Macronutrients, the microbiome, and illness-induced feeding behavior: Do macronutrients shape avian immune responses?

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Abstract

Macronutrients, such as proteins and fats, play a vital role in host immunity and can influence host-pathogen dynamics, potentially through dietary effects on gut microbiota. To increase our understanding of how feeding behavior and macronutrient selection are influenced by a direct and perceived immune threat and whether shifts in macronutrient intake affect the composition of the gut microbiome, we conducted two experiments. First, we determined if zebra finches (*Taeniopygia guttata*) exhibit shifts in physiology and gut microbiota when fed diets differing in macronutrient ratios. Second, we simulated an infection in birds using the bacterial endotoxin lipopolysaccharide (LPS) and quantified feeding behavior in immune challenged and control individuals, as well as birds housed near either a control pair (no immune threat), or birds housed near a pair given an immune challenge with LPS (social cue of heightened infection risk). We also examined whether social cues of infection alter physiological responses relevant to responding to an immune threat, an effect that could be mediated through shifts in feeding behavior. In the first experiment, protein diets decreased the abundance of the bacterial Phylum Campylobacterota. Further, diet treatment disrupted relationships between gut microbiota alpha diversity and physiological metrics. In the second experiment, LPS induced a reduction in caloric intake driven by a decrease in protein, but not fat consumption. No evidence was found for socially induced shifts in feeding behavior, physiology, or gut microbiota. However, fat consumption decreased gut microbial diversity regardless of treatment. Our findings carry implications for host health, as sickness-induced anorexia and diet-induced shifts in the microbiome could shape host-pathogen interactions.

Introduction

Nutrition is critical to the immune system and can influence how hosts respond to infection (Cunningham-Rundles et al. 2005, Ponton et al. 2011, Amar et al. 2007). Among ecological studies, the effect of the availability of resources on immune processes and disease outcomes has received significant attention (Becker et al. 2018, Strandin et al. 2018, Moyers et al. 2018), but the quality of those resources and their nutritional make-up (e.g., macronutrient content) are also important (Povey et al. 2013, Cotter et al. 2011).

Macronutrients like lipids, carbohydrates, and protein vary in their biological availability and can influence processes ranging from cellular function to whole organism performance (Warne 2014). Diet macronutrient content can influence physiological processes important in responding to pathogens, including hormonal and immune responses. For example, kittiwake chicks fed a low-lipid diet had higher baseline and stress-induced concentrations of the hormone corticosterone (Kitaysky et al. 2001), which plays a role in responding to energetic demands and is known to both stimulate and suppress immune responses in different contexts (Roberts et al. 2007, Da Silva 1999). Dietary nutrients can also impact other hormones known to influence immune function, such as testosterone (Roberts et al. 2007, Da Silva 1999). For example, humans consuming higher levels of dietary fat have higher baseline testosterone concentrations, while subjects consuming higher levels of dietary protein have lower testosterone levels (Volek et. 1997).

Diet can also directly affect immunity without the mediation of hormones. In captive white ibis, birds that were fed anthropogenic dietary items like white bread had reduced bacterial killing ability, but corticosterone levels and the other immune parameters investigated were unaffected by diet treatment (Cummings et al. 2020). Other work has found that restriction of dietary protein can limit immune activity (Lee et al. 2006, Povey et al. 2009) and high lipid diets can increase mortality rates during infection (Adamo 2008, Adamo et al. 2010). The macronutrient composition of diets can also differentially affect different components of the immune system. For example, in insects, the optimal macronutrient composition of diets varies for different immunological parameters (Cotter et al. 2011). Thus, optimal diet selection may vary based on the type of immune threat that organisms are experiencing.

Despite the apparent need for nutritional resources to mount an immune response, some organisms respond to infection with sickness-induced anorexia (Adamo et al. 2007, Adelman and Martin 2009, Povey et al. 2013). This reduction in food intake following an immune challenge is thought to reduce the risk of ingesting additional infectious agents or may function to starve pathogens and parasites of key nutrients (Kyriazakis et al. 1998, Adamo et al. 2007, Adelman and Martin 2009). Further, caloric restriction during illness can improve host health and recovery (Cheng et al. 2017; Wang et al. 2016).

In addition to influencing the quantity of food that animals consume, infection can also alter diet selection. In caterpillars challenged with a viral infection, infected individuals select diets with a higher protein to carbohydrate ratio when compared with control individuals, and infected individuals placed on a high protein diet are more likely to survive infection (Povey et al. 2013). Shifts in diet preference during infection that optimize recovery and survival are referred to as "self-medication" behaviors and have been documented in several taxa (Huffman and Seifu 1989, Hutchings et al. 2003, Povey et al. 2013). Thus, animals can shift feeding behaviors and selectively feed on foods with desirable macronutrient composition in response to an immune threat.

Shifts in diet caused by environmental availability and selective feeding can affect the immune system through shifts in the gut microbiome (Zheng et al. 2020). The gut microbiome regulates multiple aspects of host health, including metabolism and the development of the host immune system (Hird 2017, Kau et al. 2011, Zheng et al. 2020). Gut microbial communities can also influence how hosts respond to disease, as disrupting microbial communities with antibiotics can alter immunity and increase susceptibility to bacterial and parasitic infections in humans and wildlife (Buffie et al. 2012, Zheng et al. 2020, Knutie et al. 2017). Host diet plays an important role in shaping the composition and diversity of gut microbial communities (Pan and Yu 2014, Singh et al. 2017, Bodawatta et al. 2021) and diet-induced shifts in gut microbiota can alter host immune responses (Zheng et al. 2020). For example, in humans and mice, a high-fat westernized diet alters gut microbial communities and increases inflammation (Agus et al. 2016, Statovci et al. 2017). Shifts in diet can also cause changes in the gut microbiome that increase host resistance to parasites. In nestling bluebirds, food supplementation with mealworms increased gut bacterial diversity and the abundance of *Clostridium* spp., which was associated with higher nestling antibody responses and lower numbers of nest parasites (Knutie 2020). These studies provide evidence that diet can alter host responses to parasites through shifts in the gut microbiome. Thus, organisms may be able to optimize responses to infection through shifts in feeding behavior that alter gut microbial composition. Despite the clear implications for host health and wildlife disease dynamics, few studies have investigated how dietary macronutrients, gut microbiota, physiology, and feeding behavior interact to shape host responses related to infection.

Infection-induced shifts in feeding and activity can also be detected by conspecifics and viewing a sick neighbor can result in healthy individuals altering their feeding behavior in a way that optimizes or primes the immune system to fight off infection (Castella et al. 2008; Povey et al. 2013). Further, recent research in social organisms indicates that behavioral and physiological changes can occur in response to public information (Cornelius et al. 2018; Cornelius 2022, Schaller, et al. 2010, Stevenson et al. 2011, 2012; Love et al. 2021). In red crossbills, social information from conspecifics that is indicative of low food-abundance reduces the expression of glucocorticoid and mineralocorticoid receptors in the brain (Cornelius et al. 2018). Additionally, red crossbills that observed food-restricted neighbors before becoming food-

restricted themselves ate more food, conserved more body mass, and were in better condition than birds without this predictive social information (Cornelius 2022). Since nutritional state is known to greatly impact disease outcomes (Murray et al. 1998, Chandra 1996, Lochmiller & Deerenberg 2000), prophylactic behaviors involving macronutrient selection in response to cues of disease could help prepare organisms for an impending immune threat.

The goals of this study were to 1) enhance our understanding of how dietary macronutrients affect host physiology and the gut microbiome, 2) characterize how feeding behavior and macronutrient selection are influenced by immune threats that are direct (i.e., immune activation) or perceived (e.g., observing a sick conspecific), and 3) provide insight into whether shifts in feeding behavior alter the gut microbiome and physiological processes relevant to disease susceptibility and transmission. We explored these relationships in zebra finches (*Taeniopygia guttata*) through two separate experiments investigating 1) how diet macronutrient content affects immunity, hormonal responses, and the gut microbiome, and 2) how perceived and actual immune threats shape feeding behavior (caloric intake and diet macronutrient selection). Additionally, because social information from conspecifics regarding food (Cornelius et al. 2018; Cornelius 2022) and disease risk (Schaller, et al. 2010, Stevenson et al. 2011, 2012; Love et al. 2021) can alter physiology, we also investigated whether perceived risk of infection (seeing immune-challenged conspecifics) alters physiological responses pertinent to immune function and feeding behavior, specifically, complement activity, and corticosterone and testosterone blood plasma concentrations. We predicted that the different diets would induce changes in the gut microbiome and physiology. We also predicted that birds given an immune challenge and birds with a social cue of heightened infection risk would increase protein consumption, as studies in insects indicate that high protein diets are associated with increased immune capabilities and high lipid diets are associated with increased mortality during infection (Povey et al. 2013, Adamo 2008, Adamo et al. 2010). Additionally, we predicted that perceived risk of infection (seeing sick conspecifics) would alter physiological responses, and that this effect might be mediated through shifts in feeding behavior and subsequent changes in the gut microbiome. Identifying and understanding the factors that contribute to variation in avian responses to infection is of broad interest and integral to improving our understanding of avian epidemiology, especially since birds are hosts for diseases relevant to wildlife, domestic animals, and human health (Reed et al. 2003).

Materials and Methods

Experiment 1 –Timeline for Diet Manipulation Experiment

In the first experiment, zebra finches were given one of three diet treatments that varied in protein and lipid content. We investigated how diet influenced complement activity, baseline and stress-induced corticosterone concentrations, and the gut microbiome. Birds were kept on a 14 L: 10 D light cycle and individually housed in 24”x16”x16” cages. Each cage had two perches, a water dish, and one food dish in which birds were fed *ad libitum* . Diets consisted of hulled millet, egg white, egg yolk, vegetable oil, and sorbic acid (preservative) in agar blocks. Birds were placed on an acclimation diet with equal ratios of lipid and protein for 5 days prior to experimental treatment. Birds were then randomly assigned to one of three diet treatments (Figure 1; day 0), which they remained on for eight additional days. All diets were isocaloric and only varied in the ratio of protein and lipid content. All diets contained similar concentrations of carbohydrates (62-66 % of the total metabolizable energy of the diet (TME)). Diet treatments consisted of a high fat diet (25% lipid and 13% protein of TME; high fat diet: n=11, 7 males, 4 females), a diet with equally balanced ratios of lipid and protein (i.e., the acclimation diet; 17% lipid and 17% protein of TME; equal ratio diet: n=12, 8 males, 4 females) and a high protein diet (13% fat and 25% protein of TME; high protein diet: n=12, 8 males, 4 females). Three desiccation controls for each diet type were also weighed daily and the average desiccation values for each diet type (high fat, equal ratio, high protein) were subtracted from feeding values to account for daily changes in food mass due to desiccation.

To assess the effects of diet on physiological endpoints and gut microbiota, blood samples and cloacal swabs were collected on the day that acclimation diets were switched to manipulation diets and eight days after the manipulation diets were implemented. Body mass and fat score data were recorded for each

bird at all sampling time points. All blood samples were collected between 0830 – 0930 CDT to avoid differences in physiological endpoints due to variation in daily rhythms. Within 3 min of entering the room, a baseline blood sample of 120 uL was collected from the wing vein of each individual to be used to determine baseline concentration of plasma corticosterone and hemolytic complement activity. Next, we used sterile swabs to swab the cloaca of each bird. Swabs were placed in 300 µl RNAlater (Invitrogen, Thermo Fisher Scientific) and frozen at -80°C. Birds were then weighed and placed into a paper bag. Thirty minutes after entering the room, a second blood sample was collected to determine stress-induced concentrations of plasma corticosterone. All blood samples were collected into heparinized capillary tubes and immediately placed on ice. Tubes were centrifuged for 3 minutes to separate blood plasma from erythrocytes and plasma samples were stored at -20°C. All research protocols were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Experiment 2 –Timeline for Diet Selection Experiment

To investigate whether birds alter feeding behavior to optimize responses to infection we conducted a second experiment, in which we simulated an infection in established zebra finch pairs using the bacterial endotoxin lipopolysaccharide (LPS), and quantified feeding behavior in immune challenged and control individuals, as well as birds housed near either a control pair (no immune threat), or birds housed near a pair given an immune challenge with LPS (social cue of heightened infection risk). To investigate how an immune challenge and perceived immune threat shape macronutrient preference, birds were provided with two isocaloric diets with varied lipid and protein ratios and their consumption was recorded. Birds were kept on a 14 L: 10 D light cycle and housed in 24"x16"x16" cages that were divided down the center into two separate 12"x16"x16" cage sections that each housed one pair of birds. Birds were housed in previously established pairs with one female and one male per cage section. Each cage section had two perches, a water dish, and two food dishes in which birds were fed *ad libitum* . Birds were given a choice of diet where each cage contained one high lipid food block and one high protein food block, whose composition was the same as in Experiment 1. Diet type was randomly assigned to either the left or right food dish to avoid confounding effects of cage side preference. To assess whether an immune threat altered the amount and type of diet that birds consumed, birds were provided with a choice in diet for seven days prior to experimental treatment and for five days following experimental treatment. Diets were weighed daily and replaced every other day. Three desiccation controls for each diet type were also weighed daily and the average desiccation values for each diet type (high lipid or high protein) were subtracted from feeding values to account for daily changes in food mass due to desiccation.

Pairs housed on one side of the cage were injected with either LPS or Saline (Figure 1; LPS-injected: N=24 birds or Saline-injected: N=24 birds), whereas pairs housed on the other side of the cage were unmanipulated (focal pairs; LPS-focal: N=24 birds or Saline-focal: N=24 birds). Injected pairs provided social cues to the focal pair. Solid opaque dividers were placed on both sides of each 24 x 16 x 16 cage to ensure that birds housed in each double cage could only see one another. To assess how an immune challenge and social cue of heightened infection risk shape feeding behavior, we injected previously established pairs with either lipopolysaccharide (LPS), a non-replicating antigen that activates the immune system and induces sickness behaviors, or a saline solution (control). Specifically, we injected stimulus birds intra-abdominally with either 50 µL of 2 mg/kg LPS (Sigma-Aldrich #L7261, *Salmonella enterica* serotype typhimurium) or 50 µL of phosphate-buffered saline (sham control, Sigma-Aldrich #P3813). Body mass and fat score data were collected on all stimulus and focal birds prior to treatment and on days 1, 2, and 5 post-treatment.

To assess whether heightened infection risk altered complement activity or baseline corticosterone concentrations, we collected blood samples from all focal birds 3 days prior to stimulus bird injections and 1, 2, and 5 days following stimulus bird injections. All blood samples for baseline corticosterone were collected within 3 minutes of entering the room. We collected additional blood samples from all focal males to assess changes in plasma testosterone concentrations in response to a heightened cue of infection 4 days prior to and 3 days following injection of the stimulus pairs. Immediately after collection, blood samples were centrifuged and blood plasma was separated and frozen at -20°C. To determine how treatment affected zebra

finch gut microbial diversity and composition, cloacal swab samples were collected from all birds prior to treatment (day 0) and on day 5 following stimulus bird injections. Cloacal swabs were placed into 300 µl of RNAlater (Invitrogen, Thermo Fisher Scientific) and frozen at -80. All research protocols were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Hemolytic Complement Activity Assay

To assess if diet macronutrient content (experiment 1) and social cues of disease (experiment 2) influenced the complement pathway, we conducted a CH50 complement assay that measures the ability of proteins in the plasma to lyse sheep red blood cells (MP Biomedicals, Cat#55876). We measured complement activity following the methods outlined in Sinclair and Lochmiller (2000). Briefly, we ran duplicate 80 µl samples of 1:20 and 1:40 plasma dilutions. Hemolytic complement activity was expressed as CH50 units/ml plasma, where one CH50 unit signifies the reciprocal of the dilution of plasma needed to lyse 50% of the sheep red blood cells (French et al. 2010).

Corticosterone and Testosterone Assays

In the first experiment, we quantified baseline and stress-induced plasma corticosterone concentrations using an Arbor Assays Corticosterone ELISA Kit (Cat#: ADI-900-065). Samples were run in duplicate at a 1:50 dilution following treatment with 1% steroid displacement buffer. Each plate contained a standard curve run in triplicate. Absorbance was measured at 405 nm using a SpectraMAX 190 spectrophotometer (Molecular Devices). We compared the mean value of the duplicates for each sample to a standard curve that contained known amounts of corticosterone. A 250 pg/mL standard was run in triplicate on each plate. The intra-assay coefficient of variation and inter-assay coefficient of variation were 12.8% and 14.0% respectively. Cross-reactivity of the testosterone antibody was as follows: androstenedione 7.2%, estradiol 1%, dehydroepiandrosterone 1%, dihydrotestosterone 1%, and progesterone 1% (Enzo Life Sciences).

In the second experiment, baseline plasma corticosterone concentrations were measured in duplicate following standard radioimmunoassay techniques (Wingfield et al., 1992). To determine the coefficient of intra- and inter-assay variation, four standard samples were prepared with 200 pg of corticosterone and plasma and standard sample tubes were prepared with 500 µl ddH2O and 2000 dpm of tritiated corticosterone (NET-399) from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Samples were equilibrated overnight at 4 °C. Corticosterone was extracted from plasma using 4 ml of diethyl ether and dried in a 37 °C water bath with the aid of nitrogen gas. Following extractions, samples were suspended in 500 µl of phosphate buffered saline and refrigerated overnight at 4 °C. The following day, we determined individual extraction efficiency using 50 µl of each sample. Corticosterone concentrations were corrected for individual extraction efficiency (mean recoveries were 83%). For the assay, each sample was allocated into two duplicates, each consisting of 200 µl. 100 µl of corticosterone antibody (B3-163; Endocrine Sciences, Calabasas, CA, USA) and 100 µl of tritiated corticosterone were added to each sample and standard tube. We compared the mean value of the duplicates for each sample to a standard curve (also run in duplicate) that contained known amounts of corticosterone (C2505 corticosterone standard, Sigma-Aldrich, St. Louis, MO, USA). The intra-assay coefficient of variation and inter-assay coefficient of variation were 11.4% and 13.3% respectively.

Testosterone was only measured in the second experiment, in which plasma samples were collected from focal male zebra finches 4 days prior to and 3 days following experimental treatment (LPS-focal: cue of disease, saline-focal: no cue of disease). Plasma testosterone concentrations were measured using an Enzo Testosterone ELISA Kit (Cat#: ADI-900-065). Samples were run in duplicate at a 1:20 dilution following treatment with 1% steroid displacement buffer. Each plate contained a standard curve run in triplicate. Absorbance was measured at 405 nm using a SpectraMAX 190 spectrophotometer (Molecular Devices). The intra-assay coefficient of variation and inter-assay coefficient of variation were 12.8% and 14.0% respectively. Cross-reactivity of the testosterone antibody was as follows: androstenedione 7.2%, estradiol 1%, dehydroepiandrosterone 1%, dihydrotestosterone 1%, and progesterone 1% (Enzo Life Sciences).

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from cloacal swabs using DNeasy PowerPlant Pro kits (Qiagen). Both the swab and the RNAlater (Invitrogen, Thermo Fisher Scientific) that the swab was stored in were added to the PowerBead tubes, then the extraction proceeded following the instructions in the manufacturer's protocol. We quantified the DNA concentration for each sample using a Qubit fluorometer and froze samples at -20 until samples could be shipped for sequencing. The V4 region of the 16S rRNA gene was sequenced at the University of Texas's Genome Sequencing and Analysis Facility (GSAF) using an Illumina MiSeq platform. Samples with less than 1,000 total sequences were excluded from subsequent analyses. After quality filtering, we had 63 samples for further analyses in the diet manipulation experiment (experiment 1). Of these samples, 22 were from birds fed a high fat diet (day 0: n=11, day 8: n=11), 21 were from birds fed diets with equal ratios of protein and lipid (day 0: n=9, day 8: n=12), and 20 were from birds fed a high protein diet (day 0: n=11, day 8: n=9). For the second experiment, some samples failed during sequencing or after quality filtering, yielding 69 total cloacal microbiota samples for further analyses. Of these samples, 38 were from injected birds (LPS day 0: n=9, LPS day 5: n=8; saline day 0: n=10, saline day 5: n=11) and 31 were from focal birds (LPS-focal day 0: n=6, LPS-focal day 5: n=8; saline-focal day 0: n=7, saline-focal day 5: n=10). We used *DADA2* (v.1.16) in R (v.4.2.0) to process our 16S sequence data. ASVs were assigned to taxonomy using the Silva 132 bacterial reference database (Quast et al. 2013). Sequences identified as a chloroplast or mitochondria were removed from the dataset. The*DECIPHER* package (v. 2.24.0; Wright 2015) was used to create a multiple sequence alignment and a generalized time-reversible maximum likelihood tree of the remaining ASVs was constructed with the*phangorn* package version 2.9.0 (Schliep 2011). The ASV table, taxonomic information, phylogeny, and sample metadata were joined for bacterial community analyses using the package *phyloseq*(McMurdie and Holmes 2013).

Gut microbiome diversity, composition, and relative abundance analyses

To account for uneven sequencing depth across samples, samples were rarefied to the depth of our lowest sample (5,691 for the diet manipulation experiment; 1,077 for the diet selection experiment) for alpha and beta diversity analyses. We used the *vegan* package (Oksanen et al., 2022) to quantify two measures of alpha diversity, the observed number of ASVs (richness) and the Shannon diversity index (which accounts for both richness and evenness). To determine whether the observed microbial richness varied in response to treatment, we ran a generalized linear mixed models (GLMM) fitted with a negative binomial (nbinom1) distribution using the package *glmmTMB* (Brooks et al. 2017) with treatment, day, and the interaction between treatment*day as fixed effects. For the Shannon diversity index, we ran a GLMM fitted with a gaussian structure and included treatment, day, and the interaction between treatment*day as fixed effects. Because birds were sampled over time, we included bird ID as a random effect in all models. We used the *DHARMa* package (Hartig 2019) to plot residuals and confirm the suitability of each model and the Anova function in the*car* package (Fox and Weisberg 2018) to determine significance. To assess beta diversity, we first subjected the unrarefied dataset to Cumulative Sum Scaling (CSS) normalization (Paulson et al. 2013) using the metagenomeSeq package following the methods outlined in Maraci et al. 2021. To account for compositional variations in each dataset, data were log (x+0.0001)-transformed and later corrected by subtracting the log of the pseudo count (Thorsen et al. 2016). For beta diversity analyses, we computed Bray-Curtis (Bray and Curtis 1957), unweighted UniFrac (Louzupone and Knuight 2007) and weighted UniFrac (Lozupone et al. 2007) distances. To visualize dissimilarities between treatments, we used a principal coordinate analysis (PCoA) using the ordinate function in *phyloseq* (McMurdie & Holmes, 2013). To assess similarity between treatments and over time for each beta diversity metric, we conducted PERMANOVA tests using the adonis2 function in the *vegan*package (Oksanen et al., 2022). Treatment, day sampled, and the interaction between treatment and day were included as fixed effects in all PERMANOVA tests. To account for repeated sampling over time, bird ID was included under the adonis2 strata option. All P-values were adjusted for false discovery rate with a Benjamini–Hochberg correction where significance was determined as $P_{adj} < 0.05$.

Finally, to investigate whether bacterial taxa differ in abundance across treatments, we calculated the relative abundances of phyla and genera from the unrarefied datasets. For analyses, phyla and genera with mean abundances <1% were lumped into an "Other" category. This data stringency limited analyses to

the top four most abundant phyla (Campylobaterota, Firmicutes, Proteobacteria, Actinobacteriota) and the top seven most abundant genera (*Campylobacter* ,*Weissella* , *Helicobacter* , *Acinetobacter* ,*Ligilactobacillus* , *Pseudomonas* , *Achromobacter*) in experiment 1 (diet manipulation). In experiment 2 (diet selection), this data stringency limited analyses to the five most abundant phyla (Campylobaterota, Firmicutes, Proteobacteria, Actinobacteriota, Bacteroidota) and the top ten most abundant genera (*Campylobacter* , *Helicobacter* , *Corynebacterium* ,*Ureaplasma* , *Ligilactobacillus* , *Atopobium* ,*Catellicoccus* , *Gallibacterium* , *Veillonella,Weissella*) in injected birds (LPS, saline), and the top thirteen most abundant genera (*Campylobacter* , *Helicobacter* ,*Pseudomonas* , *Serratia* , *Veillonella,Gallibacterium* , *Ligilactobacillus* , *Atopobium* ,*Corynebacterium* , *Enterobacter* , *Sphingobacterium* ,*Staphylococcus* , *Weissella*) in focal birds (LPS-focal, saline-focal). To compare abundances of bacterial taxa between treatments and over time, we ran non-parametric Kruskal-Wallis tests using the kruskal.test function in R. P-values were adjusted for false discovery rate with a Benjamini–Hochberg correction where significance was determined as $P_{adj} < 0.05$.

Statistical Analyses

Statistical analyses were conducted in R version 4.2.0 (R Core Team 2022). All data were checked for normality and homoscedasticity. To determine the effect of diet treatment and time (day 0, day 8) on body mass, fat score, corticosterone concentrations, and complement (CH50) activity in experiment 1, we ran GLMMs using the *glmmTMB* package in R (Brooks et al. 2017). For body mass, we ran a GLMM fitted with a gaussian distribution, while the models for the remaining endpoints (fat score, corticosterone, and complement activity) were fitted with a tweedie distribution. All models included treatment, time (day 0, day 8), and the interaction between treatment and time as fixed effects with bird ID as a random effect. When testing the effect of diet treatment and time on corticosterone responses, we also included sample type (baseline or stress-induced) as a fixed effect in the model. To test whether the average grams consumed per day varied based on diet treatment, we ran a generalized linear model (GLM) fitted with a gaussian distribution with grams consumed as the response variable and treatment as a fixed effect. Because physiology can both mediate and respond to shifts in gut microbiota composition (Noguera et al. 2018, Williams et al. 2020, Yoshiya et al. 2011) and diet might influence these interactions, we also ran a separate GLMM for each alpha diversity metric (observed richness, Shannon diversity) which considered the interactions between diet treatment and time (day 0, day 8) with each physiology metric (complement activity, baseline corticosterone, stress-induced corticosterone). P-values from these models were adjusted for false discovery rate with a Benjamini–Hochberg correction where significance was determined as $P_{adj} < 0.05$. We used the *DHARMa* package (Hartig 2019) to plot residuals and confirm the suitability of each model and the Anova function in the*car* package (Fox and Weisberg 2018) to determine significance.

To examine how feeding behavior and diet preference changed over time with treatment in experiment 2 (diet selection), we ran separate GLMs fitted with a gaussian distribution to test for differences in the stimulus (LPS, saline) and focal (LPS-focal, saline-focal) groups, respectively. Because birds were housed in pairs for this experiment (Figure 1), pre- and post-treatment feeding behavior was analyzed by cage rather than by individual. To examine how baseline corticosterone concentrations and hemolytic complement activity varied in the focal birds, we ran GLMMs for each response variable where we included treatment (LPS-focal or saline-focal), time, an interaction between treatment and time, and sex as predictors. Testosterone samples were collected from males only, so sex was not included in this model. For body mass and fat score, we ran a GLMM fitted with a gaussian distribution, while the models for the remaining endpoints (baseline corticosterone, complement activity, and testosterone) were fitted with a tweedie distribution. To investigate whether differences in feeding behavior affected the gut microbiota, we also ran a separate GLMM for each alpha diversity metric (observed richness, Shannon diversity), which considered the interactions between treatment and time (day 0, day 5) as well as the average amount of grams of each diet type (fat diet, protein diet) consumed per day pre- and post- treatment. P-values from these models were adjusted for false discovery rate with a Benjamini–Hochberg correction where significance was determined as $P_{adj} < 0.05$. Due to power limitations associated with low sample sizes for focal birds that had both physiological and gut microbiota samples for days 0 and 5 (n=2-9 depending on the physiological metric), we were unable investigate interactions between physiology (complement, baseline corticosterone, testosterone), treatment,

and time for gut microbial metrics as we did in the first experiment.

Results

Experiment 1 – Diet manipulation

The average amount of food that birds consumed did not vary by diet treatment ($\chi^2 = 1.75$, $P = 0.416$). Body mass decreased over the course of the experiment (day: $\chi^2 = 49.49$, $P < 0.0001$), but did not vary by treatment (Figure S1; $\chi^2 = 0.36$, $P = 0.836$) or the interaction between treatment and time ($\chi^2 = 0.38$,$P = 0.829$). We observed a non-significant trend where fat score also decreased over the course of the experiment (Figure S2; day: $\chi^2 = 3.61$, $P = 0.058$). Fat score did not vary by treatment ($\chi^2 = 0.47$, $P = 0.792$) or the interaction between treatment and time ($\chi^2 = 1.29P = 0.526$). Corticosterone concentrations significantly increased after 30 min, being higher in stress-induced samples (Figure S3; sample type: $\chi^2 = 150.99$, $P < 0.0001$), but were not affected by diet treatment ($P = 0.124$) or time sampled ($P = 0.060$). Complement activity was also unaffected by treatment, day, and the interaction between treatment and day (Figure S4; all P

Bacterial richness and bacterial diversity of the gut microbiota did not vary across diet treatments, over time, or in response to the interaction between treatment and time (Table S1; Figure S5; allP_{adj}). However, when considering the interactive effects of each physiological metric (Table S2; complement, baseline corticosterone, stress-induced corticosterone) with diet treatment and time (day 0, day 8), we found that complement activity and changes in complement activity over time significantly affected microbial richness and Shannon diversity. Higher complement activity levels in blood plasma were associated with greater richness (Figure 2; complement:$P_{\text{adj}} = 0.0280$) and higher Shannon diversity (Figure 2; complement: $P_{\text{adj}} = 0.0492$). This relationship was strongest prior to treatment (day 0), and appeared to be disrupted over the course of the experiment (complement*day; Richness: $P_{\text{adj}} = 0.0023$, Shannon diversity:$P_{\text{adj}} = 0.0118$). Shannon diversity was also influenced by a significant three way interaction between treatment, time, and stress-induced corticosterone concentrations (Figure 3; treatment*day*SIcort: $P_{\text{adj}} = 0.0276$). Higher stress-induced plasma corticosterone concentrations were generally associated with higher Shannon diversity, however this positive association was not apparent in birds on a protein diet prior to treatment (day 0) or birds fed a high fat diet for 8 days. We did not find a significant effect of diet, time, or the interaction between diet and time on any beta diversity metrics (Table S3, Figure S6; allP_{adj} d that birds on the high protein diet had a significant reduction in the abundance of the phylum Campylobacterota (Figure 4; $\chi 2 = 6.75$, $P_{\text{adj}} = 0.0469$) when comparing samples collected on day 0 (pre-treatment) to samples collected on day 8 (post-treatment). Conversely, there were no significant changes in relative abundance at the phylum level in the high fat (all P_{adj} [?] 0.870) or equal ratio (allP_{adj} [?] 0.603) diet treatments when comparing pre- and post- treatment samples (Table 1). We did not detect any significant changes in any of the three diet treatment groups at the genus level when comparing pre- and post- treatment samples (Table 1, Figure S7; allP_{adj} [?] 0.143).

Experiment 2 – Diet selection

Stimulus Birds

Birds given an LPS immune challenge significantly reduced the total grams of food they consumed post-injection (Figure 5, day*treatment: $\chi 2 = 7.22$, $P = 0.007$). This reduction in overall food intake was also macronutrient specific. Specifically, LPS-challenged birds did not alter their consumption of the high lipid diet (Figure 5A, all P[?] 0.557), but significantly decreased consumption of the high protein diet (Figure 5B, day*treatment: $\chi 2 = 5.43$, $P = 0.020$). Body mass also differed over time in the two treatments (Figure S8, day*treatment: $\chi 2 = 10.37$, $P = 0.001$), where LPS-injected birds lost weight in the days following the immune challenge. Fat score generally increased over time in saline-injected birds but was more variable over time in LPS-injected individuals (Figure S9, day*treatment: $\chi 2 = 4.09$, P= 0.043). Body mass and fat score did not vary between male and female finches (sex: all P [?] 0.263).

Birds given an LPS immune challenge had lower bacterial richness (Figure S10; treatment: $\chi 2 = 10.048$, P

$_{adj} = 0.008$) and bacterial diversity (Figure S10; treatment: $\chi 2 = 11.446, P_{adj} = 0.004$) than saline-injected birds, however this difference was already apparent prior to experimental treatment (Table S4). Neither the amount of fat diet consumed or protein diet consumed influenced observed richness or Shannon diversity (all P[?] 0.218). There was no effect of treatment, sampling timepoint, or interaction between treatment and time on the Bray-Curtis dissimilarity index, community membership (unweighted UniFrac), or community composition (weighted UniFrac) (Table S5, Figure S11: PERMANOVA, allP_{adj} [?] 0.657). When examining how treatment affected the abundance of different bacterial taxa, birds in the control group (saline) did not have any significant changes in bacterial relative abundances at the phylum (Figure S12) or genus (Figure S13) level between day 0 and day 5 samples (Table S6: allP_{adj} [?] 0.381). Additionally, there were no significant effects of an immune challenge with LPS on bacterial abundances at the phylum (Figure S12) or genus (Figure S13) level when comparing pre-treatment (day 0) samples to post-treatment (day 5) samples (Table S6: all P_{adj} [?] 0.775).

Focal Birds

LPS-focal and saline-focal birds did not differ in diet intake in terms of quantity of food consumed or macronutrient composition of food consumed (Figure 5, all P [?] 0.480). Regardless of treatment group, focal bird body mass decreased over the course of the experiment (Figure S14, day: $\chi 2 = 100.55$, $P < 0.0001$), however, furcular fat scores were not influenced by treatment or time (Figure S15, all P [?] 0.126). Body mass and fat score did not vary between male and female finches (sex: all P [?] 0.282). Focal bird physiology was also not influenced by a cue of infection. Specifically, complement activity, baseline corticosterone concentrations, and testosterone concentrations did not vary by treatment or an interaction between treatment and time (Figure 6, all P [?] 0.180). Additionally, complement activity and baseline plasma corticosterone concentrations did not vary between male and female finches (sex: all$P$ [?] 0.279). Regardless of treatment, testosterone concentrations (collected from males only) decreased over time (day: $\chi 2 = 13.78 P = 0.0002$).

LPS-focal birds had lower bacterial richness (Figure S16; treatment: $\chi 2 = 7.381$, $P_{adj} = 0.018$) than saline-focal birds, however this difference was already apparent prior to experimental treatment (Table S7). Shannon diversity did not vary significantly between treatments ($\chi 2 = 2.938$, $P_{adj} = 0.216$). Regardless of treatment in the focal birds, higher fat consumption was associated with lower observed richness (Figure 7; $\chi 2 = 7.242, P_{adj} = 0.018$) and although non-significant, there was a similar trend for lower Shannon diversity (Figure 7; $\chi 2 = 6.356, P_{adj} = 0.059$). There was no significant effect of treatment (LPS-focal, saline-focal), sampling timepoint (day 0, day 5), or the interaction between treatment and time on any metrics of beta diversity in focal birds (Table S8, Figure S17; PERMANOVA, allP_{adj} [?] 0.464). Abundance of bacterial taxa at the phylum (Figure S18) or genus level (Figure S19) did not change over time in the control (saline-focal) treatment group (Table S9: allP_{adj} [?] 0.455) or in birds exposed to a social cue of infection (LPS-focal) when comparing pre- (day 0) and post-treatment (day 5) samples (Table S9: all P_{adj} [?] 0.838).

Discussion

We investigated how dietary macronutrients affected host physiology and gut microbiota and characterized how feeding behavior and macronutrient selection were influenced by both immune activation and perceived infection risk. We found that shifts in dietary macronutrients can affect the abundance of some bacterial taxa and potentially alter relationships between gut microbial communities and immune and stress physiology. Additionally, a direct immune threat but not a perceived risk of infection altered the feeding behavior of birds through reducing the consumption of specific macronutrients, which could have implications for responding to and recovering from infection. Taken together these results suggest that both diet-induced shifts in the microbiome and infection-induced shifts in macronutrient selection occur in songbirds, and interactions between macronutrients, gut microbiota, and feeding behavior likely have important consequences for host health.

Effects of diet macronutrient content on avian feeding behavior, physiology, and gut microbiota.

Diet macronutrient content did not affect feeding behavior, with birds consuming similar amounts of food regardless of diet treatment. Surprisingly, we did not find an effect of diet on complement activity or baseline or stress-induced corticosterone concentrations. Contrary to our results, several studies have found a relationship between diet and various metrics of the complement pathway. In fish, alternative complement activity (ACH50) is higher when fish were fed diets with 8% or higher lipid levels when compared with fish fed a lipid-free control diet (Lin and Shiau 2003). Similarly, in mice, a high-fat diet induces complement activation and proinflammatory cytokine production (Doerner et al. 2016). Previous work investigating the effects of dietary macronutrients on stress physiology found that kittiwake chicks fed a low-lipid diet had higher baseline and stress-induced concentrations of corticosterone (Kitaysky et al. 2001). Conversely, but similar to our results, corticosterone concentrations were unaffected by dietary supplementation with anthropogenic items like bread in captive white ibis (Cummings et al. 2020). It is possible that more dramatic shifts in macronutrient ratios between diet treatments or having longer time frames on the diets is required to detect large effects on host immune and endocrine physiology.

While we did not find a direct effect of diet treatment on complement activity or corticosterone concentrations, the gut microbiome is capable of both influencing and responding to these physiological endpoints (Williams et al. 2020), which can occur independently of changes in diet, or possibly be indirectly influenced by diet through another metric that was not measured in the present study. For example, higher glucocorticoid levels are associated with lower bacterial diversity in squirrels (Petrullo et al. 2022) and gulls (Noguera et al. 2018). Complement activity has also been linked to changes in gut microbiota. Depletion of gut commensal bacterial inhibits complement activation in mice (Yoshiya et al. 2011), and in tilapia fish complement C3 is positively correlated with the Shannon's and Simpson's index of gut microbiota (Zhu et al. 2020). In our study, complement activity was positively correlated with observed richness and Shannon diversity on day 0, but this relationship was disrupted on day 8. Baseline corticosterone concentrations did not significantly affect microbial richness or diversity, while stress-induced concentrations were generally associated with higher Shannon diversity. However, the positive association between stress-induced corticosterone and Shannon diversity was not apparent in birds on a protein diet prior to treatment (day 0) or birds fed a high fat diet post-treatment (day 8). For both complement and stress-induced corticosterone, birds in the high fat diet treatment shifted from having a positive association between physiology and gut microbiota alpha diversity metrics at day 0 to a negative association between metrics following 8 days on a high fat diet. This suggests that diet macronutrient ratios can influence the relationship between gut microbial communities and physiology and that the fat content of diet might be important in altering these relationships.

While the diet treatments in experiment 1 (diet manipulation) did not have major effects on overall gut microbiota composition, we found that protein content can shift the abundance of particular taxa. While community-level changes in gut microbiota can have important implications for host health, smaller changes in specific taxa can also affect host physiological processes (Hooper et al. 2012, Round and Mazmanian 2009). Specifically, we found that birds fed a high protein diet for 8 days had lower proportions of the phylum Campylobacterota. Previous work in mice found that individuals fed a high fat diet have a higher relative abundance of the genus *Campylobacter* (a member of the Phylum Campylobacterota), and Campylobacter abundance was positively correlated with serum lipid levels (Mu et al. 2020). The association between a high fat diet and members of Campylobacterota might explain why we observed a decrease in the abundance of Campylobacterota in birds on the high protein diet but not birds fed the high fat diet or diet with equal ratios of fat and protein.

Macronutrient-specific feeding behaviors after an immune threat.

The goals of the second experiment (diet selection) were to: 1) investigate how an immune threat influences feeding behavior and macronutrient selection, 2) explore if social cues of disease can alter feeding behavior and immune and endocrine responses relevant to disease susceptibility, and 3) determine whether an immune threat and behavioral shifts in feeding alter the gut microbiome. Based on research in invertebrates, we predicted that birds given an immune challenge would either increase protein intake or maintain consistent levels of protein consumption while reducing lipid intake (Adamo 2008, Adamo et al. 2010, Povey et

al. 2013, Cotter et al. 2010). Conversely, we found that birds given an immune challenge with LPS engaged in macronutrient-specific sickness-induced anorexia by maintaining consumption of the high lipid diet while significantly reducing consumption of the high protein diet. However, we did not detect shifts in the microbiome that were driven by LPS exposure. Consistent with sickness-induced anorexia, immune challenged individuals lost weight but did not have any detectable changes in furcular fat stores. Caloric restriction during illness can improve host health and recovery in some cases (Cheng et al. 2017; Wang et al. 2016); thus, the observed reduction in caloric intake in LPS-challenged birds may be an adaptive response to an immune threat.

The finding that sickness-induced anorexia in LPS-injected birds was driven by a macronutrient-specific reduction in protein intake is interesting given the apparent importance of protein to immune function and responding to and surviving infection (Lee et al. 2006, Povey et al. 2013). Prior research in insects suggests that individuals should benefit from reducing lipid intake during infection, however we saw no change in lipid intake in birds given an immune challenge. For example, infected caterpillars assigned to a high-lipid diet have higher mortality rates than infected individuals feeding on water or sucrose (Adamo et al. 2007). Further, research in crickets identified a tradeoff between immunity and lipid-transport, suggesting that reducing lipid consumption can maximize immune responses (Adamo et al. 2010). It is unknown whether a tradeoff between lipid-transport and immunity exists in vertebrates (Demas and Nelson 2012). However, our finding that LPS-challenged birds reduce protein consumption but not lipid consumption challenges this idea and warrants further investigation in avian and other vertebrate systems.

Although studies examining the relationship between infection and dietary macronutrient preference are uncommon in vertebrates, one study in mammals found a similar reduction in protein intake following LPS immune-challenge. Specifically, rats injected with LPS voluntarily decreased protein intake while lipid intake remained unchanged, however this study also observed a significant increase in carbohydrate consumption in LPS-treated individuals (Aubert et al. 1995). Coupled with our finding that LPS-challenged birds selectively reduce protein but not lipid intake, this suggests that reduced protein consumption may be a common behavioral response to an immune threat in vertebrate species, although the function of this shift in macronutrient preference is still unclear. It is possible that a reduction in protein consumption occurs in response to an immune challenge because protein is more likely to contain iron than other macronutrients. Although iron is essential to host immune function, it is also used by pathogens. Thus, limiting iron intake could interfere with pathogen growth and help limit infection (Soyano and Gómez 1999, Kluger and Rothernberg 1979). Further work is needed to determine if reduced protein consumption during infection is adaptive for hosts in terms of responding to and overcoming infection, and whether these effects are mediated through shifts in micronutrient intake such as iron.

Heightened risk of infection does not alter macronutrient-specific feeding behavior.

Because shifts in behavior and pathology associated with infection could act as social cues of heightened infection risk to uninfected conspecifics, we also examined if perceived risk of infection (seeing sick conspecifics) could alter feeding behavior and macronutrient selection. Separate lines of evidence indicate that social cues can influence feeding behavior and alter physiological responses relevant to immune function (Cornelius et al. 2018, Schaller et al. 2010, Stevenson et al. 2011, 2012; Love et al. 2021, Gormally and Lopes 2023). Thus, we predicted that birds exposed to a heightened risk of infection would have physiological responses relevant to responding to an immune threat and shift feeding behavior in a way that maximizes immunity. Contrary to our predictions, we found no evidence for shifts in feeding behavior, macronutrient intake, gut microbiota, or physiological responses in birds exposed to a cue of heightened infection risk. It is possible that the cue of infection was not sufficient to stimulate physiological changes or alter the feeding behavior of focal birds because we used a simulated infection (injection with LPS) in this study. The behavioral effects of LPS typically only last between 2-4 days following injection (Sköld-Chiriac et al. 2014, Love et al. 2023), thus the cue of infection elicited by LPS-injection is temporally limited. It is also possible that we failed to capture the relevant timing of shifts in physiology following exposure to sick conspecifics. Recent work in Japanese quail (*Coturnix japonica*) found that females exposed to LPS-challenged males for 3 hours had

an upregulation of immune genes in the blood, suggesting that physiological responses to LPS-challenged individuals might happen rapidly post-cue (Gormally and Lopes 2023). Additionally, it might be less costly for organisms to employ behavioral defenses (such as avoidance behavior) rather than physiological immune defenses in response to an immune threat in some settings. For example, we previously documented that zebra finches housed across from LPS-challenged conspecifics reduce flight activity and increase preening behavior (Love et al. 2023), and there might be trade-offs in how birds invest in behavioral versus physiological immune defenses (Zylberberg et al. 2012). Future work should explore whether an immune challenge or infection that elicits stronger and longer-lasting behavioral and physiological signs of disease is capable of influencing feeding behavior, gut microbiota, and physiological responses in healthy individuals, as this could have implications for host disease susceptibility and disease transmission potential.

Although we did not detect an effect of perceived infection risk on any of the physiological parameters examined in this study, we did observe changes in testosterone concentrations in male zebra finches over the course of the experiment. Specifically, we observed a decrease in testosterone levels in males 3 days after the injection of stimulus birds, and this decrease occurred in both LPS-focal and saline-focal males. Physiological stress is known to have inhibitory effects on the release of testosterone (Da Silva 1999), so it is possible that the observed decrease in testosterone concentrations in males is related to handling stress associated with repeated blood sampling. However, we did not observe a corresponding increase in circulating plasma corticosterone in birds over the course of the experiment that might be indicative of acute or chronic stress.

While a heightened risk of infection did not influence feeding behavior or gut microbiota composition, higher fat consumption was associated with lower observed richness and lower Shannon diversity across both treatment groups (LPS-focal, saline-focal). Consistent with this result, high fat diets in mice result in lower gut microbiota richness and diversity (Zhang et al. 2012). Similarly, house sparrows experimentally fed a high-fat urban diet have decreased gut microbiota diversity (Teyssier et al. 2020). Conversely, protein consumption by focal birds was unrelated to microbial richness and diversity. Interestingly, neither fat nor protein consumption predicted richness or Shannon diversity in the infected birds (LPS-injected, saline-injected). It is unclear why fat consumption predicted microbial alpha diversity in focal birds but not injected birds, however larger sample sizes might be required to confirm this trend and determine the biological underpinnings of these differences.

The present study extends our understanding of how immune activation can influence feeding behavior and diet selection in vertebrates. We did not detect any shifts in feeding behavior, nor any immune or endocrine changes in response to social cues of infection in the present study. However, we did detect macronutrient-specific illness-induced anorexia in LPS-challenged birds, where birds decreased protein but not lipid intake. Shifts in feeding behavior in sick individuals can affect both host and parasite fitness and ultimately influence disease severity, which is inherently related to disease transmission (Hite and Cressler 2019, Povey et al. 2013). Models indicate that sickness-induced anorexia, like the reduction in caloric intake observed in LPS-birds in the present study, is capable of enhancing or diminishing disease severity depending on dietary context (Hite and Cressler 2019), suggesting that the interactions between infection, resource availability, and host macronutrient selection can have important consequences for disease dynamics and deserve further attention.

Conclusions

We found that diet macronutrient content can alter the abundance of some bacterial taxa in the gut and potentially alter the relationships between gut microbial communities and physiological responses important for responding to infection. Additionally, we found that immune-challenged birds alter feeding behavior by selectively reducing protein but not fat intake, however this change in feeding behavior did not correspond with detectable differences in gut microbiota communities. Contrary to our predictions, heightened infection risk (being housed near immune-challenged conspecifics) did not alter feeding behavior, gut microbiota, or immune and endocrine physiology. However, regardless of treatment, higher fat consumption in these individuals was associated with lower gut microbial richness and diversity. Our findings carry implications for host health and epidemiology, as diet-induced shifts in the microbiome and physiology could shape disease

transmission dynamics through nutritionally driven shifts in host-pathogen interactions. Future work should explore if reduced protein consumption during infection is adaptive for hosts in terms of responding to and overcoming infection, as this could have implications for disease transmission.

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Data accessibility: Supporting information has been made available online. Data are available at

FigShare (doi: available upon acceptance) and sequences have been uploaded to GenBank (BioProject accession number: available upon acceptance).

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Conflict of interest : The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1. Experimental design and timelines for the diet manipulation and diet selection studies. In the first experiment, zebra finches were given one of three diet treatments that varied in protein and lipid content and blood and fecal samples were collected to assess how diet influenced complement activity, baseline and stress-induced corticosterone concentrations, and the gut microbiome. In the second experiment, we simulated an infection in established zebra finch pairs using the bacterial endotoxin lipopolysaccharide (LPS), and quantified feeding behavior in immune challenged and control individuals, as well as birds housed near either a control pair (no immune threat), or birds housed near a pair given an immune challenge with LPS (social cue of heightened infection risk). To investigate how a direct and perceived immune threat shape macronutrient feeding preferences, we created two isocaloric diets with varied lipid and protein ratios and quantified how much of each diet birds consumed. Fecal samples were collected from all individuals to assess changes in gut microbiota and blood samples were collected from focal individuals (LPS-focal, Saline-focal) to quantify changes in complement activity, corticosterone, and testosterone (males only).

Figure 2. Effect of diet treatment (high fat, equal ratio, high protein), time (day 0, day 8), and hemolytic complement activity (CH50) on (a) observed richness and (b) Shannon diversity index of zebra finch microbiotas in zebra finches fed diets differing in macronutrient content (experiment 1).

Figure 3. Effect of diet treatment (high fat, equal ratio, high protein), time (day 0, day 8), and stress-induced corticosterone concentrations (ng/ml) on (a) observed richness and (b) Shannon diversity index of zebra finch microbiotas in zebra finches fed diets differing in macronutrient content (experiment 1).

Figure 4. (a) Proportional abundance of bacterial phyla across diet treatment groups (high fat, equal ratio, high protein) and time (day 0, day 8) in zebra finches fed diets differing in macronutrient content (experiment 1). Each bar represents a sample from an individual bird. Phyla with less than 1% relative abundance are collapsed into the category < 1%. (b) Relative abundance of the four most common phyla where individual points represent the relative abundance of each phylum from an individual bird. Black circles denote the mean (+-SE) relative abundances across treatments.

Figure 5. Grams of high lipid and high protein diet consumed per day in zebra finches challenged with lipopolysaccharide (LPS) or saline (a, b) and in focal birds that were either housed next to sick-conspecifics (LPS-focal) or healthy conspecifics (saline-focal) (c, d). Data are reported as means +- standard error.

Figure 6. Physiological responses of focal birds housed in view of conspecifics injected with saline or LPS (experiment 2). (a) Hemolytic complement activity (CH50), (b) plasma corticosterone concentrations, and (c) plasma testosterone concentrations in zebra finches that were housed next to healthy (no cue of infection, saline-focal) or sick-conspecifics (cue of infection, LPS-focal) in experiment 2. All data are reported as means +- standard error.

Figure 7. Alpha diversity metrics are negatively associated with consumption of the high fat diet in focal birds (LPS-focal, saline-focal). Panels depict (a) the number of observed ASVs (observed richness) and (b) Shannon diversity. Points represent individual birds. Pre-treatment (day 0) samples are indicated by circles and post-treatment samples (day 5) are represented by triangles.

Tables

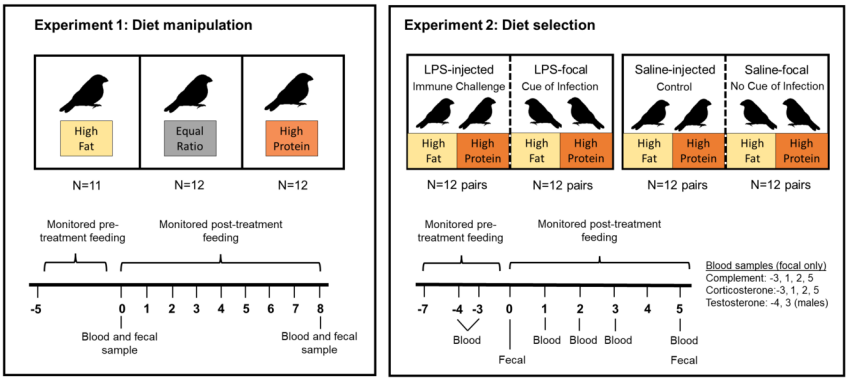
Table 1. Kruskal-Wallis chi-square ($\chi 2$) test statistics and p-values for comparisons of bacterial taxa over time (day 0, day 8) in each diet treatment group (high fat, equal ratio, high protein) in the diet manipulation study (experiment 1). *P* -values were adjusted for false discovery rate with a Benjamini–Hochberg correction where significance was determined as $P_{adj} < 0.05$. Significant differences between groups are shown in bold.

Taxonomic Group	**High fat diet**	**High fat diet**	**Equal ratio diet**	**Equal ratio diet**	**High protein diet**	**High protein diet**
	χ^2	P_{adj}	χ^2	P_{adj}	χ^2	P_{adj}
Phylum						
Actinobacteriota	0.481	0.870	0.336	0.603	0.875	0.583
Campylobacterota	0.087	0.870	0.653	0.603	6.750	**0.047***
Firmicutes	0.312	0.870	0.270	0.603	4.563	0.082
Proteobacteria	0.027	0.870	0.403	0.603	0.441	0.633
Other (< 1%)	0.186	0.870	1.784	0.603	0.000	1.000
Genus						
Achromobacter	0.355	0.871	0.863	0.907	0.110	0.772
Acinetobacter	1.812	0.712	0.225	0.907	0.084	0.772
Campylobacter	0.010	0.922	1.203	0.907	3.413	0.259
Helicobacter	0.092	0.871	0.170	0.907	0.861	0.471
Ligilactobacillus	1.232	0.712	0.427	0.907	1.270	0.416
Pseudomonas	2.710	0.712	0.056	0.908	2.310	0.343
Weissella	0.182	0.871	0.013	0.908	5.606	0.143

Other (< 1%)	0.570	0.871	0.270	0.907	1.268	0.416

Figures

Fig 1.



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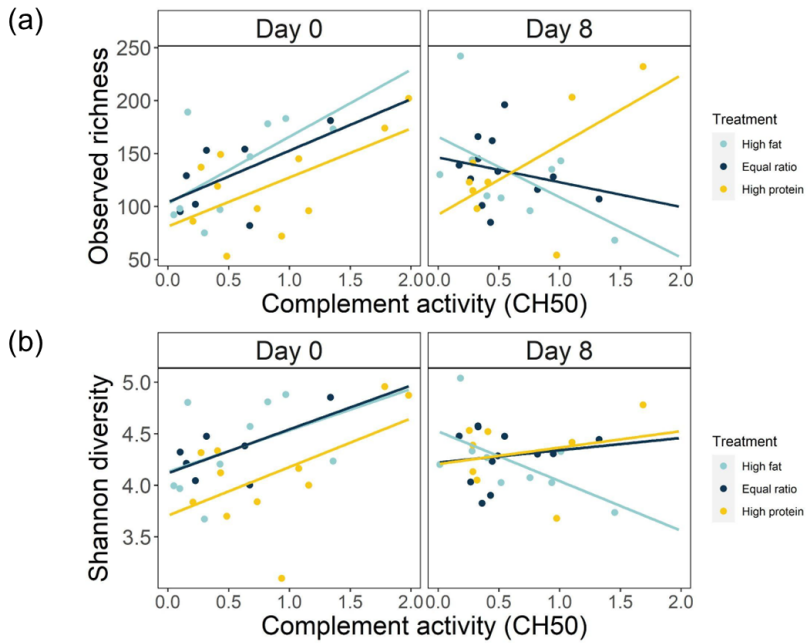

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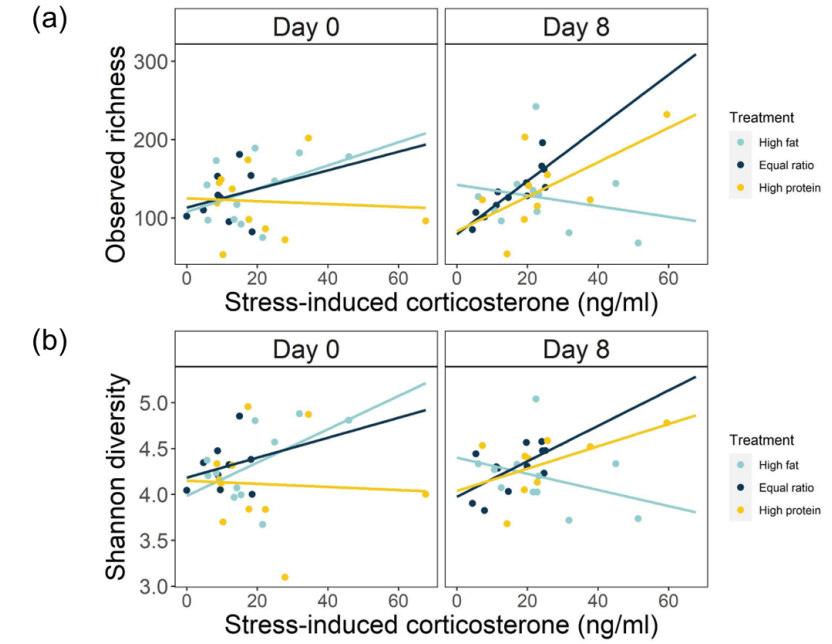


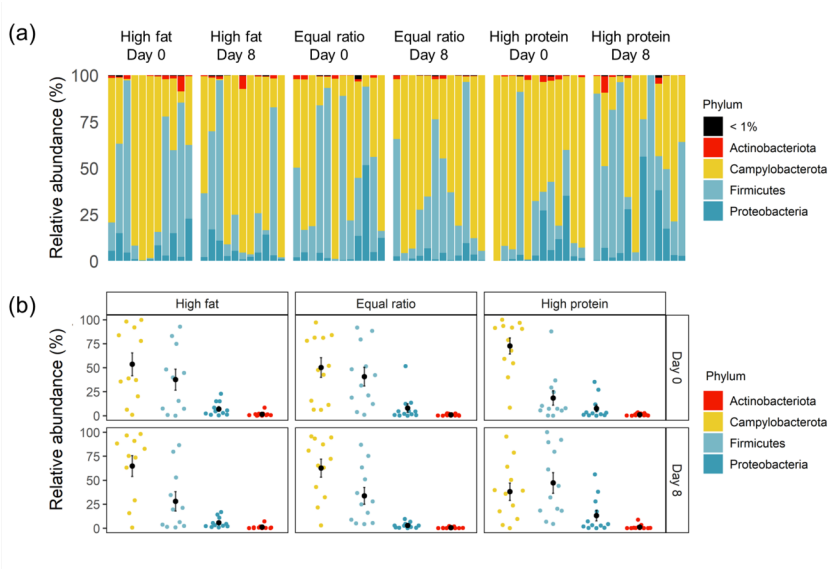
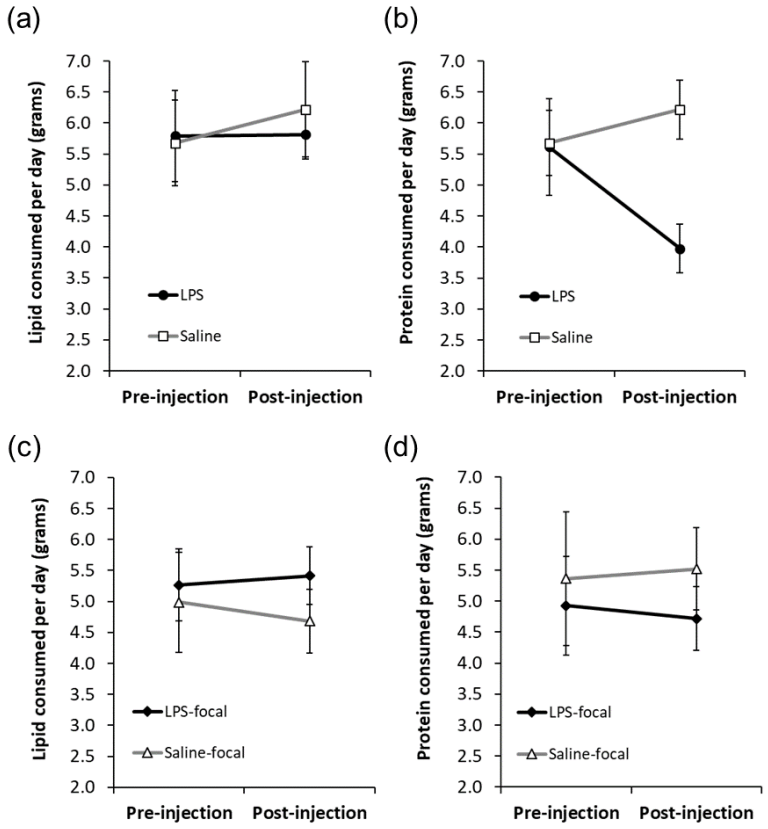


Fig 4.

Fig 5.



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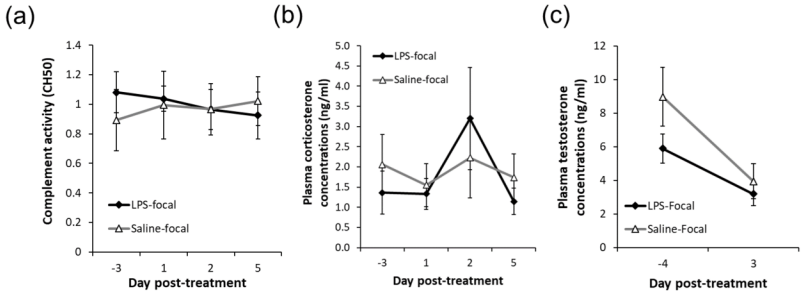

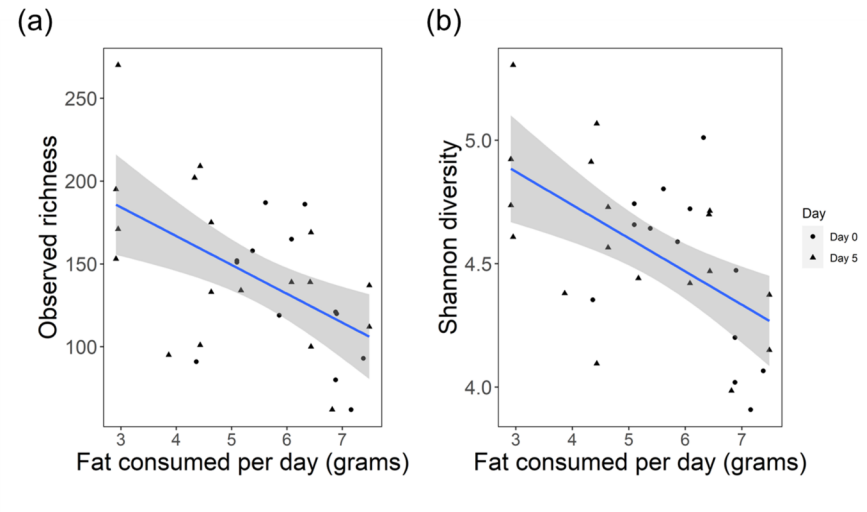

Fig 7.