	AST(IU*1-1)	$F_{2,18} = 4.97$, $p = 0.02$ **	$F_{1,6} = 8.17$, $p = 0.03$ **
Enzyme Ratio	AST:ALT Ratio	$F_{2,18} = 0.99$, $p = 0.39$	$F_{1,7} = 0.33$, $p = 0.56$
Enzyme	LDH (IU*1-1)	$F_{2,18} = 4.89$, $p = 0.02$ **	$F1_{,5} = 6.31$, $p = 0.05$ **
Hormone	CORT (ng*mL-1)	$F_{2,7} = 0.79$, $p = 0.49$	$(_{1,7} = 7.41$, $p = 0.03$ **

Immune Defense

We evaluated two proteins (IL-6 and α_2-M) thought to play important roles in innate immunity and early defense responses in tortoises. IL-6 is a pro-inflammatory cytokine protein activated during cell damage and subsequent inflammatory signaling. α_2-M is a large plasma protein that serves as an acute phase protein, and is often used clinically to assess various pathologies such as nephrotic and hepatic stress, disease, and cellular dysfunction (Gressner et al. 2007). Western

blot statistical analysis revealed no overall differences in IL-6 or α_2-M among diets or months (Table 3.1.1; Figure 3.3.2). However, tortoises consuming native forbs had higher and more variable IL-6 protein availability and α_2-M was notable higher in tortoises eating commercial diet compared to other groups (Figure 3.3.3).

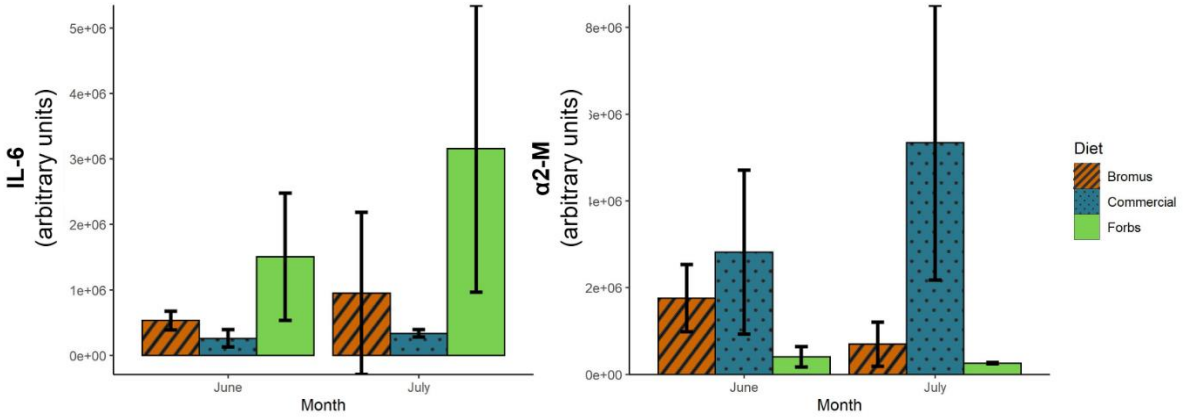

Figure 3.3.3. Mean + 95% confidence intervals for plasma (left) interleukin-6 (IL-6;) and (right) alpha-2-macroglobulin (α_2-M) proteins measured in juvenile Mojave desert tortoises (*Gopherus agassizii*) consuming a diet of the invasive grass *Bromus rubens*, commercial tortoise food, or a mixture of native forb plants. Samples were evaluated in June and June 2018. Protein levels were similar among diet groups and sampling periods.

Physiological Stress

Indicators of physiological stress such as heat shock proteins (HSP70; HSP90), metabolic enzymes (AST, ALT, AST:ALT Ratio; LDH), and glucocorticoid hormones (CORT) were used to evaluate changes in plasma relative to diet and temporal sampling events. Heat shock proteins are chaperone proteins that assist in proper protein folding during stress. We found similar HSP70 and HSP90 levels among diet groups throughout our study (Table 3.1.1; Figure 3.3.4). HSP90 was similar between sample periods; however, HSP70 levels decreased for all diet groups in July (Table 3.1.1; Figure 3.3.4).

We evaluated three intracellular enzymes (ALT, AST, and LDH) that often are released into blood or increase during physiological stress, cellular damage, and tissue injury as well as the stress hormone - CORT. Overall enzymatic activity was generally higher in animals consuming commercial diet (Figure 3.3.5). Both AST and LDH enzymatic activity levels varied by diet group and month, with lower activity among *Bromus* and forb diet groups (Table 3.1.1; Figure 3.1.5). ALT was similar among diets and marginally lower for each group in July (Table 3.1.1). We evaluated the ratio of AST:ALT activity levels and found similar ratios among groups and sampling periods (Table 3.1.1; Figure 3.3.5). We found plasma total CORT levels were similar among diet groups, yet varied by month (Table 3.1.1; Figure 3.3.6). CORT decreased in July for all individuals, likely from changes in metabolic capacity, animal behavior, and temporal and

seasonal influences on this hormone (Drake et al. 2012). This drop is likely associated with temperature/seasonal change in this hormone as well as the tortoises' metabolic capacity and behavior.

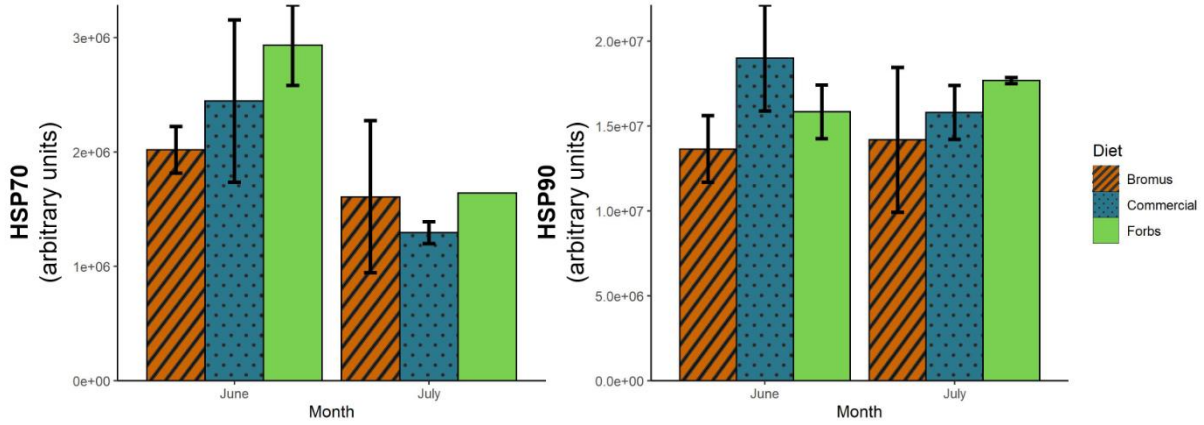

Figure 3.3.4. Mean + 95% confidence intervals for heat shock proteins -70 (HSP70) and HSP90 in juvenile Mojave desert tortoises (*Gopherus agassizii*) consuming a diet of the invasive grass *Bromus rubens*, commercial tortoise food, or a mixture of native forb plants. Samples were evaluated in June and June 2018. Protein levels were similar among diet groups, yet lower for HSP70 in July.

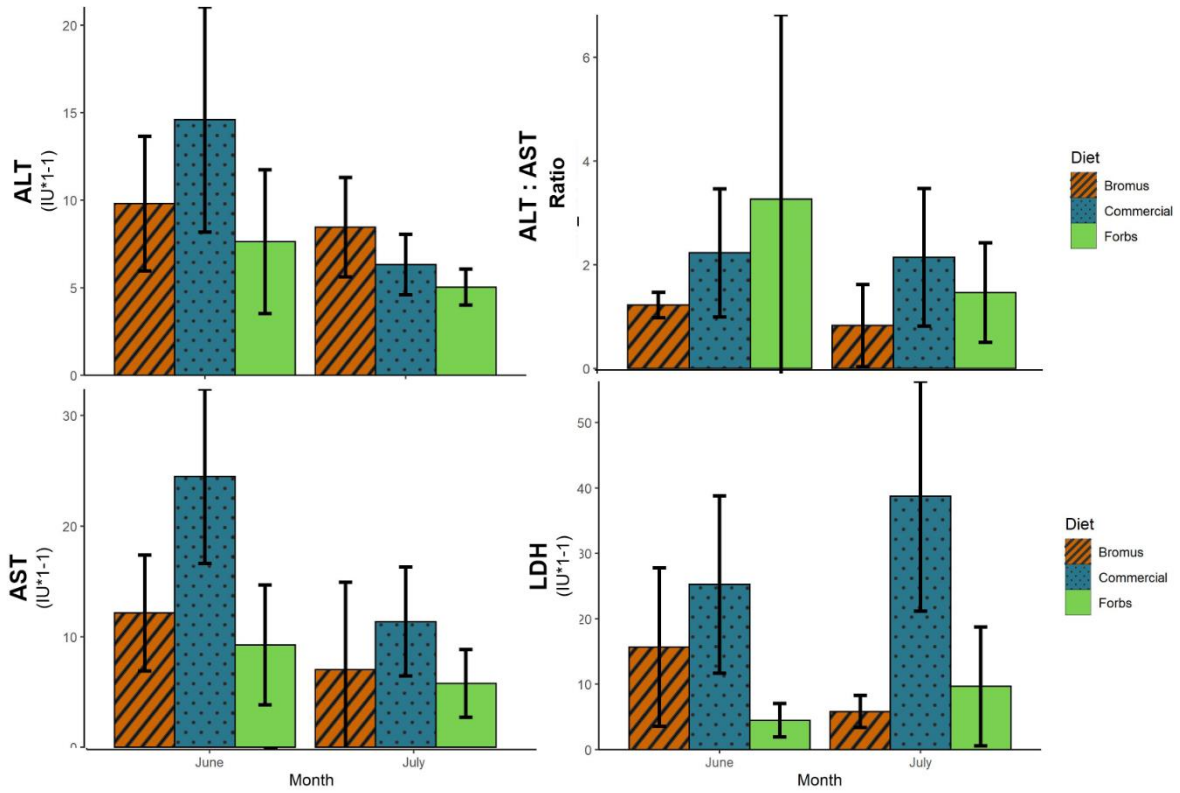

Figure 3.3.5. Mean + 95% confidence intervals for intracellular enzymatic activity for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) for juvenile Mojave desert tortoises consuming 3 diets in June and July. AST:ALT ratios are also presented.

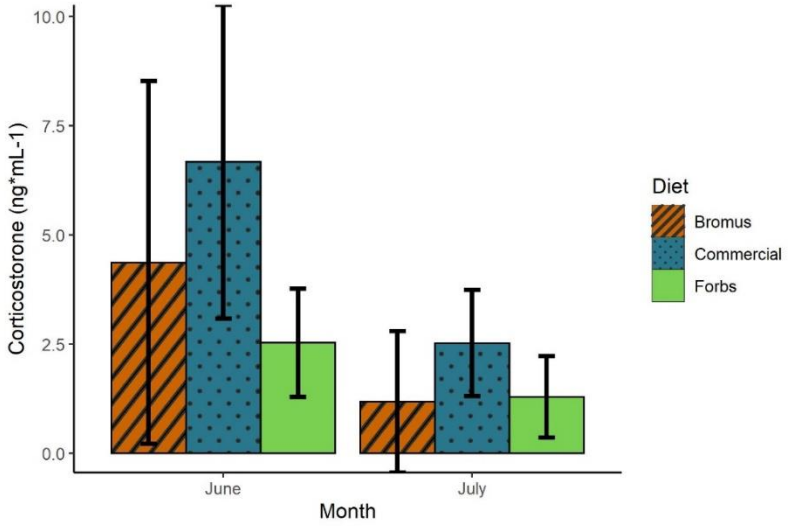

Figure 3.3.6. Mean + 95% confidence intervals for total plasma corticosterone (CORT; ng/mL) measured for juvenile Mojave desert tortoises consuming diets of either *Bromus rubens*, commercial food, or mixture of native forb plants in June and July. CORT was similar among groups, but levels were lower in July.

Exploratory Analyses

As an exploratory exercise, we evaluated measured plasma analytes relative to animal mass and shell size in our experiment. We found that protein levels are positively associated with cumulative changes with mass ($R^2 = 0.34$, $p < 0.01$) and plastron length ($R^2 = 0.34$, $p < 0.01$; Figure 3.3.7) and best predicted dietary group in this study. Changes in mass and plastron length often scale together; however, mass in tortoises can fluctuate widely depending on hydration opportunities, animal activity, and bladder voiding prior to tissue collection. We considered that including both metrics relative to plasma protein may be useful for future studies. Tortoises consuming commercial diet represent animals with higher protein concentrations and the most increases in body size. Animal survival was not evaluated with respect to protein and other blood analyte values, as most tortoises were alive during our sampling events (early June and July).

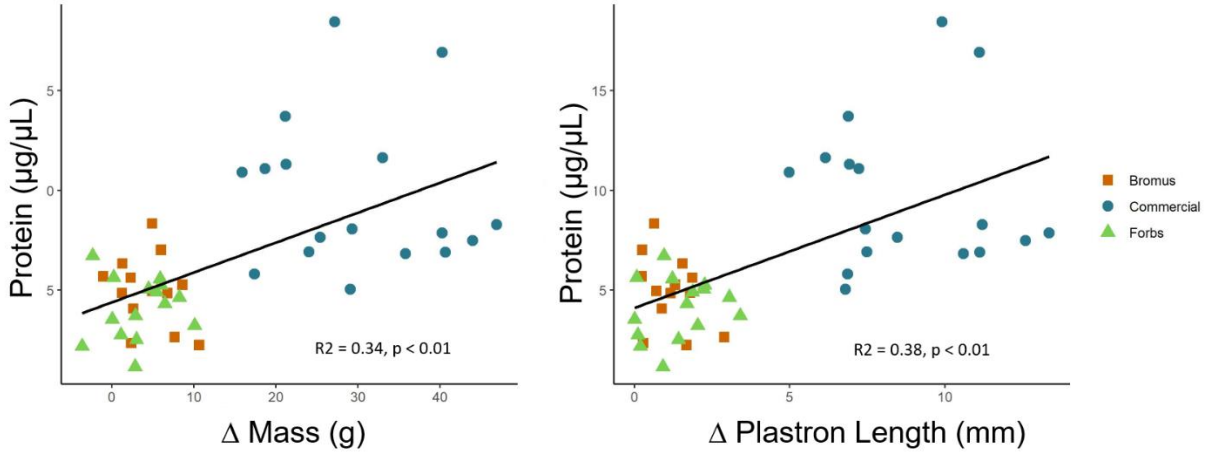

Figure 3.3.8. Comparisons of associations of total plasma protein (µg/µL) concentrations with changes in animal mass (left) and shell size (right; plastron length) for juvenile Mojave desert tortoises consuming diets of either *Bromus rubens*, commercial food, or mixture of native forbs. Higher concentrations of protein were observed in tortoises with increased growth.

3.4. DISCUSSION

Wildlife diagnostic blood panels (e.g. hematology, biochemistry, serology and cytology) and physical examinations are routinely used on reptiles to investigate conditions that may affect blood cells or cause a change in blood cell composition including anemia, inflammation, hematopoietic disorders and parasitemia (Christopher et al. 1999; Christopher 1999; Sheldon et al. 2016; Drake et al 2017). Comprehensive panels often require more blood or plasma than can be safely collected from wild or smaller reptiles, and the interpretation of these data can be challenging because of the low number of studies and the lack of reference values for certain species or wild populations (Lloyd et al. 2016). We optimized laboratory techniques and procedures so that ~75 µL (0.075 mL) or less of plasma can be used to evaluate a comprehensive blood panel to compare indicators of stress and immune response in Mojave desert tortoises. Furthermore, we explored several primary and secondary antibodies involved in western blotting approaches from model-organisms (goat, rabbit, mouse) and successfully found matches that adhere well to targeted proteins in tortoise plasma (see methods). This work greatly contributed

to on-going efforts to develop a more robust quantitative panel of blood analytes that can be used to evaluate animal stress and physiological condition in tortoises and similar taxa under a variety of scenarios.

Experimental Responses

Growth differences were documented among diet groups in our study, as tortoises foraging on commercial diet grew nearly 3x the rate as other diet groups. We expected growth and survival in tortoises eating native forbs would be higher than those fed the *Bromus* diet each month (Drake et al. 2016; Drake and Esque unpublished data); however, performance (growth and physiological responses) were largely similar among these groups. We suspect that potential parasitism from *Ornithordorus* ticks, unknown pathogens, or underlying health or disease conditions may have confounded growth and performance in this study.

Understanding physiological response mechanisms in tortoises and most ectotherms is challenging. Tortoises have adapted metabolic modulations to enhance survival when environmental conditions are physiologically challenging and resources limited (Drake et al. 2012). We don't fully understand the genetic and phenotypic plasticity involved with these metabolic modulations; however, appreciate their complexity and benefit to this species. We expected indicators of oxidative stress, such as [MDA] to increase in nutritionally challenged animals. Yet, tortoise response was limited and did not vary by diet group. Immune responses such as cytokine production (IL-6) and acute phase protein defenses (a_2-M) also did not vary in our study by diet group, yet IL-6 was more variable and a_2-M higher in tortoises consuming native forbs. Comparisons of immune protein activity and clinical health profiles suggest inflammatory responses were present in native forb tortoises, possibly from frequent tick parasitism or other unknown pathogens or disease. Classic stress response indicators such as heat shock proteins, intracellular enzymes (ALT, AST, LDH), and CORT were marginally reactive in this study. AST and LDH varied by diet group but were increased for tortoises consuming commercial diet and individuals with robust growth and health profiles in our study.

The most robust outcome of our study was the association of protein concentration and animal growth. Plasma total protein concentrations increased in animals with more growth, and proved to be our best predictor of diet group. Conversely, you could argue that commercial diet tortoises responded with normal protein concentrations, and animals that stopped eating or ate nutritionally deficient plants reduced detectable protein levels. Hypoproteinemia, or lower than normal levels of protein in the body, may complicate an animals' ability to assimilate nutrients and fight pathogens, has been associated with reduced wound healing, wound dehiscence, reduced immune responsiveness, delayed gastric and cecum emptying, and reduced intestinal motility and absorption. Hypoproteinemia is not necessarily an indication of malnutrition but a reflection of the severity of metabolic stress. As the stress subsides, it is only with adequate nutritional support that circulating protein synthesis levels can return to normal (Newton et al. 2006). We assume that anemia and hypoproteinemia for animals consuming poor nutritional plants, fighting infection, or are heavily parasitized can create scenarios of diminishing returns in vulnerable age classes.

This study highlights the complex and counterintuitive responses of tortoises to environmental and nutritional stress. We provided several scenarios to explain our results. We hypothesized that

1) tortoises may not stress in a "traditional context" to the point where their physiological profiles change at detectable levels, 2) metabolites (proteins, enzymes, etc.) in response to stressful stimuli were missed in blood panel, or 3) important biological and environmental components may be missing from our study. For example, metabolic output (measured as oxygen intake and carbon dioxide output) in relation to body temperature may be necessary to interpret subtle changes and to be able to detect and accurately measure tissue dysfunction and physiological stress in this species. We are optimistic that inclusion of plasma protein concentrations, in combination with measures of metabolism and body temperature may greatly inform future studies on tortoises and related species.

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