Title

High sugar diet alters immune function and the gut microbiome in juvenile green iguanas *(Iguana iguana)*

Running Title

Sugar, immunity, and the microbiome

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Summary Statement

We provide evidence that a high sugar diet has differential effects on components of the green iguana immune system and alters the gut microbiome.

Abstract

The present work aimed to study whether a high sugar diet can alter immune responses and the gut microbiome in green iguanas. Thirty-six iguanas were split into four treatment groups using a 2x2 design. Iguanas either received a sugar supplemented diet or a control diet, and either received a lipopolysaccharide (LPS) injection or a phosphate buffer solution (PBS) injection. Iguanas were given their respective diet treatment through the entire study (~3 months) and received a primary immune challenge one month and two months into the experiment. Blood samples and cloacal swabs were taken at various points in the experiment and used to measure changes in the immune system (bacterial killing ability, lysis and agglutination scores, LPS specific IgY concentrations), and alterations in the gut microbiome. We found that sugar diet reduces bacterial killing ability following an LPS challenge, and sugar and the immune challenge temporarily alters gut microbiome composition while reducing alpha diversity. While sugar did

not directly reduce lysis and agglutination following the immune challenge, the change in these scores over a 24-hour period following an immune challenge was more drastic (it decreased) relative to the control diet group. Moreover, sugar increased constitutive agglutination outside of the immune challenges (i.e., pre-challenge levels). In this study, we provide evidence that a high sugar diet affects the immune system of green iguanas (in a disruptive manner) and alters the gut microbiome.

Introduction

Simple sugars are an energy-rich macronutrient, but too much of it in some organisms can lead to physiological or microbial imbalances leading to negative health outcomes (French, Hudson, et al., 2022; Kawano et al., 2022; Ruxton et al., 2010). Sugar content in the natural diet varies considerably among organisms, with sugar being the predominant nutrient in the diet of some species (e.g., frugivores, nectarivores), while other species (e.g., carnivores) consume very little sugar. Accordingly, organisms have evolved physiological processes that reflect their usual dietary composition (Kohl et al., 2016). For example, nectar feeding bats (*Glossophaga soricina)* can have blood glucose levels that would be considered pathologically high in other mammals of similar size (Kelm et al., 2011). However, despite high spikes in post-prandial blood glucose, these bats show adaptations to tolerate potentially harmful sugar levels and channel it towards their metabolically costly flight (Kelm et al., 2011). Additionally, both nectar feeding birds and bats are metabolically adapted to high sugar diets as found in their unique enzymes and metabolomic profile (Potter et al., 2021). Despite this diversity in the physiological adaptations to diets with very high sugar levels, there is potential for environmental change to create imbalances in how organisms respond to food.

One common cause of mismatches between diet composition and physiological capabilities is anthropogenic foods. There is evidence that humans are increasingly providing unnatural foods to wild animals, but the effects on health and survival as well as the mechanisms underlying these effects are understudied. For example, there are nutritional health risks for dolphins in Florida fed bananas, candy, beer, and potato chips (Bryant, 1994). Fishes in the Great Barrier Reef fed inappropriate foods by tourists have an increase in liver fat deposits (Great Barrier Reef Marine Park Authority, 1993). Kangaroos in Australia fed breads, sausages, and other unnatural foods often have “lumpy jaw” disease (Burger, 1997). In all of these cases, supplemental feeding has been shown to contribute to increased mortality in these wild populations (Orams, 2002). These altered diets usually include unnaturally high or artificial sources of sugar (i.e., candy, bread, beer, banana), and high sugar diets are known to lead to deficiencies in blood sugar regulation in a diverse array of species including humans (Kawahito et al., 2009), fruit flies (Musselman et al., 2011), and iguanas (French et al., 2022). However, the effects of high-sugar diets on other physiological processes have received much less attention.

The immune system of free-living animals is still not well understood, including how it is impacted by diet. Previous research provides conflicting information regarding the effects of sugar on immunity across species. Glucose is found to have pro-inflammatory effects on human

monocytes via increasing toll-like receptors (Dasu et al., 2008). Conversely, high blood sugar decreases complement activation (a component of the immune system responsible for inflammation, opsonization, and lysis) in rats and impairs phagocytic ability in *Drosophila* (Mauriello et al., 2014; Yu et al., 2018). This suggests that the effects of sugar on immunity are likely context-dependent and nuanced, affecting various cells and molecules differently. The innate response is very robust and heavily relied upon in many reptilian species yet it is not well understood how the immune system may respond differently upon subsequent infections (Ademokun & Dunn-Walters, 2010; Wright & Schapiro, 1973). With the exception of a study demonstrating agglutination and antibody titer responses to subsequent challenges in desert iguanas, *Dipsosarus dorsalis*, little work has investigated responses to multiple immune challenges in reptiles (Wright & Schapiro, 1973). Thus, we intend to investigate the effects of a high sugar diet on physiology after multiple immune challenges. Clarifying the mechanisms by which diet modulates immunity across multiple challenges may provide a better understanding of why there is considerable immune variation in response to a high sugar diet.

One potential mechanism underlying the link between diet and immune function is the effects of diet on the microbiome (Burr et al., 2020; Siddiqui et al., 2022). Diet directly influences the establishment and maintenance of an organism's microbiome and the microbiome is related to the quality and development of the immune system (Amenyogbe et al., 2017; O'Sullivan et al., 2013; Tamburini et al., 2016). Therefore, the microbiome may be an important pathway by which diet can modulate immune function. For example, the establishment of the microbiome at an early age is important for development of the immune system such that chicken hatchlings raised in a germfree environment were found to lack T and B cells in certain parts of the body and had altered cytokine expression (Broom and Kogut, 2018). When the human microbiota is altered via antibiotics, neutrophil extracellular trap activity and the concentration of antimicrobial peptides is elevated, but macrophage phagocytic killing is inhibited (Konstantinidis et al., 2020; Yang et al., 2017). In mice, however, antibiotic changes to the gut microbiome results in the up-regulation of cytokines and an increased Th1 response (Sun et al., 2019). Other studies have demonstrated how bacterial metabolites influence immune regulation. Metabolites such as short chain fatty acids (i.e., gut microbes produce these during sugar catabolism) can regulate innate immune cells and B and T cells (C. H. Kim, 2018; Siddiqui et al., 2022). However, the link between diet, immunity, and the dysregulation of the microbiome has not been studied at the population level, particularly regarding a high dose of sugar supplementation.

In the present study, we tested the effects of a sugar supplemented diet on the immune function, gut microbiome, and energy metabolites of captive green iguanas (*Iguana iguana).* Green iguanas are a good model for testing the effects of unnaturally high levels of sugar in the diet as they are herbivores, and their natural diet has a low glycemic content (Rand et al., 1990). Furthermore, previous studies indicate iguana physiology is consistently altered via a high sugar diet. For example, in green iguanas (French et al., 2022) and rock iguanas (*Cyclura cychlura)* (French et al., 2022, 2023), sugar alters glucose metabolism, energy metabolites, immune

function, and blood chemistry. However, no work has investigated how diet affects acquired immunity in reptiles, and so we tested the effects of diet on immunity via two consecutive immune challenges to quantify both innate and acquired immune responses over time.

Our study tested a central hypothesis that sugar supplementation alters physiological processes and the gut microbiome. Specifically, we have three not mutually exclusive predictions related to the physiological effects of an unnatural, high sugar diet.

1) Iguanas fed an unnaturally high sugar diet will have higher levels of circulating energy metabolites (glucose, triglycerides, free glycerol).
2) An unnaturally high level of sugar in the diet will differentially alter subsequent immune responses to a lipopolysaccharide (LPS) challenge.
3) There will be changes in the composition and diversity of the gut microbiome in iguanas fed a high sugar diet.

Materials and Methods

Study Animals and Husbandry

Thirty-six wild-caught, male, juvenile green iguanas (mean snout-to-vent length SVL = 12.0 ± 0.1 cm, mean mass 54.1 ± 2.5 g) were obtained from Underground Reptiles (Deerfield Beach, FL, USA) in March 2021. For the duration of the study, the iguanas were housed singly in clear polycarbonate cages (47.6 cm D X 26.0 cm W X 20.3 cm H) covered by fine-mesh metal lids. Each cage was provided with a sheet of Techboard paper (Shepherd Specialty Products, Watertown, TN, USA) on the cage floor, a water bowl, and a wooden perch set at a 45-degree incline directly under a 25 W incandescent light bulb at one end of the cage to provide a basking site and thermogradient. The housing room was kept at 25.6°C, and the room's fluorescent lights and heat lamps were on from 0700 to 1900 daily. Except when noted below, the iguanas were fed daily a combination of diced romaine lettuce and moistened grain food pellet (Tortoise LS Diet, Mazuri, St. Louis, MO, USA). The iguanas had a 7-day acclimation period before the start of the study. All treatments in this study were approved by the IACUC of Arizona State University (18-1658R)

Dietary sugar treatment and sampling

First, the impact of a high-sugar diet on the gut microbiome was investigated. Specifically, dextrose was used as it is a readily available corn-based simple sugar that is chemically identical to glucose. After the acclimation period, we randomized sampling order to balance any circadian effects on blood glucose levels; however, the time of bleeding may still have contributed to variation in our metrics. Blood was collected in under 3 minutes from the caudal vein using a heparinized 1ml syringe with a 25 g X 1.6 cm needle within the hr of 0700 and 1200, prior to the animals being fed that day. Immediately after being collected, a drop of blood was used to determine blood glucose concentration in mg dl^{-1} (EvenCare G2 Blood Glucose Monitor, #MPH1540), and the remaining blood stored in a 1.5 ml sterile microcentrifuge tube on ice until all samples were collected. To obtain the colon sample, a sterile swab was moistened with sterile water and then gently inserted through the vent and cloaca. Once in the colon, the swab was swirled while moving it approximately 1cm proximally and then

distally. The swab was then removed from the iguana, its tip broken off and sealed into a 1.5 ml sterile microcentrifuge tube that was first put on ice until all samples were collected and then placed in a -20°C freezer. Finally, each iguana's mass (using a platform scale) was measured (Fig 1). After all collections were complete, blood samples were centrifuged at 3000 rpm for 4 min. Following centrifugation, the blood plasma was separated into 0.5 ml microcentrifuge tubes and then frozen at -20°C.

The day after sample collection, each iguana was assigned to either a dextrose-supplemented or control diet using a mixed dispersion design based on their mass, from largest to smallest, with 18 iguanas in each assignment group (dextrose mean mass = 53.3 ± 3.4 g; control = 53.6 ± 2.8 g). The control diet was the same diet as the iguanas had been receiving during acclimation. For the dextrose-supplemented diet, 0.17 g dextrose was added to each mL of water used to soak the tortoise pellets. This dose equated to the estimated amount of sugar (on a per gram body mass basis) ingested by grape-fed rock iguanas on tourist-visited islands in the Bahamas and known to induce changes in glucose metabolism (French, et al., 2022). Iguanas remained on their assigned diets throughout the entire study.

After 30 days of the iguanas being on their assigned diets, mass and SVL (using a rigid ruler) were re-measured, and a blood sample (for blood glucose, plasma, and cells) and a colon swab were collected again (Fig. 1). We measured blood glucose despite treating the iguanas with dextrose as both have the same empirical formula and the glucose monitor measures dextrose as glucose (we will refer to dextrose as sugar for the rest of the paper).

Immune Challenges

Next, the effects of a high-sugar diet on the immune response to a simulated pathogen via the use of LPS (L3129, Sigma-Aldrich) were investigated. The iguanas in each of the two diet treatment groups were subdivided equally (n = 9) into two challenge groups using a mixed dispersion design and given either an intracelomic injection of 15 μg LPS g^{-1} body mass or an equal volume (0.50 – 0.85 ml) of phosphate-buffered saline (PBS) as a control. Body mass, SVL, colon swabs and blood samples were measured throughout the study (Fig. 1). We evaluated the iguanas' response to a primary immune challenge (~30 days from the start of the experiment) and their responses to a secondary immune challenge (~60 days from the start of the experiment, ~30 days after the primary immune challenge). These time points were chosen based on the response time to LPS injection in other tetrapod ectotherms, including *Lithobates catesbeianus* and *Uta stansburianna* (Figueiredo et al., 2021; Smith et al., 2017). In addition, as reptiles rely heavily on an innate response and very few studies have investigated how the immune system may respond differently in a subsequent infection, we chose to use two consecutive immune challenges (Ademokun & Dunn-Walters, 2010; Wright & Schapiro, 1973) to understand how subsequent responses may differ over time.

DNA extraction

Genomic DNA was extracted from colon swabs using DNeasy PowerSoil Kits (Qiagen Inc. 12888-100) according to the manufacturer's protocol. DNA was extracted from a total of 238 swabs in sets of eleven with one blank per extracted set to control contamination. Blanks

were treated like swabs from the start of the extraction process by mimicking movement of the swab to a beaded tube with flame sterilized tweezers. DNA yield was measured with a Qubit 2.0 Fluorometer (Invitrogen by Life Technologies, Singapore) using the High Sensitivity assay. Extracted DNA was stored at -80°C until sequencing.

Sequencing

Library preparation and sequencing was performed at the Shedd Aquarium Microbial and Molecular Ecology Lab (Chicago, IL, USA). Bacterial DNA was amplified using primers 515f (Parada et al. 2016) and 806rB (Aprill et al. 2015) targeting the V4 region of the 16S rRNA gene. The primer constructs contain Illumina specific adapters followed by 12bp Golay barcodes on each forward primer, primer pads and linkers, and finally the template specific PCR primer at the 3' end (Walters et al., 2016). PCR was performed in replicate 25 µl reactions containing 12.5 µl Phusion Hot-Start Flex 2X MasterMix (New England Biolabs), 0.2 µM final primer concentrations, 2 µl of template DNA and nuclease free water to equal 25 µl. Mock microbial community DNA standards (Zymo Research) and negative controls containing no template DNA were prepared with each PCR replicate. Thermal cycling conditions were carried out as follows: 98°C for 30 seconds, 30 cycles at 98°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec, with a final extension of 5 minutes at 72°C. After PCR, replicate amplicons were combined and 5 µl of each were electrophoresed in 1.8% agarose gels to confirm amplification of the V4 region. Twenty-five µl of each amplicon library was then cleaned and normalized using the SequalPrep™ Normalization Plate Kit (Applied Biosystems), and equal volumes of each normalized library were pooled together. The pooled amplicon library was quantified using a Qubit™ 3.0 fluorometer and Qubit™ dsDNA HS Assay Kit (Life Technologies). The molarity of the pooled library was calculated, then denatured and diluted to a loading concentration of 5.15 pM. Paired-end sequencing for a total of five hundred cycles was conducted on the Illumina MiSeq platform using custom sequencing primers described previously (Caporaso et al., 2012) with addition of 10% PhiX Control library (Illumina).

Sequence Processing

The 16s rRNA sequences were processed in the QIIME2 (v2019.4) environment after visual inspection of quality score distribution. The 'trim-paired' cutadapt function was used to trim adapters for the 515F and 806R primers. Then, DADA2 was used to join, denoise, and dereplicate sequences, including the removal of chimeras and singletons. Forward and reverse reads were truncated at 248 nts and 219 nts, respectively, based on the earliest location at which the median quality score dropped below 30 in either dataset. The amplicon sequence variants (ASVs) were classified with the SILVA 16S rRNA database (v132), using the 7-level taxonomy file and 99% identity. Reference reads were extracted based on our 515F/806R primer pairs and length 100-400 nts. The ASVs were then classified with 'classify-sklearn'. Sequences were aligned with MAFFT and a rooted phylogenetic tree was generated with FastTree using 'align-to-tree-mafft-fasttree'. Code is available at https://github.com/kapheimlab.

Energy metabolites

Free glycerol and triglycerides were measured via an enzymatic color endpoint assay (F6428, T2449 and G7793, Sigma- Aldrich, Missouri, USA) based on a modified protocol (Webb et al., 2019). The absorbance was measured at 540 nm for both glycerol and triglyceride concentration (mg mL^{-1}) (xMark; Bio- Rad, California, USA). The inter-assay variation was 6.09% for glycerol and 4.62% for triglycerides.

Bacterial Killing Ability (BKA)

This assay enables us to understand a functionally relevant, integrative immune function to a common pathogen, *E. coli*. It is important as it characterizes the overall response of immune components such as phagocytes, opsonizing proteins, and natural antibodies, which are critical to innate immunity which reptiles rely heavily upon. Assays were performed under sterile conditions using a laminar flow hood and autoclaved materials. The assay procedures followed are outlined in French and Neuman-Lee (2012) with modifications for use in a 96-well microplate with positive and negative controls. Pipetted into each well in duplicate were: 5 µl of plasma, 13 µl of CO_2-independent media (Gibco, Grand Island, NY) plus 4 mM L-glutamine (Sigma-Aldrich), and 6 µl of *E. coli* (EpowerTM Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN). The plate was incubated for 30 min at 37°C, then 125 µl of tryptic soy broth was added (Sigma-Aldrich NO. T8907;15 g broth/500 ml nano-pure water) and a background absorbance was taken at 300 nm (xMark; Bio- Rad, California, USA). The plate was incubated for 12 hr at 37°C and then read at 300 nm in the spectrophotometer again. The intra-assay variation was 0.46% for assay 1, 1.34% for assay 2, 0.75% for assay 3, and 6.71% for assay 4; the inter-assay variation was 4.93%.

Lysis and Agglutination

This assay measures a form of innate constitutive humoral immunity (natural antibodies, complement proteins, and other antimicrobial proteins). The agglutination and lysis assay (which are also encompassed in part of the BKA) enables us to parse out how specific immune components within the broader BKA functional response are altered by our treatments. Thus, using both assays we can see how the overall functional response changes together with how specific components within that response contribute to the integrated response. Heparinized sheep red blood cells (HemoStat Laboratories, SBH050) were washed 5-7 times to eliminate dead cells. In a microplate, 20 µl of PBS was added to each well followed by 30 µl of plasma added to just the first column. The samples were serially diluted with a multi-channel pipette down the plate through column 12. Finally, 20 µl of a 1% sheep red blood cell solution was added to all wells. The plate was incubated for 90 min at 37°C and another 20 min at room temperature, after which the plate was scanned (Epson Perfection V750 Pro) for agglutination. The plate was incubated at room temperature for another 70 min and scanned again for lysis. Three people independently scored the scanned pictures for the agglutination and lysis scores (ranging from 0 to 12) and scores were averaged.

IgY

IgY is a type of immunoglobulin that is part of the humoral response found in reptiles and akin to IgG in mammals. This is a way to measure acquired immunity in response to a specific

antigen and is a direct measure of the LPS-induced responses. The concentration (mg ml^{-1}) of LPS-specific iguana IgY was quantified via our newly developed iguana-specific ELISA. On day 1, the plates were coated with LPS carbonate buffer and incubated at 4°C overnight. On day 2, the plates were washed with PBS/Tween20 solution, then 3% milk powder buffer was added, and the plate was incubated at 4°C overnight. On day 3, the plates were washed with PBS/Tween 20 solution and diluted plasma samples (1:20) were added. Again, plates were incubated at 4°C overnight. On day 4, plates were washed and diluted rabbit-anti-iguana-IgY (SouthernBiotech, custom antibody SBCS-58) with HRP was added. The antibody was diluted 1:1000 in 1% milk powder solution. The plate was incubated for 1 hr at 37°C, and then the plates were washed. TMB substrate was added, and the plates were incubated in the dark for 30 min at room temperature. Finally, stop solution was added and plates were read at 450 nm. The inter-assay CV was 0.17%.

Statistical Analysis

All statistical analyses of physiological measures were performed in R, version 4.1.1 (R Core Team 2021), using the packages: "tidyverse" v 1.1.4 (Wickham et al. 2019), "rstatix" v0.7.0 (Kassambara 2021), "betareg" v2.0.0. (Cribari-Neto F, 2010). Tests were only used if assumptions were met (checked using diagnostic plots). An alpha level of 0.05 was used for all tests. The code for all analyses is available on https://github.com/KiClaudia/greeniguana.

Physiology – Dietary Sugar Treatment

The effects of one month of diet treatment, before the immune challenge, were tested with Welch's t-test. The change between pre-diet and post-diet was calculated for both treatment groups (sugar group and water group). Then, the change over time was compared between the two treatment groups using a 2-tailed, independent t-test.

Physiology – Immune Challenge

The effects of diet and the immune challenges for each physiological variable were tested using a three-way repeated mixed measures ANOVA (time, diet, and immune challenge) over the 4-week period of each immune challenge (where time is referring to the multiple timepoints throughout the study that we measured physiological variables (Fig 1)). Separate ANOVAs were used for the primary and secondary challenges. Upon a significant interaction amongst any combination of variables, a post hoc multiple comparison Benjamini-Hochberg adjusted test was used. Agglutination and lysis scores have ordinal data; thus, a Mann-Whitney test was used to detect effects of diet and the immune challenge (separately) at each time point. Additionally, the change between pre-injection and several post-injection scores for diet and immune challenge was compared. Bacterial killing ability values are percentages and violated assumptions of normality. Thus, a beta regression was used (BKA data from the primary challenge was not used due to methodological issues in the assay). These data were transformed using the formula (y*(n-1) + 0.5) / n), where n is sample size and y is the data point, as recommended by the "betareg" package. Six different models were created (different combinations of the three variables: immune challenge, diet, and time) and the top model as indicated by Akaike information criterion (AIC) was the diet model.

Microbiome

Phyloseq v.1.40.0 (McMurdie and Holmes 2013) was used to perform statistical analysis of iguana microbiomes in R v.4.2.3 (R Core Team 2019). R code is available at https://github.com/kapheimlab. Decontam v.1.6.0 (Davis et al. 2017) was used to identify and remove 11 potential contaminants as those ASVs that were more prevalent in negative controls (i.e., extraction blanks, no template controls) than in experimental samples. Those ASVs classified as mitochondria (627) or chloroplast (3) and those which could not be classified at the Phylum level (0) were removed as well as those not found at least 10 times in at least 1% (2) of samples. Samples (12) with fewer than 1,000 reads were also removed. The final dataset included 170 samples and 1,636 ASVs. The data were rarefied to an even depth of 1,159 reads per sample. Given the ongoing debate about the value of rarefaction (McMurdie and Holmes 2014), more than one normalization method was employed where appropriate. The final dataset was subdivided into two phyloseq objects for individual analysis. First, the effects of the sugar diet alone were explored, prior to any immune challenge (n = 69). A separate phyloseq object was made to look at the effects of diet and immune challenge together 24 hr (n = 29) and 72 hr (n = 34) post-primary immune challenge, and 4 weeks post-secondary challenge, approximately two months after the first immune treatment (n = 36). These time points were chosen based on the response time to LPS injection in other reptiles such as *Lithobates catesbeianus* and *Uta stansburianna* (see methods above) (Figueiredo et al., 2021; Smith et al., 2017). Upon visual inspection of Principal Coordinates Analysis (PCoA) plots and comparison with field and laboratory notes, two outliers were removed from the 72 hr post LPS dataset. The following set of analyses were performed separately for each of these datasets.

Overall differences in microbial communities were visualized across treatments with PCoA applied to Bray-Curtis distance matrices of log-transformed abundance data. Variance in the multivariate microbiome community was partitioned by variables of interest with adonis2 in vegan (Jari Oksanen et al. 2019) based on a Bray-Curtis distance matrix of relative abundances over 9,999 permutations. To assess the effects of the glucose diet independent of immune challenge, the model included diet and time, as well as their interaction, and was stratified across iguana ID to account for repeated sampling of the same animal at two time points. Then, pairwise comparisons between diet-time levels using 9,999 permutation MANOVAs and a Benjamini-Hochberg (BH) correction of p-values were used. To assess the combined effects of diet and immune challenge at different times, a model that included diet, immune treatment, and time point (24 hr, 72 hr, 4week post immune challenge), as well as their interactions, stratified across iguana ID was used. This revealed significant effects of each factor, as well as their two-way interactions; thus, a single variable that encoded diet, immune treatment, and time point to look for pairwise differences was used. ASVs with significant differences in abundance were identified for samples collected 24 h past the primary immune challenge. Counts were transformed to a geometric mean and DeSeq2 (v1.39.8) was applied to a model that included diet + immune treatment. P-values were adjusted using the Benjamini-Hochberg method.

Differences in beta diversity was tested as a function of diet-time or diet-immune treatment-time with independent iterations of betadisper in vegan followed by pairwise comparisons with the Tukey Honest Significant Difference method (TukeyHSD) (Anderson et al. 2006).

Alpha diversity was estimated with the Shannon index and observed species richness, computed with the estimate_richness function in the 'vegan' R package using non-filtered datasets. Then, the Shannon index and observed species richness were modelled as a function of diet-time with iguana ID included as a random effect for the first dataset using lmer in the lme4 package (v. 1.1-30) (Bates et al. 2015). For the second dataset, alpha diversity was modeled separately for each time point as a function of diet-immune challenge. Model assumptions were tested via visual inspection of sample distributions and an Anderson-Darling test for normality, and Tukey adjusted pairwise tests were used for posthoc comparisons. Box-Cox transformation was used to fit linear models where necessary (MASS v. 7.3, Venables & Ripley 2002).

The correlations between microbiome composition and physiology were explored using the 'associate' function in the microbiome package (v.1.18-0) (Lahti & Shetty 2019). All correlations were run with method = "spearman" and p.adj.method = "BH". To investigate the relationship between bacterial diversity and blood physiology, 'cor.test' was used to calculate Spearman's rank correlations between Shannon index and observed species richness at 24 hr and 72 hr post immune challenge with agglutination at the same time points.

Results

Immune Metrics

BKA

We use a beta regression model with a logit link to analyze the relationship between the BKA values and diet (while accounting for random effects amongst iguanas). The model was a good fit to the data with an AIC of -27.6 and a BIC of -14.1 and preferred above other models (different combinations of diet, time, and immune challenge). A high sugar diet was associated with reduced bacterial killing independent of immune challenge or time point ($X2 = 13.56$, $df = 3$, $p < 0.001$). There was a significant effect of diet ($z = 3.72$, $df = 212$, $p < 0.001$) on BKA during the second immune challenge (Fig. 2). The sugar group had a bacterial killing of 49.1% ± 3.50% while the control diet group performed better, at 68.7% ± 2.83% killing. We omitted results for BKA from the first immune challenge due to procedural issues.

Agglutination

A significant effect of diet on agglutination was detected for the timepoint before the first ($W = 194.5$, $p = 0.034$) and the second ($W = 204$, $p = 0.039$) challenge (Fig. 1, and 3). The median for the sugar group was higher than that of the control diet group before both challenges. In a separate model examining differences in the change in agglutination scores pre and 24 hr post injection between diet groups, we detected significant differences during both challenges. In the first challenge, the sugar group had a larger reduction in agglutination score 24 hr after the immune challenge relative to the control diet group ($W = 186$, $p = 0.029$). In the second challenge, the control diet group had a larger increase in agglutination score than the sugar group

(W = 165, p = 0.03) (Fig. 3B). There was a significant effect of the immune challenge on agglutination that was independent of both diet and time (time referring to the multiple time points we measured agglutination throughout the experiment (Fig 1)). Significant effects of the immune challenge first appeared at 72 hr following the primary injection, when the LPS treated iguanas had 3-fold higher agglutination scores than the PBS treated iguanas (72 hr W = 61.5, p = 0.006; 1 week W = 29, p = 0.002; 2-week W = 76.5, p = 0.019) (Fig 3A). LPS iguana sustained an increase in agglutination through the first challenge into the second challenge (24 hr W = 62, p = 0.002; 72 hr W = 41, p = 0.001; 1 week W = 71.5; p = 0.021; 2-week W = 89, p = 0.034).

Lysis

We detected significant differences during the first challenge but not the second when examining differences in the change in lysis scores pre and 24-hr post injection between diet groups. Iguanas given the sugar diet had a larger reduction in lysis 24 hr after the primary immune challenge than did the control diet group (W = 181, p = 0.047). In a separate model, there was a significant main effect of the immune challenge on lysis independent of diet and time, but it was not detected until 24 hr after the second immune injection (W= 97.5, p = 0.041). Specifically, groups injected with LPS had significantly higher lysis scores. In

IgY antibodies

There were no effects of diet across both challenges. For the first immune challenge, immune challenge ($F_{1,15} = 25.5$, $p < 0.001$), time ($F_{5,75} = 13.4$, $p < 0.001$), and their interaction ($F_{5,75} = 8.9$, $p < 0.001$) had a significant effect on IgY concentrations. One week after the first challenge, IgY concentrations increased and remained elevated above that of the PBS-treated iguanas for the 4 weeks that the animals were evaluated (Fig. 4A). During the second immune challenge, there was a main effect of LPS ($F_{1,10} = 14.7$, $p = 0.003$) with the LPS group having higher IgY concentrations than the PBS group for all time points. IgY concentrations were already elevated before the second challenge, apparently a residual effect from the first challenge, so there was no effect of time (Fig. 4B).

Energy Metabolites

After 1 month of diet treatment (before any immune challenge), there was a significant effect of diet for plasma glucose levels, glycerol, total triglycerides, and mass (Table 1). Iguanas on a high sugar diet increased blood glucose by 1.5-fold, glycerol levels by 14-fold, and total triglycerides levels by >2.5-fold relative to the control group. Iguanas that received a high sugar diet gained approximately 4 times as much weight as control iguanas after a month of the altered diet.

In terms of blood glucose levels after the first immune challenge, there was only a significant effect of time and no effect of immune challenge nor diet. Iguanas in all treatment groups had decreased circulating blood glucose 72 hr after the first immune challenge ($F_{3,96} = 4.3$, $p = 0.007$). Glucose concentrations, regardless of treatment, were about twice as high pre-immune challenge than 72 hr after. After the second immune challenge, there were no effects of time, diet, nor immune challenge.

In terms of total triglycerides, there were significant effects of diet and an interaction of the immune challenge and time during the first challenge. Iguanas in the sugar group had a 1.5-fold increase in concentration relative to the control diet group ($F_{1,25} = 19.7$, $p < 0.001$). The interaction between LPS and time takes place at 24 hr after the first challenge; the iguanas injected with LPS had fewer circulating total triglycerides than the PBS-injected iguanas ($F_{5,125} = 3.2$, $p = 0.01$) regardless of diet (Fig. 5A). After the second immune challenge, there continued to be significant effects of diet at every timepoint ($F_{1,25} = 19.8$, $p < 0.001$). The ANOVA also detected an interaction between the immune challenge and time but the pairwise test was unable to determine in which groups where the significance is ($F_{1,25} = 3.59$, $p = 0.005$). From Figure 5B., it would appear to be at the 24 hr post injection time point, similar to the first challenge.

Morphometrics

There was a significant 3-way interaction between diet, immune challenge, and time ($F_{5,160} = 2.3$, $p=0.047$). Specifically, before the first immune challenge, iguanas fed a sugar diet and injected with PBS had the highest mass (g) relative to the other groups (sugarPBS: 60.2±3.78; sugarLPS: 55.9±4.14; controlPBS:55.0±3.06; controlLPS: 53.8±5.50). This was also the only time point in which there was a difference between the groups as any differences in mass were not detected during the rest of the primary challenge. During the second immune challenge, there was a significant effect of time regardless of diet or LPS treatment ($F_{5,160}= 106.94$, $p < 0.001$). Specifically. all iguanas weighed more at the end of the experiment relative to any time points throughout the second challenge. In terms of SVL, there was a significant effect of time but not diet nor immune challenge ($F_{2,64} = 10.1$, $p < 0.001$). All iguanas were ~4cm longer by the end of the experiment relative to the initial measurement at the beginning of the experiment.

Microbiome

General characteristics of the green iguana microbiome

The filtered combined dataset had 170 samples from 36 iguanas sampled at 3-6 time points, each with an average of 24,740.82 ± 629.62 s.e. reads. The green iguana hindgut microbiome in this dataset was composed primarily of bacteria from 11 Phyla (Fig. 6), and the mean number of ASVs per sample was 238.69 ± 111.70 s.e. There were no significant correlations between blood physiology (glucose, total triglycerides, agglutination, or lysis) with microbial relative abundance (ASVs or Families) at the start of the experiment (Table S1, S2).

Effects of a high sugar diet on the microbiome

Both diet and time were significant sources of variance in microbiome composition of the green iguana gut microbiome. In an analysis of community-wide differences, both diet and time (but not their interaction) were a significant source of variance in community composition ($F_{diet} = 1.96$, $p_{diet} = 0.0001$; $F_{time} = 6.69$, $p_{time} = 0.0001$). The interaction between diet and time was not a significant predictor of relative abundance ($F_{dietXtime} = 1.07$, $p_{dietXtime} = 0.27$). Pairwise comparisons suggested that iguanas given a sugar diet versus a control diet did not have significantly different microbiomes at the start of the experiment, as would be expected (BH-adjusted $p = 0.26$). The microbiome composition of iguanas in both diet treatments changed

significantly over time (sugar baseline vs 1-month BH-adjusted $p = 0.0003$; water baseline vs 1-month BH-adjusted $p = 0.0006$). However, significant differences (BH-adjusted $p = 0.05$) in community composition between sugar and water-treated iguanas after a month of diet treatment suggest that diet influenced temporal shifts in the microbiome. This pattern was consistent with the visual pattern observed with a PCoA of log-transformed abundances (Fig. 7). Overall and pairwise results were consistent when this analysis was repeated on rarefied data ($F_{diet} = 1.95$, $p_{diet} = 0.0001$; $F_{time} = 6.65$, $p_{time} = 0.0001$; $F_{dietXtime} = 1.04$, $p_{dietXtime} = 0.30$).

No direct relationships between bacterial abundance and blood physiology were detected. There were no ASVs or bacterial families for which abundance was significantly correlated with blood glucose, or total triglycerides after one month on a high sugar diet or the change in blood physiology during the month-long diet treatment (BH-adjusted $p > 0.1$; Table S3, S4). Abundance of any particular taxa or bacterial family was not predictive of blood physiology throughout the one month of diet treatment, the primary immune challenge, and the secondary immune challenge (~3 months) on a high-sugar diet (BH-adjusted $p > 0.1$; Table S3, S4).

Analyses of diversity revealed the microbiome was highly consistent among individuals in each diet group. There were no significant differences in multivariate dispersion between diet-time groups, which is a measure of beta diversity ($F = 1.18$, $p = 0.32$). There were also no significant differences in alpha diversity, measured with either the Shannon index or the observed species richness (Shannon: $X^2 = 1.89$, $p = 0.59$; Richness: $X^2 = 0.94$, $p = 0.82$; Fig S1). Results were similar with rarefied data (Shannon: $X^2 = 4.67$, $p = 0.20$; Richness: $X^2 = 2.05$, $p = 0.56$).

Effects of diet and immune challenge on the microbiome

The composition and diversity of the microbiome was affected by both diet and immune challenge (IC in subscript of results), but this effect was short-lived. Diet, immune challenge, time, and the interaction between immune challenge and diet or time point were significant sources of variance in microbiome composition ($F_{diet} = 3.78$, $p_{diet} = 0.0001$; $F_{IC} = 4.09$, $p_{IC} = 0.0001$; $F_{time} = 2.86$, $p_{time} = 0.0001$; $F_{diet\ X\ IC} = 1.90$, $p_{diet\ X\ IC} = 0.0007$; $F_{time\ X\ IC} = 1.86$, $p_{time\ X\ IC} = 0.0002$). The interaction between diet and time did not have a significant effect on microbiome composition ($F_{diet\ X\ time} = 1.09$, $p_{diet\ X\ IC} = 0.09$), nor did the three-way interaction between diet, immune challenge, and time ($F_{diet\ X\ IC\ X\ time} = 0.85$, $p_{diet\ X\ IC\ X\ time} = 0.29$). This pattern was consistent with that revealed by visual assessment of a PCoA at each time point (Fig. 8). Pairwise comparisons revealed these results were largely driven by the effects of the immune challenge (Fig. S2). Overall and pairwise results were consistent when this analysis was repeated on rarefied data ($F_{diet} = 3.72$, $p_{diet} = 0.0001$; $F_{IC} = 3.95$, $p_{IC} = 0.0001$; $F_{time} = 2.78$, $p_{time} = 0.0001$; $F_{diet\ X\ IC} = 1.90$, $p_{diet\ X\ IC} = 0.001$; $F_{time\ X\ IC} = 1.84$, $p_{time\ X\ IC} = 0.0004$; $F_{diet\ X\ time} = 1.12$, $p_{diet\ X\ IC} = 0.08$; $F_{diet\ X\ IC\ X\ time} = 0.85$, $p_{diet\ X\ IC\ X\ time} = 0.30$).

There were 13 ASVs with significant differences in abundance related to diet 24 hr after the immune challenge. These ASVs belonged to nine families from four Phyla, with all but one (Bacteroidaceae) decreasing in abundance on a sugar diet (Table S5). There were 32 ASVs with significant differences in abundance related to the immune challenge at 24 hr. These immune-

responsive ASVs belonged to 16 families from 7 Phyla (Table S6). Most notably, five ASVs from the Bacteroidaceae were significantly reduced following the immune challenge, and four ASVs from the Micrococcaceae were significantly increased.

Bacterial diversity was most affected by the short-term response to an immune challenge. There were no significant differences in multivariate disperson, a metric of beta diversity, between diet and immunity treatments at any time point ($F = 0.64$, $p = 0.79$). There were significant differences across treatment groups in alpha diversity at 24 hours post-injection, measured as both the Shannon index ($X^2 = 40.25$, $p = 9.45e\text{-}9$) and observed number of ASVs ($X^2 = 36.44$, $p = 6.04e\text{-}8$). In fact, the immune challenge reduced the median number of observed ASVs after 24 hr by a factor of 3 in iguanas on the sugar diet (363 vs 115) and by nearly an order of magnitude in the iguanas on the water diet (298.5 vs 31) (Fig. 9). However, there were no longer significant differences in alpha diversity by 72 hr and 4 weeks post-injection ($p > 0.05$). Results were consistent when repeated with rarefied data (Shannon: $X^2 = 52.12$, $p = 2.59e\text{-}7$; Observed ASVs: $X^2 = 58.83$, $p = 1.53e\text{-}8$).

There were no significant correlations between microbial abundance and iguana blood physiology. There were no ASVs or families for which relative abundance was significantly correlated with blood glucose or total triglycerides at 24 hr post immune challenge (BH-adjusted $p > 0.05$). The initial correlation analysis identified three families with marginally (BH-adjusted $p = 0.05$) significant correlations with agglutination 24 hr after the immune challenge. However, visual inspection revealed this to be driven by one sample with unusually high agglutination levels. There were also no ASVs or families for which relative abundance was significantly correlated with glucose, total triglycerides, lysis, or agglutination 72 hr after the immune challenge (BH-adjusted $p > 0.64$). The set of 32 immune responsive (differentially abundant) ASVs at 24 h were not significantly correlated with any metrics of physiological immunity at 24 h -- agglutination, lysis, BKA (BH-adjusted $p > 0.71$). This was also true when the ASVs were agglomerated at the family level. There were also no correlations between alpha diversity (Shannon index or observed ASVs) and agglutination 24 hr ($\rho_{Shannon} = 0.44$, $p_{Shannon} = 0.17$; $\rho_{richness} = 0.16$, $p_{richness} = 0.64$) or 72 hr ($\rho_{Shannon} = -0.08$, $p_{Shannon} = 0.79$; $\rho_{richness} = -0.16$, $p_{richness} = 0.57$) after the immune challenge.

Discussion

General Overview

This study tested the effects of a sugar-supplemented diet on the energy metabolites, immune function, and gut microbiome of green iguanas. As expected, sugar treatment elevated plasma energy metabolites, glucose, and total triglycerides during the first month of the experiment. While the effects of diet on blood glucose did not persist beyond the first month, the effects of diet continued to elevate total triglycerides through both immune challenges (second and third month of the experiment). Our results also establish a link between diet and immune response, showing that a high sugar diet alters bacterial killing, agglutination, and lysis (but not IgY levels) following a primary or secondary immune challenge relative to the control group, and agglutination prior to the immune challenges. Diet treatment significantly affected the

558 overall composition of the gut microbiome, but not the diversity, following one month of
559 treatment. Thus, sugar supplementation was successful in altering both the gut microbiome and
560 physiology in just one month. Following the immune challenge, there were overall shifts in
561 microbial community composition and a reduction in alpha diversity in response to the immune
562 challenge, and these effects were exaggerated on a high sugar diet. By 72 hr post LPS injection,
563 the microbiome was mostly recovered. However, a lack of significant correlations between
564 microbial relative abundance and blood physiology indicates the two responses may be
565 unrelated. While we expected significant interactive effects between diet and immune challenge
566 (specifically with a sugar diet exacerbating effects of the immune challenge), we did not find
567 such results in the physiological changes over the course of the experiment. These results
568 highlight the complex relationships among sugar, physiology, and the gut microbiome.
569 Specifically, sugar affects composition of the microbiome and separately alters overall immune
570 function.
571 *Energy Metabolites*
572 While sugar-treated iguanas were expected to have higher circulating glucose, there
573 were no effects of diet or LPS treatment on circulating glucose. This may be due to the timing of
574 the sampling occurring first thing in the morning before the iguanas were fed. It is also possible
575 that this is the result of physiological compensation, where the sugar fed animals developed a
576 greater ability to manage glucose intake throughout the experiment There was, however, an
577 increase in total triglycerides in the sugar groups during both immune challenges. Similar to
578 humans, reptiles ingesting higher amounts of sugar have been found to have higher triglyceride
579 levels as seen in the Northern Bahamian rock iguana (*Cyclura cychlura)* (French et al., 2022).
580 There was also an interaction between LPS and time during the first challenge. Specifically, 24
581 hr after the first LPS challenge, the LPS group had lower total triglyceride levels. This decrease
582 in total triglyceride levels could be due to the breakdown of triglycerides to use in immune
583 activation because of the LPS injection. Mounting an immune response is energetically
584 demanding which could explain the increase in circulating energy metabolites like triglycerides
585 which is one of the main fuel sources for immune cells (Demas & Nelson, 2011; Ganeshan &
586 Chawla, 2014; Hudson et al., 2021).
587 *Immune Status, diet, and the Gut Microbiome*
588 Following the immune challenge, there was an elevated response in agglutination, lysis,
589 and IgY, demonstrating activation of the immune system in response to this simulated bacterial
590 infection. In terms of response and timing, our findings are similar to those from other
591 ectotherms, including bullfrogs (*Lithobates catesbeianus*) and side-blotched lizards (*Uta*
592 *stansburiana)* (Figueiredo et al., 2021; Hudson et al., 2021)**.** While there was a significant effect
593 of LPS during the first and second immune challenge on both agglutination and IgY, as
594 expected, there was not an effect of LPS on lysis until the second challenge. This may be due to
595 the relatively slower immune responses that have been documented in reptiles (Rios &
596 Zimmerman, 2015). Specifically, during subsequent exposures, the latent period for immune
597 responses often shortens, which may explain why we did not observe a significant effect of LPS

on lysis activity until the second challenge (Rios & Zimmerman, 2015; Zimmerman et al., 2010). Alternatively, the first challenge may prime the immune system such that a greater, detectable response occurred during the second challenge. Moreover, there was an elevated response for agglutination and IgY across the entire second challenge, even prior to LPS-injection. This was likely a continuation of the immune activation following the first challenge. However, the present study provides evidence that green iguanas can mount specific antibody (IgY) responses as early as 1 week following an immune challenge and peaking at 2 weeks (as opposed to the previously thought 6-8 weeks). This rapid antibody response is more on par with mammalian immune responses which has a latent period of about 1 week depending on the antigen (Zimmerman et al., 2010). The idea that reptiles have a long antibody response time comes mainly from studies on turtles (i.e., chelonians) (Zimmerman et al., 2010). However, as reptiles are an incredibly diverse paraphyletic clade, with turtles and iguanids being distantly related, it is unsurprising that different reptiles can have vastly different immune responses.

We found clear but differential effects of sugar on bacterial killing ability, agglutination, and lysis. Prior to the primary and secondary immune challenges, sugar groups exhibited elevated agglutination relative to controls, suggesting sugar may induce an increase in constitutive immunity in the form of inflammation. Sugar is known to have pro-inflammatory effects on human monocytes by increasing toll-like receptors and there is also evidence that sugar can increase oxidative stress, further contributing to inflammation signaling to immune cells and acute phase proteins (Dasu et al., 2008; Leung et al., 2014; Valera-gran et al., 2022; Burr et al., 2020; Kim, 2018; Yu et al., 2018). However, following the LPS challenges, there was a large reduction in agglutination and lysis scores when comparing scores before and 24 hr after the first immune challenge (relative to the control diet group) but this was not observed in the second challenge. During the second challenge, we observe that sugar group iguanas do not differ in their agglutination nor lysis ability after the immune challenge. The transient reduction in lysis and agglutination during the first challenge observed in iguanas given a high sugar diet may be explained by the documented suppressive effects of sugar on immunity. For example, sugar has been found to impede IgG and complement proteins in rats, impair phagocytosis in fruit flies (*Drosophila)*, and inhibit neutrophil migration and microbial killing in humans (Jafar et al., 2016; Mauriello et al., 2014; Yu et al., 2018). In vitro experiments support a similar trend with human neutrophils that are cultured in glucose having impaired neutrophil extracellular trap formation, and human B cells producing less IgM, reducing proliferation, and reducing cell function in response to a bacterial antigen stimulus (Joshi et al., 2013; Sakowicz-Burkiewicz et al., 2013). It is interesting, however, that during the secondary challenge, the IgY concentrations, and lysis ability of iguanas given high sugar were not affected by diet, but the overall functional bacterial killing ability was still reduced. An organism's bacterial killing ability represents the integrated immune function that encompasses agglutination, lysis, opsonizing proteins, acute phase proteins, natural antibodies, the complement system, and other peptides. These different responses between the two immune challenges demonstrate the nuanced effects that a high sugar diet can have on different components of the immune system across time. Although the sugar

group seemed to have a more robust immune system than the control group (agglutination was initially higher), when faced with an immune challenge, immune function is actually reduced (bacterial killing dropped).

Given the current focus on relationships among diet, the microbiome, health, and crosstalk between the immune system and microbiome, this study tested whether gut microbial changes corresponded with our treatments (Kim & Kim, 2017; Ooi et al., 2014; Shi et al., 2022; Siddiqui et al., 2022; Tamburini et al., 2016). The immune challenge led to rearrangement of the gut microbial community composition after 24 hr, especially in the high sugar group. Likewise, there was a transient (24 hr) reduction in alpha diversity following the immune challenge. Together, these results indicated that the immune challenge influenced how the gut microbiome was impacted by dietary sugar. Thus, there are three potential mechanisms by which a high sugar diet may be jointly interacting with the immune system and the microbiome. First, the diet may be interacting with the immune system directly, and changes in immune activity alter the gut microbiome. Sugar can directly modify immunity as it is an important fuel source and can be metabolized directly by neutrophils, T-cells, dendritic cells, and macrophages (Burr et al., 2020). While there was not a direct increase of circulating glucose due to diet or immune challenge, it is not surprising as glucose changes are highly labile and blood was sampled first thing in the morning before the daily feeding. There was, however, an increase in total triglycerides in the sugar diet iguanas, regardless of LPS treatment. Triglycerides can also be used by immune cells as fuel in the form of fatty acids which can directly activate toll-like receptor 4 (TLR4) on macrophages which activates proinflammatory pathways (Shi et al., 2006). The iguanas in the sugar group, generally, had suppressed immunity. Furthermore, there is evidence that the immune system uses antimicrobial proteins, IgA, phagocytes, and CD4 cells to control and limit contact between the gut and the systemic immune system (Alexander & Turnbaugh, 2020; Hooper & MacPherson, 2010). Iguanas in this study had an immediate decrease in gut microbial diversity 24 hr after LPS treatment, suggesting immune activation changes the gut microbiome. However, this effect was transient and disappeared after 72 hr. Thus, while diet and immunity can alter the gut microbiome, the effects are only temporary with the gut microbiome re-establishing balance after pertubation.

Alternatively, the high sugar diet may change the gut microbiome directly which then alters immunity. Our study provides evidence that a high sugar diet changes gut microbial composition, trends towards increasing alpha diversity, and hinders functional bacterial killing. However, there were no direct correlations between blood physiology and bacterial abundance, family abundance, or diversity. One potential mechanism is that the sugar-induced change in microbial diversity altered production of sugar-related metabolites such as short chain fatty acids (SCFA) ,which can modulate the immune system (Alexander & Turnbaugh, 2020). These SCFA are the products of sugar fermentation and triglyceride hydrolysis, which is notable as the sugar groups in our study had elevated levels of ciruclating triglycerides, and thus may have produced SCFA at different rates than controls (Karasov & Douglas, 2013). SCFA receptors have been identified on neutrophils, macrophages, and dendritic cells (C. H. Kim, 2018; Siddiqui et al.,

2022); thus, an increase in sugar intake may increase SCFA production, leading to more interactions with the immune system.

Finally, there is a possibility that a high-sugar diet may be altering other physiological factors such as insulin and glucagon, which can influence both immunity and gut microbiome. Together, insulin (glucose uptake) and glucagon (glucose production) regulate energy metabolism and energetic tradeoffs with the immune system. In rats and mice, hyperglycemia can impair immune response but the effect was reversible with insulin treatment (Mandel & Mahmoud, 1978; Mauriello et al., 2014). Similarly, glucagon has been found to improve intestinal immunity in mice, and normalize immune response of rats recovery from a burn injury There is some evidence that insulin and glucagon have roles in regulating energy metabolism in reptiles (similar to mammals), but there is limited evidence as to the action of these hormones so comparisons to mammalian hormones are cautioned (Marques, 1967; Penhos & Ramey, 1973; Putti et al., 1986). Squamates also tolerate a wider range of blood glucose concentrations; thus, regulation of blood glucose may have different or less impactful effects on other physiological systems.

Summary

Our study provides evidence to partly support our hypothesis that sugar supplementation alters immune responses, energy metabolites, and the gut microbiome. Total triglycerides but not glucose was elevated in the sugar groups throughout the entire study. We originally expected that glucose would also remain elevated but timing of sampling and/or physiological plasticity to accommodate higher doses of sugar may account for the fact that blood glucose did not change due to a sugar or immune challenge after the first month of the study. Additionally, a sugar diet has distinct and differential effects on immunity, such that it is markedly immunosuppressive following an immune challenge but potentially stimulatory at baseline. We see elevated agglutination prior to immune challenges with added sugar, but a decrease in bacterial killing ability and a greater reduction in agglutination and lysis ability in the first 24 hrs after an immune challenge. However, these changes are not permanent (does not always continue into the second challenge) and not all components of the immune system are affected (IgY concentrations were not affected by diet). The gut microbiome composition and alpha diversity was altered/reduced transiently after an immune challenge and the effects were exaggerated in the sugar group. We present 3 possible explanations for the relationships among diet, immune function, and microbiome; 1) diet affects the immune system which affects the gut microbiome, 2) diet affects the gut microbiome which affects the immune system, and 3) diet affects an un-measured physiological factor which then affects the immune system and the gut microbiome. While we only present 3 explanations, it is also possible that the effects of diet on the immune system and gut microbiome are completely independent of each other. Considerable further study is required to better understand the mechanism involved in these relationships.

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Competing Interests

No competing interests declared.

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Data Availability

https://github.com/KiClaudia/greeniguana

https://github.com/kapheimlab

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Table and Figure Legends

Table 1. Change between diet treatment groups after 1 month of respective diet treatment (control or sugar) for each physiological variable. Results are from the Welch's T-Test comparing the mean and standard error for the two diet treatments (control and sugar) before and after the treatment, and the change over a month. Bolded values are statistically significant. Samples are collected from iguanas in a laboratory setting and all samples were run in duplicate for each biochemical test. Sample sizes are noted within the table.

	BKA (%)	Agglut-ination	Lysis	**Glucose (mg dL^{-1})**	**Glycerol (mg mL^{-1})**	**Total TRI (mg mL^{-1})**	**Mass (g)**
n	18,18	16,15	16,15	**18,18**	**17,15**	**17,15**	**18,18**
T	-1.35	-0.43	-0.38	**2.66**	**2.45**	**4.64**	**2.17**
df	28.8	29.0	27.0	**30.9**	**16.9**	**19.4**	**32.5**
p-value	0.187	0.669	0.705	**0.012**	**0.025**	**0.0001**	**0.038**
Before sugar	80.16 ± 6.03	2.35 ± 0.33	2.69 ± 0.38	**215.50 ± 11.91**	**0.09 ± 0.04**	**1.52 ± 0.15**	**53.61 ± 2.81**
After sugar	46.81 ± 7.01	2.65 ± 0.31	2.96 ± 0.35	**342.33 ± 22.08**	**1.45 ± 0.41**	**4.10 ± 0.44**	**58.06 ± 2.77**
Δ Sugar Group	-33.35 ± 6.30	0.25 ± 0.36	0.47 ± 0.34	**126.83 ± 24.04**	**1.37 ± 0.44**	**2.54 ± 0.52**	**4.44 ± 0.98**
Before control	76.50 ± 7.51	1.19 ± 0.27	1.90 ± 0.31	**258.72 ± 17.06**	**0.04 ± 0.07**	**1.44 ± 0.13**	**53.33 ± 3.06**
After control	58.98 ± 5.30	1.65 ± 0.27	2.28 ± 0.31	**306.67 ± 17.06**	**0.35 ± 0.07**	**1.52 ± 0.13**	**54.39 ± 3.06**

Δ Control Group	-17.51 ± 9.89	0.47 ± 0.34	0.42 ± 0.44	**47.94 ± 17.31**	**0.29 ± 0.08**	**-0.002 ± 0.17**	**1.06 ± 1.22**

Day -1	Day 0	Day 30	Day 34	Day 35	Day 37	Day 41	Day 48	Day 62	Day 64	Day 65	Day 67	Day 71	Day 78	Day 92
Blood, colon swab, glucose, SVL, weight	Diet begins	Blood, colon swab, glucose, SVL, weight	Primary LPS challenge	Blood, colon swab, glucose, weight	Blood, colon swab, glucose, weight	Blood, colon swab, weight	Blood, weight	Blood, colon swab, glucose, SVL, weight	Secondary LPS challenge	Blood, colon swab, glucose, weight	Blood, colon swab, glucose, weight	Blood, colon swab, weight	Blood, weight	Blood, colon swab, glucose, SVL, weight
		Pre-1st injection		24-hour post injection	72-hr post injection	1-week post injection	2-week post injection	4-week post injection AND pre-2nd injection		24-hour post injection	72-hr post injection	1-week post injection	2-week post injection	4-week post injection

Fig. 1. Timeline of the study indicating when treatments began and when/which samples were collected.

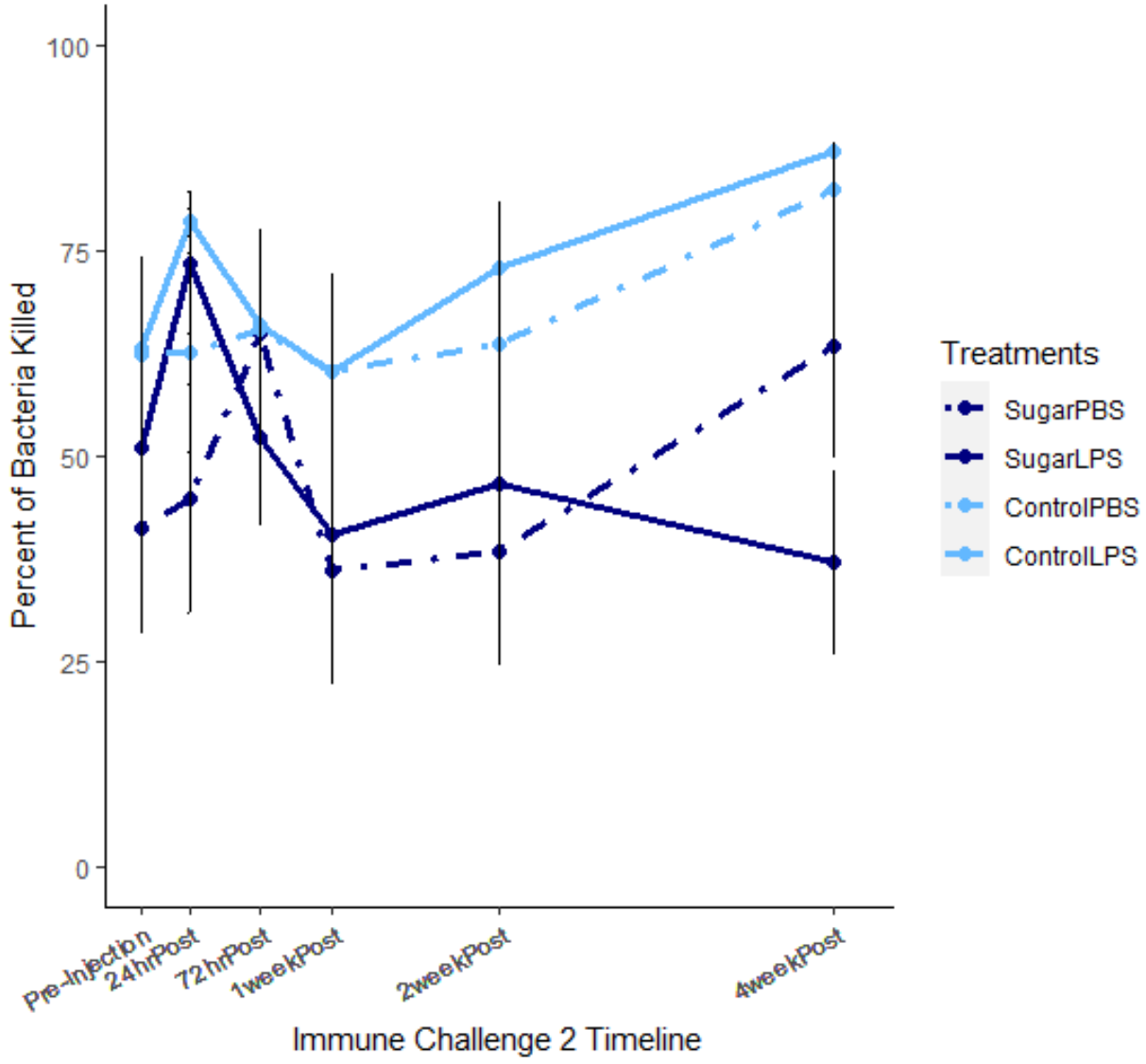

Fig. 2. Effect of diet on BKA. A beta regression reveals that iguanas treated with a high sugar diet have significantly lower BKA than iguanas on the control diet. The x-axis indicates timepoints throughout the experiment (post denoting post-LPS/PBS injection). The y-axis indicates percent of bacteria killed with 0% meaning no bacteria killed and 100% indicating all bacteria were killed. Diet treatment is denoted by color and immune challenge treatment is denoted by line type. Black vertical lines represent the standard error. There were 18 animals in each diet group and across 6 time points. Samples were collected from iguanas in a laboratory setting and all samples were run in duplicate for the BKA.

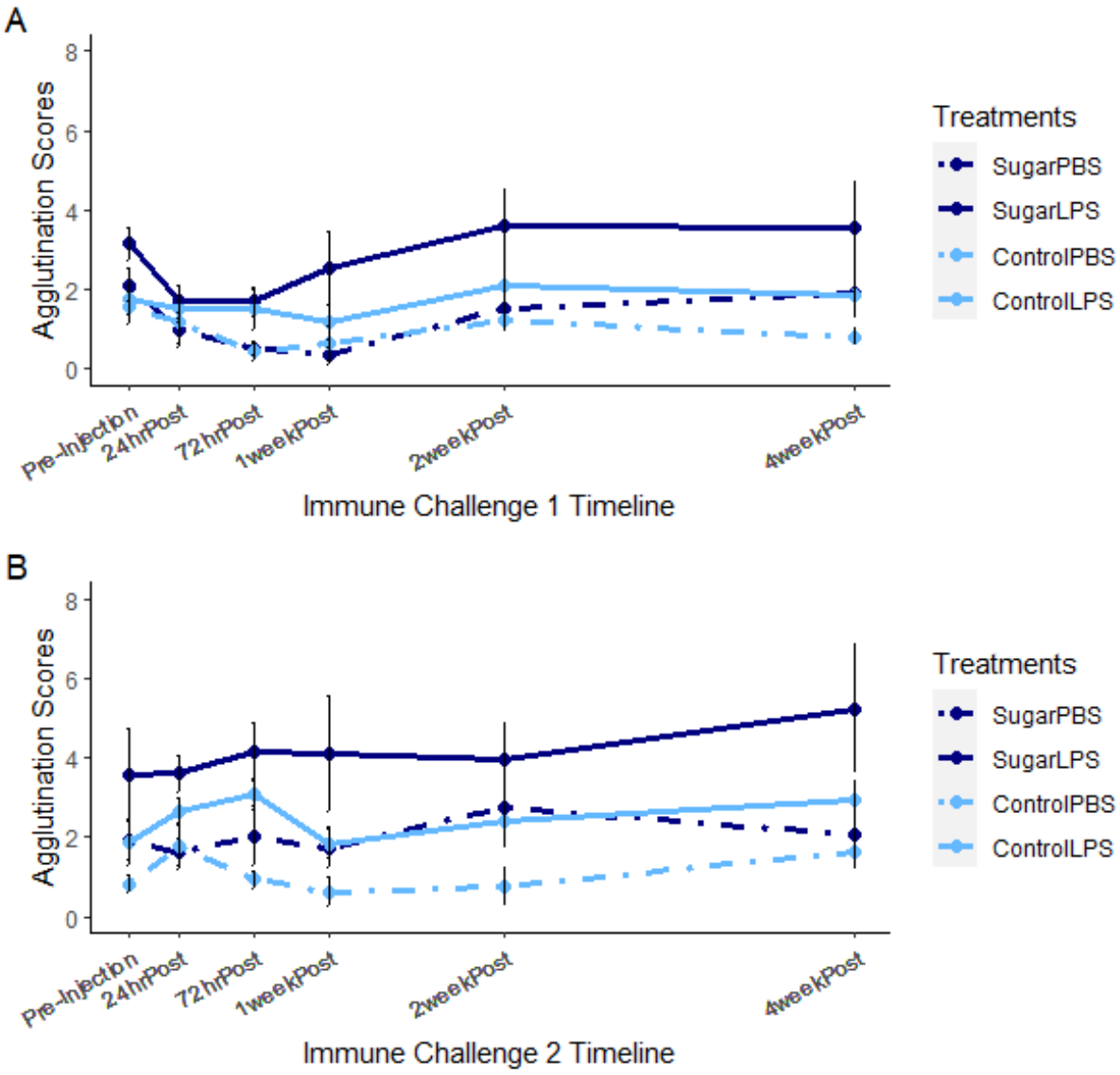

Fig. 3A Effect of diet and a primary immune challenge on agglutination. A Mann-Whitney test revealed an effect of the immune challenge with LPS iguanas (n=16) having higher agglutination values than PBS iguanas (n=17). In a separate model comparing the difference in change between pre and 24 hr post immune challenge, there is an effect of diet. Initial change in

agglutination from baseline was significantly reduced in the sugar-treated iguanas (n=16) compared to control diet iguanas (n=16). Finally, agglutination is elevated in the sugar groups (n=16) prior to the immune challenges as opposed to the control group (n=17). **B Effect of diet and a secondary immune challenge on agglutination.** A Mann-Whitney test illustrates the continued effect of the immune challenge. LPS iguanas (n=18) have higher agglutination values than PBS iguanas (n=17). In a separate model comparing the difference in change between pre and 24 hr post immune challenge, there is an effect of diet. Control diet iguanas (n=16) had a larger increase in agglutination than sugar group iguanas (n=17). Similar to the first challenge, agglutination is elevated in the sugar groups (n=18) prior to the immune challenges relative to the control group (n=16). Black vertical lines represent the standard error in both plots. Samples were collected from iguanas in a laboratory setting and all samples were run in duplicate for agglutination.

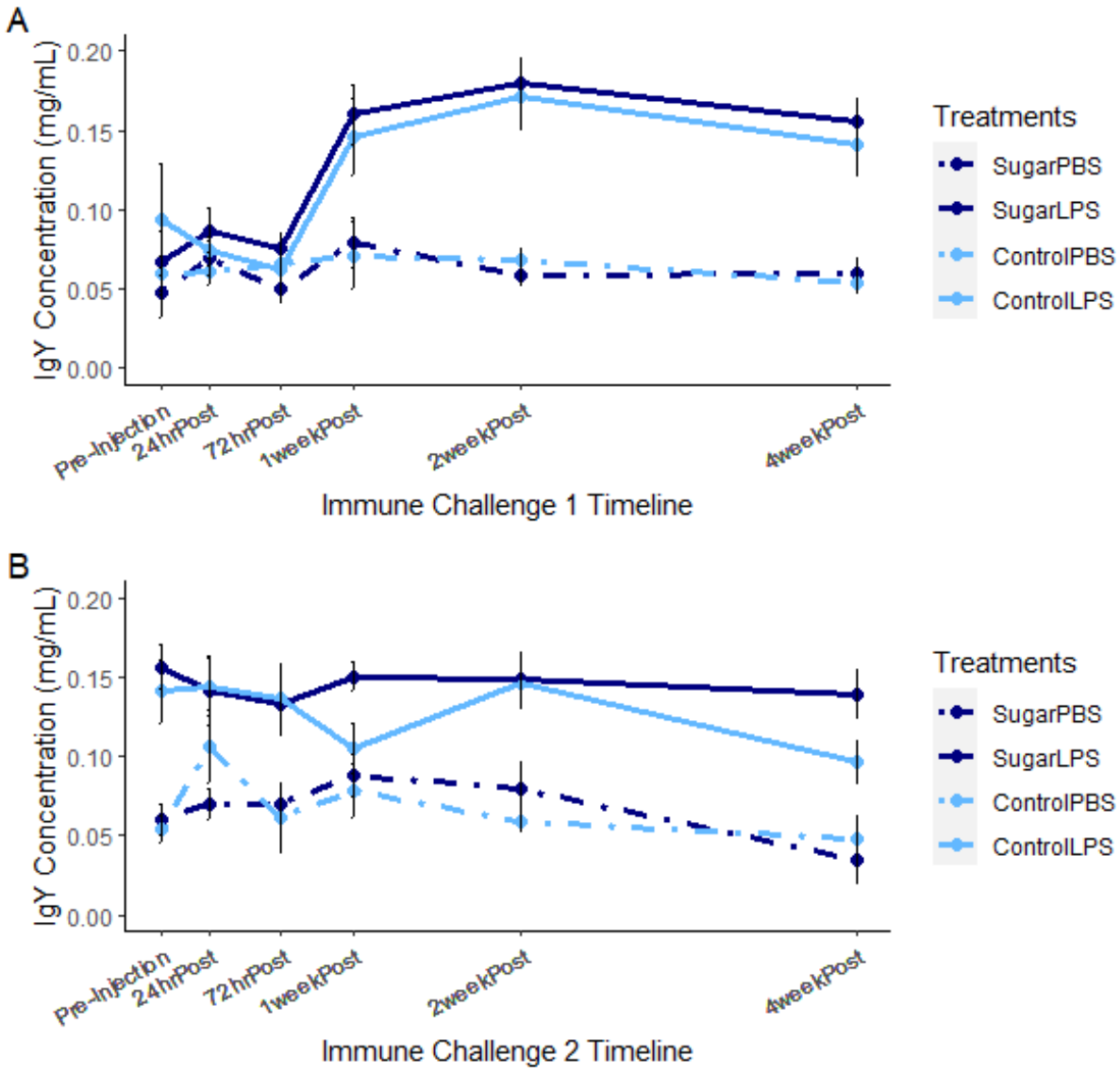

Fig. 4A Effects of a primary immune challenge on IgY. A 3-way repeated measures ANOVA found that iguanas in the LPS group (n=16) had significantly higher IgY concentrations 1 week post-LPS treatment as compared to PBS iguanas (n=15). **B Effects of a secondary immune challenge on IgY.** A 3-way repeated measures ANOVA found that LPS iguanas (n=16) had a significantly higher IgY concentration during the entire secondary treatment as compared to PBS iguanas (n=14). Black vertical lines represent the standard error in both plots. Samples were collected from iguanas in a laboratory setting and all samples were run in duplicate for agglutination.

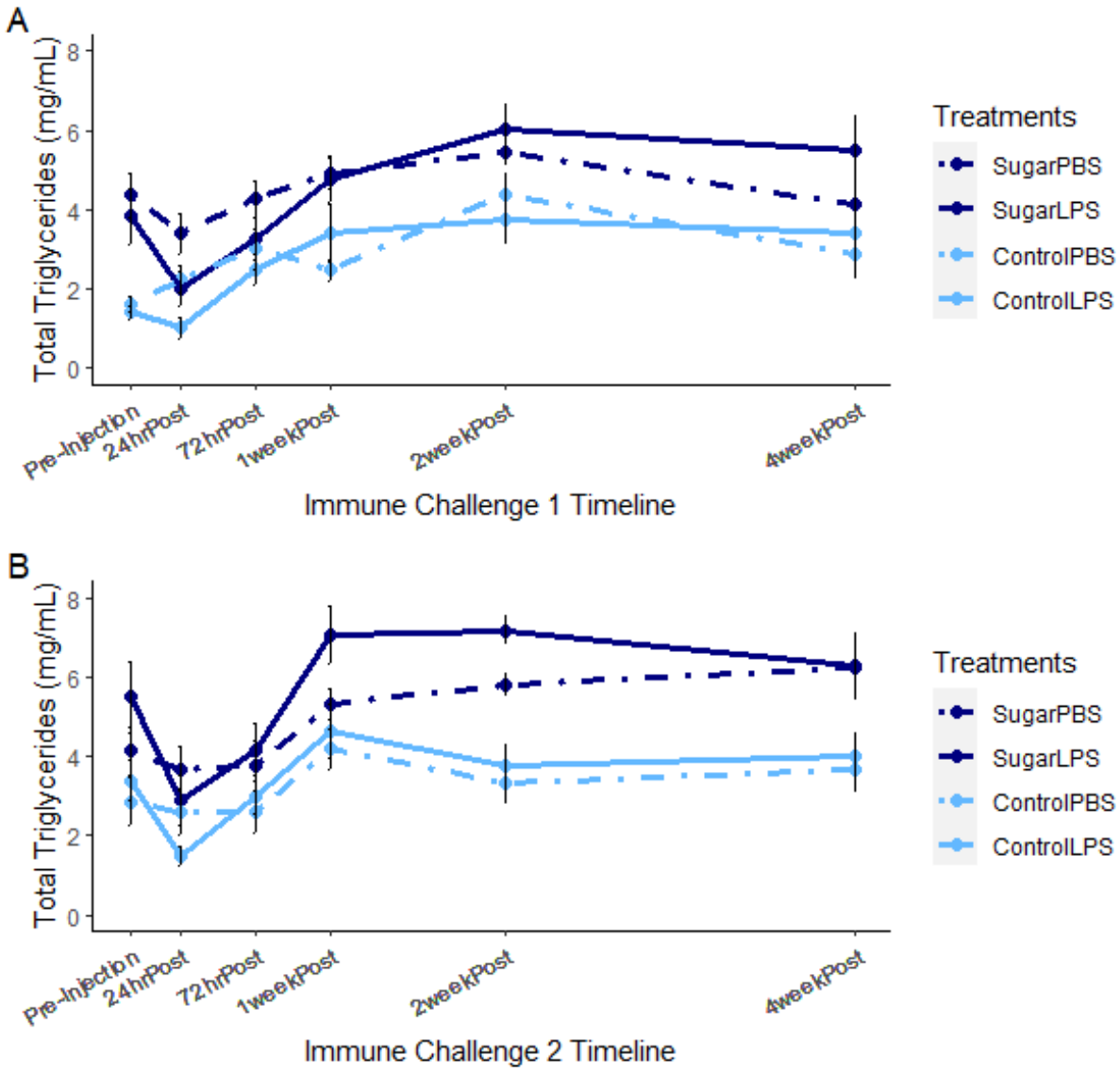

Fig. 5A Effects of diet and a primary immune challenge on total triglycerides. A 3-way repeated measures ANOVA showed that total triglycerides in the sugar groups (n=18) were higher than the control diet groups (n=17). LPS groups (n=17) had a momentary reduction in total triglycerides 24 hr after injection relative to the PBS group (n=17). **B Effects of diet and a**

secondary immune challenge on total triglycerides. A 3-way repeated measures ANOVA showed the elevation of total triglycerides of the sugar groups continued through the second challenge (In general n=18 per treatment for 6 separate time points but see methods for details). Black vertical lines represent the standard error in both plots. Samples were collected from iguanas in a laboratory setting and all samples were run in duplicate for agglutination.

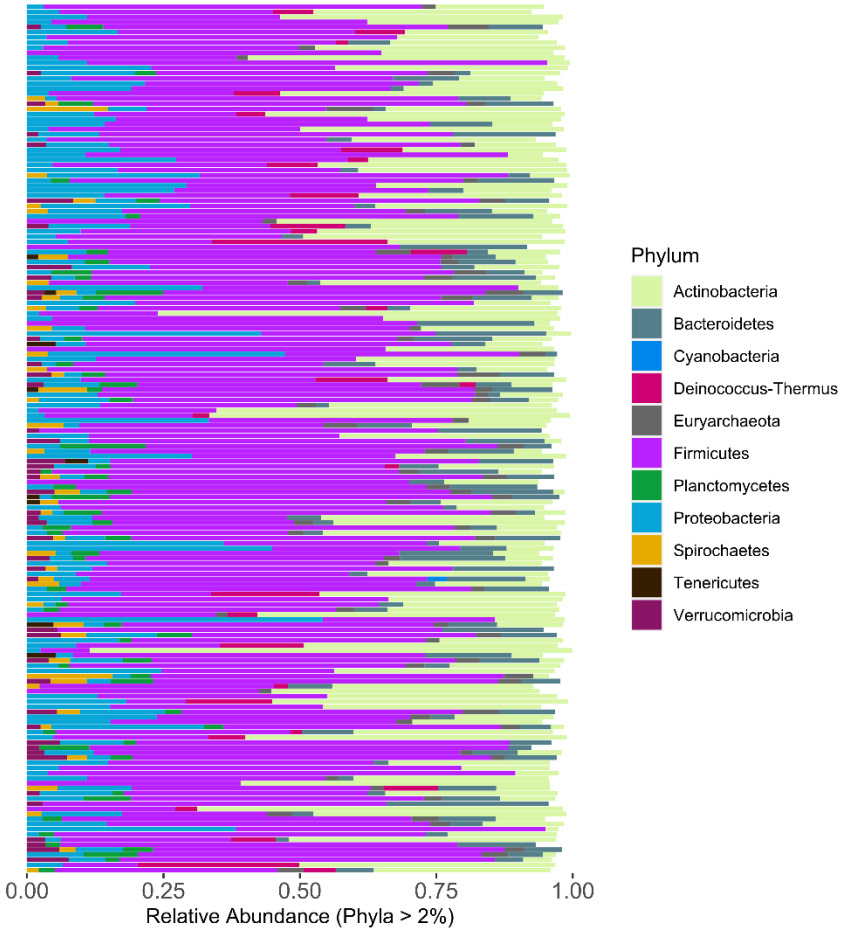

Fig. 6. Hindgut microbiome composition of green iguanas. Relative abundance of Phyla found at greater than 2% in each sample (collected via cloacal swab). Each row represents the bacterial community of a given sample, and samples include multiple timepoints for each iguana. There were 36 iguanas sampled at 3-6 times (170 samples total).

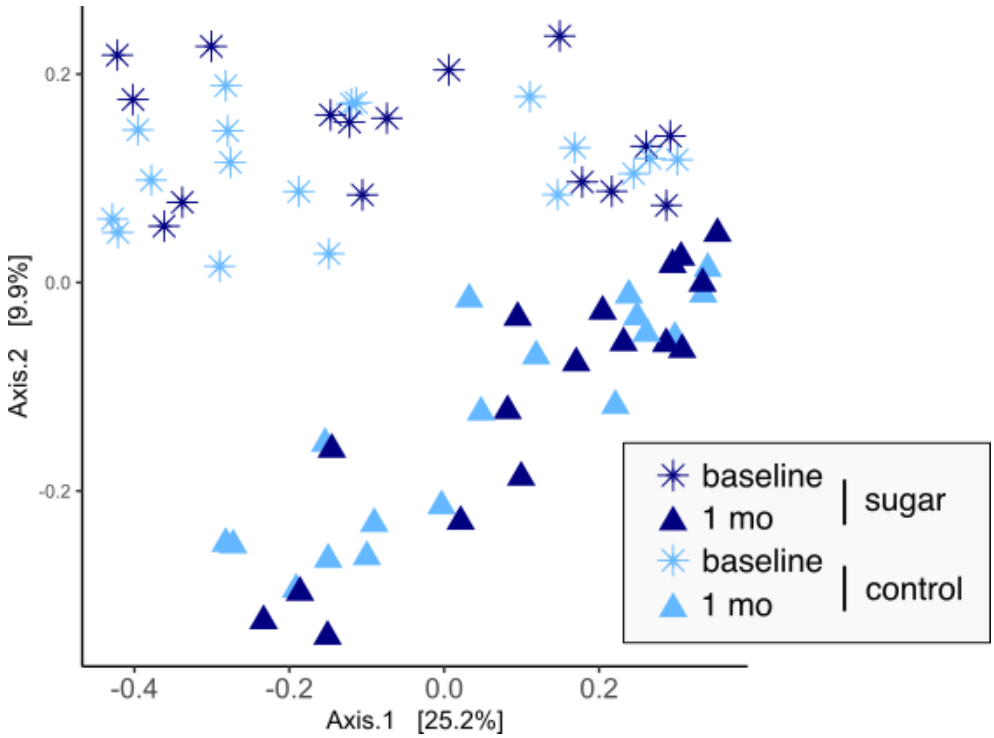

Fig 7. Principal Coordinates Analysis (PCoA) plot of Bray-Curtis dissimilarity from log-transformed abundances. Each point represents the bacterial community of an individual sample from 36 green iguanas.

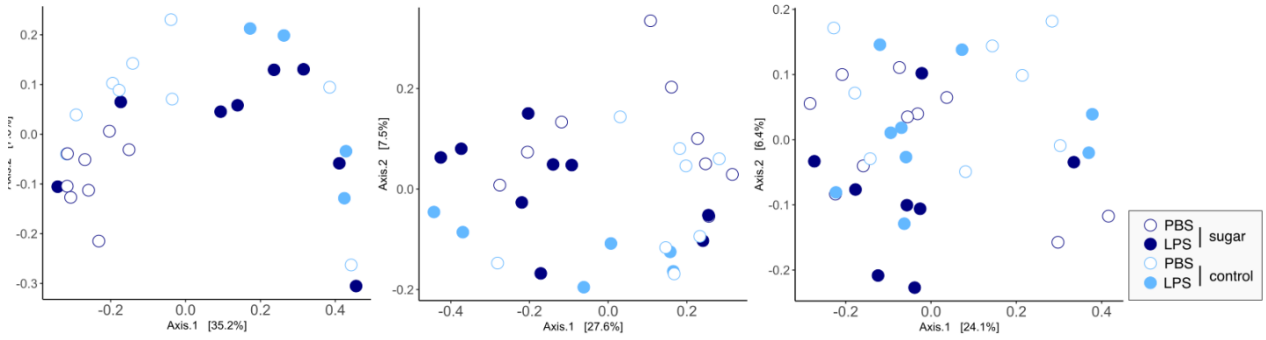

Fig 8. Principal Coordinates Analysis (PCoA) plot of Bray-Curtis dissimilarity from log-transformed abundances. Each point represents the bacterial community of an individual sample from 36 green iguanas at three time points: (**A**) 24 hr, (**B**) 72 hr, or (**C**) approximately 2 months after immune challenge. Colors indicate diet and shades of a given color indicate immune challenge treatment.

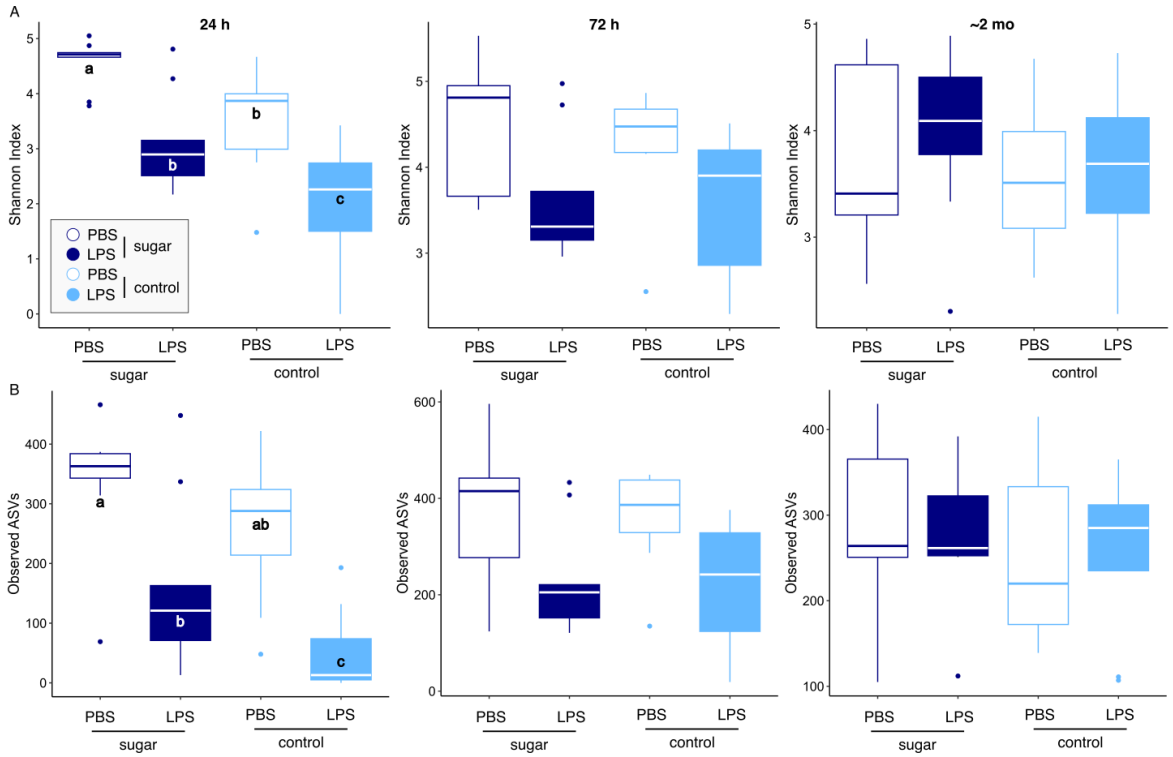

Fig 9. Alpha diversity as an effect of diet, immune challenge, and time measured as (A) Shannon index and (B) observed species richness. Boxes represent the interquartile range, with the line at the median. Whiskers extend to the smallest and largest values no further than 1.5 times the interquartile range. Outlying points are plotted individually beyond the whiskers. Letters indicate significant differences ($p < 0.05$) between groups.

Abbreviations Used

ASV – amplicon sequence variant
BKA – bacterial killing ability
IC – immune challenge
LPS – lipopolysaccharide
PBS – phosphate buffer solution
PCoA – Principal Coordinates Analysis
SCFA – short chain fatty acids
SVL – snout vent length
TLR4 – toll-like receptor 4