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Effect of dietary macronutrients and immune challenge on gut microbiota, physiology and feeding behaviour in zebra finches

Ashley C. Love[1,2,3] | Victoria Tabb[2] | Noha H. Youssef[4] | Shawn M. Wilder[2] | Sarah E. DuRant[3]

[1]Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut, USA

[2]Department of Integrative Biology, Oklahoma State University, Stillwater, Oklahoma, USA

[3]Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas, USA

[4]Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, Oklahoma, USA

Correspondence
Ashley C. Love, Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269, USA.
Email: ashley.love@uconn.edu

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Abstract

Macronutrients 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 dietary macronutrients affect physiology and gut microbiota and investigate whether feeding behaviour is influenced by an immune threat, we conducted two experiments. First, we determined whether zebra finches (*Taeniopygia guttata*) exhibit shifts in physiology and gut microbiota when fed diets differing in macronutrient ratios. We found the type and amount of diet consumed affected gut microbiota alpha diversity, where microbial richness and Shannon diversity increased with caloric intake in birds fed a high-fat diet and decreased with caloric intake in birds fed a high protein diet. Diet macronutrient content did not affect physiological metrics, but lower caloric intake was associated with higher complement activity. In our second experiment, we simulated an infection in birds using the bacterial endotoxin lipopolysaccharide (LPS) and quantified feeding behaviour 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 behaviour. 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 behaviour, physiology or gut microbiota. Our findings carry implications for host health, as sickness-induced anorexia and diet-induced shifts in the microbiome could shape host–pathogen interactions.

KEYWORDS
diet, gut microbiota, immunity, macronutrients, social cues

1 | INTRODUCTION

Nutrition is critical to the immune system and can influence how hosts respond to infection (Amar et al., 2007; Cunningham-Rundles et al., 2005; Ponton et al., 2011). Among ecological studies, the effect of resource availability on immune processes and disease outcomes has received significant attention (Becker et al., 2018; Moyers et al., 2018; Strandin et al., 2018), but the quality of those resources and their nutritional make-up (e.g., macronutrient content) are also important (Cotter et al., 2011; Povey et al., 2013). Macronutrients such as 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, such as host immunity. For example, work in a range of taxa shows that restriction of dietary protein can limit immune activity (Cheema et al., 2003; Lee et al., 2006; Lochmiller et al., 1993; Povey et al., 2009; Sakamoto et al., 1981). Similarly, high lipid diets can alter immunity and increase mortality rates during infection (Adamo et al., 2008, 2010; Strandberg et al., 2009). 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.

One way that dietary macronutrients can shape immune function is through dietary-induced shifts in immune-mediating hormone concentrations. 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 (Da Silva, 1999; Dhabhar, 2000; Roberts et al., 2007). Dietary nutrients can also impact other hormones known to influence immune function, such as testosterone (Da Silva, 1999; Roberts et al., 2007). For example, humans consuming higher levels of dietary fat have higher baseline testosterone concentrations (Volek et al., 1997), and higher testosterone concentrations can suppress immunity in mammals and birds (Da Silva, 1999; Duffy et al., 2000).

Shifts in diet can also 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; Knutie et al., 2017; Zheng et al., 2020). Host diet plays an important role in shaping the composition and diversity of gut microbial communities (Bodawatta et al., 2021; Pan & Yu, 2014; Singh et al., 2017) and diet-induced shifts in gut microbiota can alter host immune responses (Ingala et al., 2019; 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 physiological responses to infection through shifts in feeding behaviour 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 behaviour interact to shape host responses related to infection.

Since dietary nutrients can affect multiple aspects of host health, animals might be able to adjust feeding behaviour to support physiological responses that help resist or survive a given infection. For example, 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 & 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 (Adamo et al., 2007; Adelman & Martin, 2009; Kyriazakis et al., 1998). 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' behaviours and have been documented in several taxa (Huffman & Seifu, 1989; Hutchings et al., 2003; Povey et al., 2013). Thus, animals can shift feeding behaviours and selectively feed on foods with desirable macronutrient composition in response to an immune threat.

Infection-induced shifts in feeding and activity can be detected by conspecifics, and viewing a sick neighbour can result in healthy individuals altering their feeding behaviour 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 behavioural and physiological changes can occur in response to public information (Cornelius, 2022; Cornelius et al., 2018; Love et al., 2021; Schaller et al., 2010; Stevenson et al., 2011, 2012). 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 neighbours 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 (Chandra, 1996; Lochmiller & Deerenberg, 2000; Murray et al., 1998), prophylactic behaviours involving macronutrient selection in response to cues of disease could help prepare organisms for an impending immune threat.

To date, most studies investigating the effect of dietary macronutrients on immunity and infection outcomes have been conducted in laboratory rodents, domestic livestock, humans and insects (Cheema et al., 2003; Ponton et al., 2011; Sakamoto et al., 1981). However, whether macronutrients have similar effects on immunity and responses to infection outside these taxa, and whether these effects might be mediated by shifts in gut microbiota, is unclear.

Further, the effects of dietary macronutrients on immune function, hormonal responses and gut microbiota have rarely been examined simultaneously in the same study, and to our knowledge, no previous studies have investigated whether both a direct and perceived immune threat alters macronutrient-specific feeding preferences, gut microbiota and physiological responses. Thus, 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 behaviour 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 behaviour alter the gut microbiome and physiological processes relevant to disease susceptibility and pathogen transmission. Zebra finches (*Taeniopygia guttata*) are an ideal system for investigating the complex interactions between diet, physiology, gut microbiota and feeding behaviour due to their extensive history as an effective model for laboratory research on physiology and behaviour. Zebra finches are also ideal for exploring whether cues indicative of infection risk can alter physiology through shifts in feeding behaviour because prior work has demonstrated that these birds can detect and behaviorally respond to cues from sick conspecifics (Love et al., 2023). We explored these relationships in zebra finches 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 behaviour (caloric intake and diet macronutrient selection). Additionally, because social information from conspecifics regarding food (Cornelius, 2022; Cornelius et al., 2018) and disease risk (Love et al., 2021; Schaller et al., 2010; Stevenson et al., 2011, 2012) can alter physiology, we also investigated whether perceived risk of infection (seeing immune-challenged conspecifics) alters physiological responses pertinent to immune function and feeding behaviour, specifically, complement activity, and corticosterone and testosterone blood plasma concentrations. We predicted that birds fed high fat diets would have lower corticosterone levels, lower complement activity and a disrupted gut microbiota. We also predicted that birds given an immune challenge and birds with a social cue of heightened infection risk would increase protein consumption, as high protein diets are typically associated with increased immune capabilities and higher survival during infection (Povey et al., 2013; Sakamoto et al., 1981). 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 behaviour and subsequent changes in the gut microbiome. Since corticosterone can be immunostimulatory in response to acute stress (Dhabhar, 2000), we predicted that perceived infection risk would increase both corticosterone and complement activity and subsequently decrease testosterone concentrations. 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).

2 | MATERIALS AND METHODS

2.1 | Experiment 1—Timeline for diet manipulation experiment

In the first experiment, we investigated how diet influenced complement activity, baseline and stress-induced corticosterone concentrations, and the gut microbiome. Adult zebra finches were given one of three diet treatments that varied in protein and lipid content. Birds were kept on a 14 L: 10 D light cycle and individually housed in 24″ × 16″ × 16″ cages. Each cage had two perches, a water dish and one food dish in which birds were fed ad libitum. Prior to the experiment, all birds were fed the same diet, which consisted entirely of millet. Experimental 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). Diet percentages were chosen based on the nutritional composition of standard seed mixes used for captive songbirds (Harper et al., 1998). 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 8 days after the manipulation diets were implemented. Body mass and fat score data were recorded for each bird on all sampling days. Body mass was recorded by weighing birds to the nearest 0.01 g on a scale balance, and changes in body fat were assessed by scoring subcutaneous fat deposits in the furcular cavity on a scale of 0.0 (no fat visible in cavity) to 3.0 (gross bulging fat deposit). All blood samples were collected between 08:30 and 09:30 CDT to avoid differences in physiological end points due to variation in daily rhythms. To ensure that blood samples could be collected within 3 min of entering the animal room, we staggered when the experiment started so that there were three separate groups with 11–12 birds per group. Treatments were evenly distributed across the three sampling groups. Within 3 min of entering the room, a baseline blood sample of 120 μL was collected from the wing vein of each individual to be used to determine baseline concentration of plasma corticosterone and hemolytic

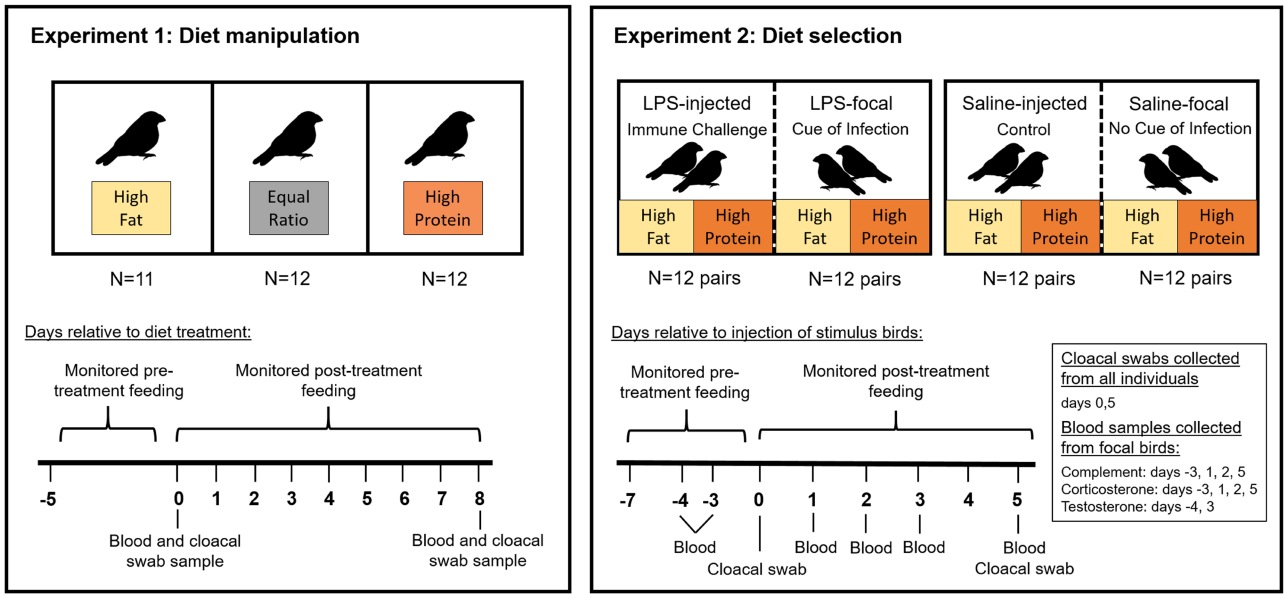

FIGURE 1 Experimental design and timelines for the diet manipulation and diet selection studies. In the first experiment, zebra finches were given one of three diet treatments that varied in protein and lipid content and blood and cloacal swab samples were collected to assess how diet influenced complement 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 behaviour in immune challenged and control individuals, as well as birds housed near either a control pair (Saline-focal: no immune threat), or birds housed near a pair given an immune challenge with LPS (LPS-focal: 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. Cloacal swab 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). [Colour figure can be viewed at wileyonlinelibrary.com]

complement activity. To collect cloacal swab samples, we carefully inserted and rotated sterile swabs in 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 min to separate blood plasma from erythrocytes, and plasma samples were stored at −20°C.

2.2 | Experiment 2—Timeline for diet selection experiment

To investigate whether birds alter feeding behaviour 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 behaviour 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). Zebra finch pairs, rather than individuals, were used in Experiment 2 because this study was run concomitantly with a study on pair-bond behaviour (Love et al., 2023) and therefore adhered to the same experimental design. 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″ × 16″ × 16″ cages that were divided down the center into two separate 12″ × 16″ × 16″ 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 was arranged identically and 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 7 days prior to experimental treatment and for 5 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 × 16 × 16 cage to ensure that birds housed in each double cage could only see one another. 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). This dose has previously been used to elicit sickness behaviours in zebra finches (Love et al., 2023; Schreier & Grindstaff, 2020). Body mass and fat score data were collected on all stimulus and focal birds 7 and 3 days prior to treatment and on Days 1, 2 and 5 post-treatment. 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, since prior work suggests that physiology is affected for at least 4–5 days following LPS immunization in zebra finches (Sköld-Chiriac et al., 2014). Cloacal swabs were placed into 300 μL of RNAlater (Invitrogen, Thermo Fisher Scientific) and frozen at −80°C.

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. 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. Blood samples for testosterone were collected on different days from the other endpoints due to constraints on how much blood can safely be collected from individuals at a given timepoint. Immediately after collection, blood samples were centrifuged and blood plasma was separated and frozen at −20°C. All research protocols were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

2.3 | Hemolytic complement activity assay

Complement activity, which plays an important role in lysing pathogen cells, has been linked to changes in dietary macronutrient content (Sakamoto et al., 1981) and changes in gut microbiota (Yoshiya et al., 2011; Zhu et al., 2020) in other vertebrate systems. To assess whether diet macronutrient content (Experiment 1) and social cues of disease (Experiment 2) influenced the complement pathway in birds, 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).

2.4 | 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-901-097). 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. The intra-assay coefficient of variation and inter-assay coefficient of variation were 5.6% and 7.6%, respectively. Cross-reactivity of the corticosterone antibody was as follows: deoxycorticosterone 21.3%, desoxycorticosterone 21.0%, progesterone 0.46%, testosterone 0.31%, tetrahydrocorticosterone 0.28%, aldosterone 0.18%, cortisol 0.046%, and <0.03%: pregnenolone, estradiol, cortisone, 11-dehydrocorticosterone acetate (Enzo Life Sciences). In the second experiment, baseline plasma corticosterone concentrations were measured using a different assay technique due to the laboratory resources available at the time the study was conducted. Samples were run in duplicate following standard radioimmunoassay techniques following the detailed methods outlined in Love et al. (2017). 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).

2.5 | 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°C until samples could be shipped for sequencing. The V4 region of the 16S rRNA gene was sequenced (primers: Hyb515F_rRNA: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTA-3′ and Hyb806R_rRNA: 3′-TAATCTWTGGGVHCATCAGGGACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-5′) at the University of Texas's Genome Sequencing and Analysis Facility (GSAF) using an Illumina MiSeq platform. Samples with less than 1000 total sequences were excluded from subsequent analyses. In Experiment 1, we had a total of 3,087,114 raw reads and 2,783,031 passed the DADA2 denoising step (90%). The average number of merged, non-chimeric reads was 29,599 ± 14,381 across all samples. For Experiment 2, we had a total of 2,941,541 raw reads and 2,676,862 passed the DADA2 denoising step (91%). The average number of merged, non-chimeric reads across all samples was 27,128 ± 3795. 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 & Holmes, 2013).

2.6 | 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 account for uneven sequencing depth across samples for alpha diversity analyses, samples were rarefied to the depth of our lowest sample (5691 for the diet manipulation experiment; 1077 for the diet selection experiment). Rarefaction reduced the number of ASVs from 985 to 923 for the diet manipulation experiment, and from 1620 to 1307 ASVs for the diet selection experiment. 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 model (GLMM) fitted with a negative binomial (nbinom1) distribution using the package *glmmTMB* (Brooks et al., 2017) with treatment, day, and the treatment × day interaction 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. 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 data set, 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 & Curtis, 1957), unweighted UniFrac (Lozupone & Kniight, 2005) 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 $p_{adj} < .05$.

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. These data stringency limited analyses to the top four most abundant phyla (Campylobaterota, Firmicutes, Proteobacteria and Actinobacteriota) and the top seven most abundant genera (*Campylobacter*, *Weissella*, *Helicobacter*, *Acinetobacter*, *Ligilactobacillus*, *Pseudomonas* and *Achromobacter*) in Experiment 1 (diet manipulation). In Experiment 2 (diet selection), these data stringency limited analyses to the five most abundant phyla (Campylobaterota, Firmicutes, Proteobacteria, Actinobacteriota, Bacteroidota) and the top 10 most abundant genera (*Campylobacter*, *Helicobacter*, *Corynebacterium*, *Ureaplasma*, *Ligilactobacillus*, *Atopobium*, *Catellicoccus*, *Gallibacterium*, *Veillonella*, *Weissella*) in injected birds (LPS, saline), and the top 13 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 nonparametric 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 $p_{adj} < .05$.

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, which is appropriate for overdispersed data containing non-integer values and zeros. 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 both the quality and quantity of diet consumed have the potential to shape gut microbiota and physiology, we also ran GLMs which considered the interactions between diet treatment and the average grams consumed per day (between Days 0 and 8) on post-treatment (Day 8) alpha diversity metrics (observed richness, Shannon diversity) and physiology metrics (complement activity, baseline corticosterone, stress-induced corticosterone).

To examine how feeding behaviour (total grams consumed and diet preference) changed over time with treatment in Experiment 2 (diet selection), we ran separate GLMMs 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 behaviour was analysed 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 behaviour 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 ratio of protein relative to the total (protein + fat) grams of 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 $p_{adj} < .05$. For all models, we used the *DHARMa* package (Hartig, 2022) to plot residuals and confirm the suitability of each model and the Anova function in the *car* package (Fox & Weisberg, 2018) to determine significance.

3 | RESULTS

3.1 | Experiment 1—Diet manipulation

The average amount of food that birds consumed did not vary by diet treatment ($\chi^2 = 1.75$, $p = .416$). Body mass decreased over the course of the experiment (day: $\chi^2 = 49.49$, $p < .0001$), but did not vary by treatment (Figure S1; $\chi^2 = 0.36$, $p = .836$) or the interaction between treatment and time ($\chi^2 = 0.38$, $p = .829$). We observed a non-significant trend where fat score decreased over the course of the experiment (Figure S2; day: $\chi^2 = 3.61$, $p = .058$). Fat score did not vary by treatment ($\chi^2 = 0.47$, $p = .792$) or the interaction between treatment and time ($\chi^2 = 1.29$, $p = .526$). Corticosterone concentrations significantly increased after 30 min, being higher in stress-induced samples (Figure S3; sample type: $\chi^2 = 150.99$, $p < .0001$), but were not affected by diet treatment ($p = .124$) or time sampled ($p = .060$). Complement activity was also unaffected by treatment, day, and the interaction between treatment and day (Figure S4; all $p \geq .375$). When considering the interactive effects of diet quality and caloric intake (grams consumed per day) on post-treatment (day 8) physiological metrics, diet macronutrient content and the interaction between diet treatment and grams consumed did not affect any physiology metrics (all $p \geq .100$). However, higher caloric intake was associated with lower complement activity (Figure 2;

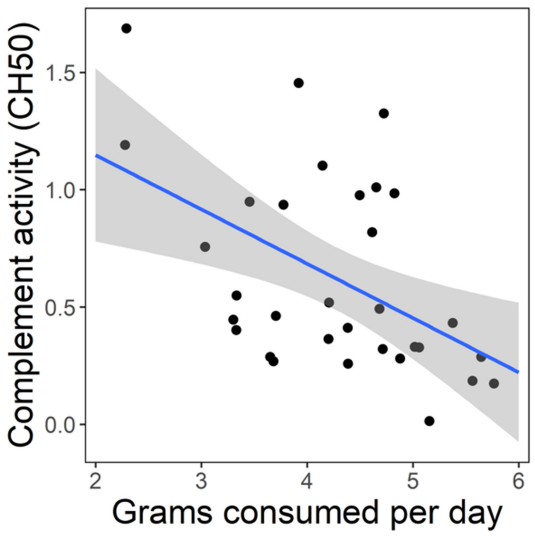

FIGURE 2 Effect of food consumption (average grams of diet consumed per day) on hemolytic complement activity (CH50) in zebra finches fed diets differing in macronutrient content for 8 days (experiment 1). The shaded area represents the 95% confidence interval. [Colour figure can be viewed at wileyonlinelibrary.com]

grams consumed: $\chi^2 = 8.45$, $p = .004$), but did not affect baseline ($\chi^2 = 0.24$, $p = .625$) or stress-induced ($\chi^2 = 0.48$, $p = .489$) corticosterone concentrations.

Bacterial richness and Shannon 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; all $p_{adj} \geq .687$). However, when considering the interactive effects of caloric intake (grams consumed per day) on post-treatment (Day 8) alpha diversity metrics, we found that diet composition and the quantity of each diet consumed significantly influenced gut microbiota alpha diversity (Table S2; diet treatment × grams consumed: ASV richness: $p_{adj} = .000002$, Shannon diversity: richness: $p_{adj} = .0004$). Specifically, elevated consumption of the high-fat diet was associated with higher microbial richness and Shannon diversity, while intake of the high-protein diet was associated with lower microbial richness and Shannon diversity (Figure 3). 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; all $p_{adj} \geq .396$). When examining the effect of diet macronutrient content on abundance of different bacterial taxa, we found that birds on the high protein diet had a significant reduction in the abundance of the phylum Campylobacterota (Figure 4; $\chi^2 = 6.75$, $p_{adj} = .047$) 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 $p_{adj} \geq .870$) or equal ratio (all $p_{adj} \geq .603$) 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; all $p_{adj} \geq .143$).

3.2 | Experiment 2—Diet selection

3.2.1 | Stimulus birds

Birds given an LPS immune challenge significantly reduced the total grams of food they consumed post-injection (Figure 5, day × treatment: $\chi^2 = 7.22$, $p = .007$). 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 $p \geq .557$), but significantly decreased consumption of the high protein diet (Figure 5b, day × treatment: $\chi^2 = 5.43$, $p = .020$). Body mass also differed over time in the two treatments (Figure S8, day × treatment: $\chi^2 = 10.37$, $p = .001$), 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: $\chi^2 = 4.09$, $p = .043$). Body mass and fat score did not vary between male and female finches (sex: all $p \geq .263$).

Birds given an LPS immune challenge had lower bacterial richness (Figure S10; treatment: $\chi^2 = 11.384$ $p_{adj} = .003$) and bacterial diversity (Figure S10; treatment: $\chi^2 = 13.808$, $p_{adj} = .002$) than saline-injected birds; however, this difference was already apparent prior to experimental treatment (Table S4). Macronutrient intake (ratio of protein consumed relative to total grams consumed) did not affect observed richness or Shannon diversity (all $p \geq .810$). 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, all $p_{adj} \geq .657$). 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

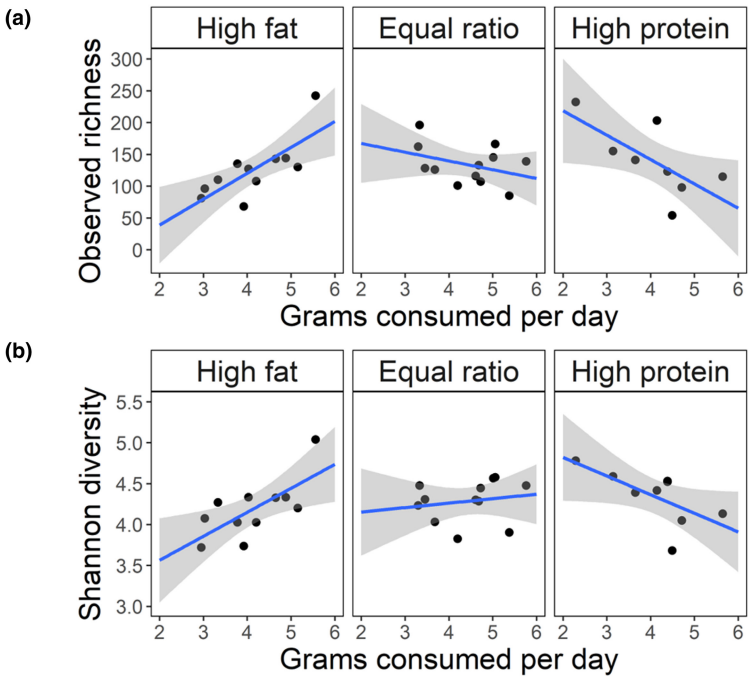

FIGURE 3 Effect of diet treatment (high fat, equal ratio, high protein) and food consumption (average grams of diet consumed per day) on the (a) observed richness and (b) Shannon diversity index of zebra finch microbiotas in birds fed diets differing in macronutrient content for 8 days (experiment 1). Shaded areas represent 95% confidence intervals. [Colour figure can be viewed at wileyonlinelibrary.com]

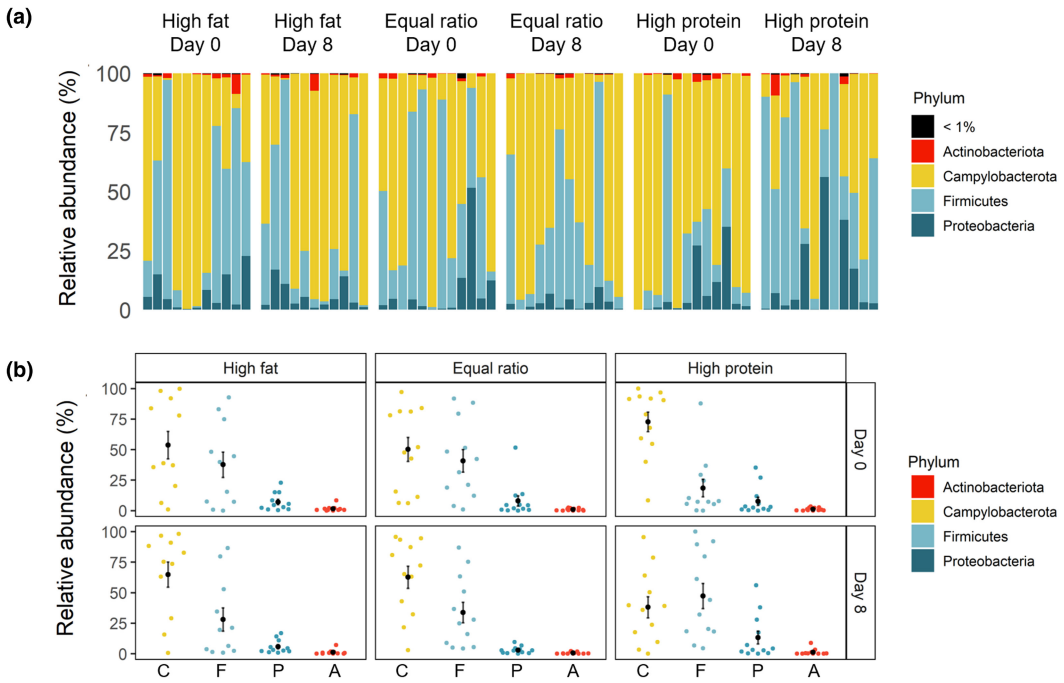

FIGURE 4 (a) Proportional abundance of bacterial phyla across diet treatment groups (high fat, equal ratio, high protein) and time (day 0, day 8) in zebra finches fed diets differing in macronutrient content (experiment 1). Each bar represents a sample from an individual bird. Phyla with less than 1% 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. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Kruskal-Wallis chi-square (χ^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).

	High fat diet		Equal ratio diet		High protein diet	
Taxonomic group	χ^2	p_{adj}	χ^2	p_{adj}	χ^2	p_{adj}
Phylum Actinobacteriota	0.481	.870	0.336	.603	0.875	.583
Phylum Campylobacterota	0.087	.870	0.653	.603	6.750	**.047***
Campylobacter	0.010	.922	1.203	.907	3.413	.259
Helicobacter	0.092	.871	0.170	.907	0.861	.471
Phylum Firmicutes	0.312	.870	0.270	.603	4.563	.082
Ligilactobacillus	1.232	.712	0.427	.907	1.270	.416
Weissella	0.182	.871	0.013	.908	5.606	.143
Phylum Proteobacteria	0.027	.870	0.403	.603	0.441	.633
Achromobacter	0.355	.871	0.863	.907	0.110	.772
Acinetobacter	1.812	.712	0.225	.907	0.084	.772
Pseudomonas	2.710	.712	0.056	.908	2.310	.343
Other Phyla (<1%)	0.186	.870	1.784	.603	0.000	1.000
Other Genera (<1%)	0.570	.871	0.270	.907	1.268	.416

Note: *p*-Values were adjusted for false discovery rate with a Benjamini–Hochberg correction where significance was determined as $p_{adj} < .05$. Significant differences between groups are shown in bold.

comparing pre-treatment (day 0) samples to post-treatment (day 5) samples (Table S6: all $p_{adj} \geq .775$).

3.2.2 | 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 $p \geq .480$). Regardless of treatment group, focal bird body mass decreased over the course of the experiment (Figure S14, day: $\chi^2 = 100.55$, $p < .0001$); however, furcular fat scores were not influenced by treatment or time (Figure S15, all $p \geq .126$). Body mass and fat score did not vary between male and female finches (sex: all $p \geq .282$). Focal bird physiology was also not influenced by a cue of infection. Specifically, complement activity, baseline corticosterone concentrations, and testosterone concentrations

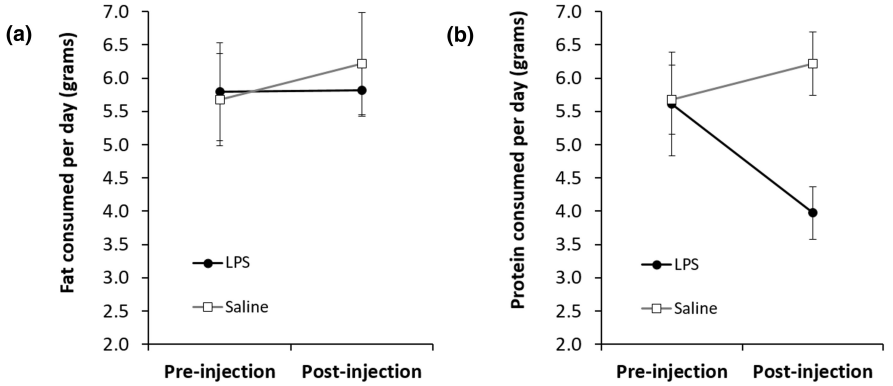

FIGURE 5 Grams of high fat and high protein diet consumed per day in zebra finches challenged with lipopolysaccharide (LPS) or saline (a, b) and in focal birds that were either housed next to sick-conspecifics (LPS-focal) or healthy conspecifics (saline-focal) (c, d). Data are reported as means ± standard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

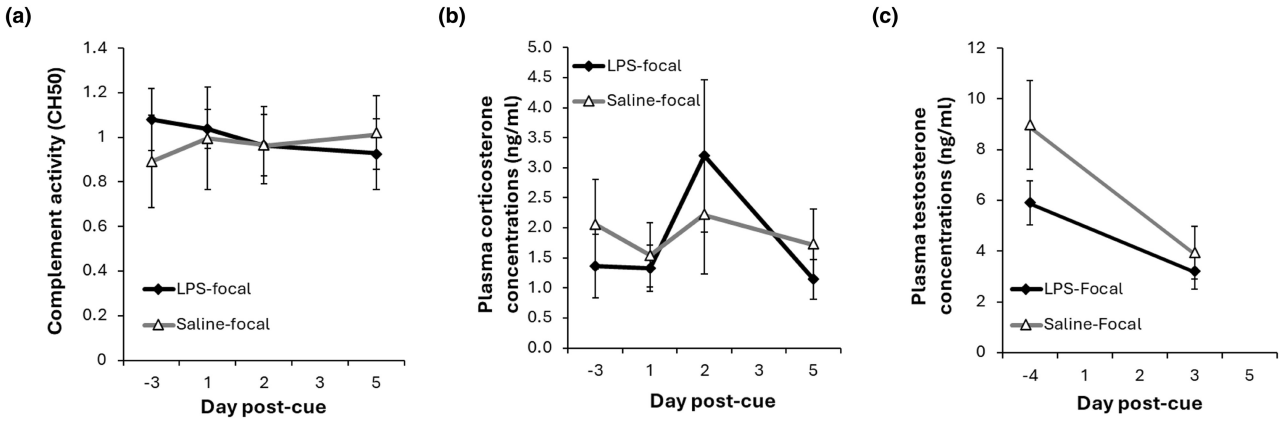

FIGURE 6 Physiological responses of focal birds housed in view of conspecifics injected with saline or LPS (experiment 2). (a) Hemolytic complement activity (CH50), (b) plasma corticosterone concentrations, and (c) plasma testosterone concentrations in zebra finches that were housed next to healthy (no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

did not vary by treatment or an interaction between treatment and time (Figure 6, all $p \geq .180$). Additionally, complement activity and baseline plasma corticosterone concentrations did not vary between male and female finches (sex: all $p \geq .279$). Regardless of treatment, testosterone concentrations (collected from males only) decreased over time (day: $\chi^2 = 13.78$, $p = .0002$).

There was a non-significant trend for LPS-focal birds to have lower bacterial richness (Figure S16; treatment: $\chi^2 = 7.400$, $p_{adj} = .052$). Shannon diversity did not vary significantly between treatments ($\chi^2 = 4.164$, $p_{adj} = .165$). Macronutrient intake (ratio of protein consumed relative to total grams consumed) did not affect richness or Shannon diversity (Table S7; all $p_{adj} \geq .345$). 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, all $p_{adj} \geq .464$). Abundance of bacterial taxa at the phylum (Figure S18) or genus level (Figure S19) did not change over time in either treatment (Table S9: all $p_{adj} \geq .838$).

4 | DISCUSSION

We investigated how dietary macronutrients affected host physiology and gut microbiota and characterized how feeding behaviour and macronutrient selection were influenced by both immune activation and perceived infection risk. We found that shifts in the type and amount of dietary macronutrients consumed can affect gut microbial communities and that reduced caloric intake was associated with higher complement activity regardless of diet macronutrient content. Additionally, a direct immune threat but not a perceived risk of infection altered the feeding behaviour 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 behaviour likely have important consequences for host health.

4.1 | Effects of diet macronutrient content on avian feeding behaviour, physiology and gut microbiota

We did not find an effect of diet macronutrient content 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 & 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). Many of these studies used diets with more extreme differences in macronutrient ratios than the present study, thus 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. Regardless of diet macronutrient treatment, increased caloric intake was associated with lower complement activity. The finding that caloric intake is negatively related to immunity is interesting given that many organisms engage in sickness-induced anorexia (Adelman & Martin, 2009; Povey et al., 2013), and suggests that birds might be capable of modifying immunity in response to immune threats through reductions in feeding.

In Experiment 1 (diet manipulation), the quality and quantity of diet consumed affected microbial richness and Shannon diversity. Both richness and Shannon diversity increased with caloric intake in birds fed a high-fat diet and decreased with caloric intake in birds fed a high protein diet. The observed interactive effects of diet quality and caloric intake could have important implications for host health given that higher gut microflora diversity is associated with more robust immune responses and parasite resistance in wild songbirds (Knutie, 2020). Contrary to our results, studies in other organisms found that high fat diets reduce gut microbiota richness and diversity (Teyssier et al., 2020; Zhang et al., 2012). However, these studies had more extreme differences in diet composition and lasted for several weeks which could explain the discrepancy in findings. While community-level changes in gut microbiota can have important implications for host health, smaller changes in specific taxa can also be important (Hooper et al., 2012; Round & Mazmanian, 2009). 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. Members of the phylum Campylobacterota, such as *Campylobacter* spp., are common avian commensals (Waldenström et al., 2002). However, some members of this genus are considered opportunistic pathogens that can cause bacterial gastroenteritis in humans and elicit the expression of inflammatory cytokines in chickens (Connerton et al., 2018; Waldenström et al., 2002). Thus, macronutrient-induced shifts in Campylobacterota could play a role in shaping intestinal inflammation and overall health.

4.2 | Macronutrient-specific feeding behaviours after an immune threat

The goals of the second experiment (diet selection) were to (1) investigate how an immune threat influences feeding behaviour, (2) explore whether social cues of disease can alter feeding behaviour and immune and endocrine responses relevant to disease susceptibility and (3) determine whether an immune threat and behavioural shifts in feeding alter the gut microbiome. Based on research in other taxa, 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 et al., 2008, 2010; Cheema et al., 2003; Cotter et al., 2011; Lochmiller et al., 1993; Povey et al., 2013; Sakamoto et al., 1981). Conversely, we found that birds given an immune challenge with LPS engaged in macronutrient-specific sickness-induced anorexia by maintaining consumption of the high fat 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). Work in other taxa 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 & 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. 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 behavioural 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 (Kluger & Rothenburg, 1979; Soyano & Gómez, 1999). 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.

4.3 | Heightened risk of infection does not alter macronutrient-specific feeding behaviour

Because shifts in behaviour and pathology associated with infection could act as social cues of heightened infection risk to uninfected conspecifics, we also examined whether perceived risk of infection (seeing sick conspecifics) could alter feeding behaviour and macronutrient selection. Separate lines of evidence indicate that social cues can influence feeding behaviour (Cornelius et al., 2018) and alter physiological responses relevant to immune function (Gormally & Lopes, 2023; Love et al., 2021; Schaller et al., 2010; Stevenson et al., 2011, 2012). 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 behaviour in a way that maximizes immunity. Contrary to our predictions, we found no evidence for shifts in feeding behaviour, 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 behaviour of focal birds because we used a simulated infection (injection with LPS) in this study. The behavioural effects of LPS typically only last between 2 and 4 days following injection in zebra finches (Love et al., 2023; Sköld-Chiriac et al., 2014), 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 h had an upregulation of immune genes in the blood, suggesting that physiological responses to LPS-challenged individuals might happen rapidly post-cue (Gormally & Lopes, 2023). Additionally, it might be less costly for organisms to employ behavioural defences (such as avoidance behaviour) rather than physiological immune defences in response to an immune threat in some settings. Experiment 2 was run concomitantly with Love et al. (2023), which found that LPS-challenged birds exhibited sickness behaviours and zebra finches housed across from LPS-challenged conspecifics reduced flight activity and increased preening behaviour (Love et al., 2023). This suggests that focal birds responded to sick conspecifics behaviorally rather than physiologically and that there might be trade-offs in how birds invest in behavioural versus physiological immune defences (Zylberberg et al., 2012). Future work should explore whether an immune challenge or infection that elicits stronger and longer-lasting behavioural and physiological signs of disease is capable of influencing feeding behaviour, gut microbiota, and physiological responses in healthy individuals, as this could have implications for host disease susceptibility and pathogen transmission potential.

5 | CONCLUSIONS

We found that diet macronutrient content and caloric intake can alter gut microbial communities and that regardless of diet quality, caloric intake is associated with complement activity but not baseline or stress-induced corticosterone concentrations. We did not detect any shifts in feeding behaviour, 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 behaviour in sick individuals can affect both host and parasite fitness and ultimately influence disease severity, which is inherently related to pathogen transmission (Hite & 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 & 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. Future work should explore whether reduced protein consumption during infection is adaptive for hosts in terms of responding to and overcoming infection, as this could have implications for pathogen transmission.

AUTHOR CONTRIBUTIONS

ACL and SED were involved in conceptualization, funding acquisition and writing—original draft; ACL, SED, SMW, NHY and VT were involved in experimental methodology; ACL was involved in analyses; ACL, VT and SED were involved in investigation; AL was involved in visualization. All authors were involved in writing—review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Supporting information has been made available online. Data are available at FigShare (Love et al., 2024; https://doi.org/10.6084/m9.figshare.25852708) and sequences have been uploaded to GenBank (BioProject accession number: PRJNA1112869).

ORCID

Ashley C. Love https://orcid.org/0000-0002-7144-7884

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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