Diet-induced changes to host gut microbiota are linked to foraging innovation in a wild bird.

Authors: Gabrielle Davidson[a,b,*] , Niamh Wiley[c,d], Amy C. Cooke[a], Crystal N. Johnson[c,d], Fiona Fouhy[c,d], Michael S. Reichert[a, e], Ivan de la Hera[a], Jodie M.S. Crane[a,f], Ipek G. Kulahci[a], R. Paul Ross[c,d], Catherine Stanton[c,d], John L. Quinn[a,*]

* Correspondence: Gabrielle Davidson email: gd339@cam.ac.uk; John Quinn email: j.quinn@ucc.ie.

Tel: +44(0)1223 747321

a. School of Biological, Earth and Environmental Sciences, Distillery Fields, North Mall, University College Cork, Cork, Ireland.
b. Department of Psychology, Downing Street, University of Cambridge, Cambridge, UK.
c. APC Microbiome Ireland, University College Cork, Cork, Ireland.
d. Teagasc Food Research Centre, Moorepark, Fermoy, Ireland.
e. Department of Integrative Biology, Oklahoma State University, USA
f. Kākāpō Recovery Programme, Department of Conservation, 7th Floor, 33 Don Street, Invercargill 9810, New Zealand.

ABSTRACT: Studies in lab rodents indicate diet alters host gut microbiome and that the gut microbiome influences behaviour. However, the ecological relevance across species and in wild animals is unclear. First we showed that problem solving performance in wild-caught great tits (*Parus major*) was weakly associated with natural variation in the gut microbiome. Then we experimentally manipulated the gut microbiome by feeding birds one of two different diets – an all insect diet or an all seed/nut diet. We presented these individuals with the problem solving task after the dietary manipulation to test whether the gut microbiome alterations influenced foraging innovativeness. Microbial communities changed substantially when given the insect, but not the seed diet. Individuals were less likely to problem-solve after being given the insect diet, and performance was positively associated with microbiome diversity. This is the first demonstration of an association between innovative problem solving and the gut microbiome in a wild animal.

Keywords: gut microbiome, gut-brain axis, diet, innovation, problem solving performance, great tits, foraging, cognition

INTRODUCTION

The enteric microbial community, frequently referred to as the gut microbiome, is an important ecosystem that contributes to host behaviour (Cryan & Dinan 2012;Sherwin *et al.* 2019). Recent evidence has pointed to a bidirectional communication link between host brain and the gut microbiome known as the gut-brain axis (e.g. Diaz Heijtz *et al.* 2011;Foster & McVey Neufeld 2013). Experimental alteration to the gut microbiome can impact on a suite of behavioural and cognitive phenotypes including learning, memory, anxiety, activity levels and social interactions (Clarke *et al.*

2012;Diaz Heijtz *et al.* 2011;Desbonnet *et al.* 2014;Hoban *et al.* 2017;Magnusson *et al.* 2015), as well as cause changes to neurogenesis (Ogbonnaya *et al.* 2015) and protein expression in the brain (Clarke *et al.* 2012;Gareau *et al.* 2011;Hoban *et al.* 2017). Neurotransmitters and short chain fatty acids released by microbes act as signals that can be communicated to the brain (reviewed in Cryan & Dinan 2012). Therefore, the extent to which signalling occurs is dependent on the microbial taxa present and their metabolic functions (Stilling *et al.* 2016). Evidence of the gut-brain axis is limited to experimental manipulations with model animals in the lab and correlational studies on mental health in humans (e.g. reviewed in Cryan & Dinan 2012;Foster & McVey Neufeld 2013). However, nothing is known about whether these findings can be applied to wild animals where we predict microbiome-host interactions to have important effects on traits that directly impact animal fitness, such as cognition (Morand-Ferron *et al.* 2016) and foraging behaviour (Stephens & Krebs 1986). We hypothesised that relationships between microbiome, diet and foraging behaviour are likely to be bi-directional if foraging determines diet, and if diet impacts on the same microbes/microbial community structures that are involved in altering host behaviour via the gut-brain axis.

Several environmental factors contribute to enteric microbial community composition in vertebrates, including geographical location (e.g. children, De Filippo *et al.* 2010; birds, Gillingham *et al.* 2019), habitat characteristics (e.g. amphibians, Costa *et al.* 2016;birds, Knutie *et al.* 2019) and seasonality (e.g. mammals, Amato *et al.* 2015;Hicks *et al.* 2018;Maurice *et al.* 2015). These temporal and spatial differences in microbiome between and within populations are most frequently attributed to variation in diet (e.g. Amato *et al.* 2015; reviewed in Lozupone *et al.* 2012). From an evolutionary perspective, host diet strongly predicts phylogenetic convergence of the gut microbiomes in several vertebrate clades (Youngblut *et al.* 2019), including humans (Muegge *et al.* 2011), mammals (Ley *et al.* 2008), fish (Sullam *et al.* 2012), and birds (Hird *et al.* 2015;Youngblut *et al.* 2019; but see Kropackova *et al.* 2017). Diet has also been shown to alter gut microbes in chickens (reviewed in Pan & Yu 2014), humans and lab rodents (reviewed in Singh *et al.* 2017). Significant alterations can occur within 24 hours of the introduction of a new dietary regime (David *et al.* 2014), dependent on dietary features such as the ratio of protein (Clarke *et al.* 2014;David *et al.* 2014), fat (Fava *et al.* 2013), and plants present in a diet (Wu *et al.* 2016;Zimmer *et al.* 2012). Therefore, we expect the effect of diet on the microbiome to be particularly important in wild animals that are subject to variations in food availability (e.g. Amato *et al.* 2015), and where individuals differ in their foraging success (Davidson *et al.* 2018).

Diet-related changes to the microbiome have been linked to changes in host cognition and behaviour. For example, mice fed beef-chow had a higher microbial diversity than those fed on normal chow, and showed improved working and reference memory (Li *et al.* 2009). Moreover, high fat and high sugar diets caused differential changes to gut microbial taxonomic groups in mice. Compared to control animals on calorie balanced chow, both diets resulted in poorer behavioural flexibility on a reversal learning task, but only the high sugar diet impacted on spatial memory (Magnusson *et al.* 2015). These studies show, at least in model lab organisms, phenotypic plasticity in cognitive performance can occur in parallel with gut microbiome alterations. Observational studies also point to a relationship between diet, cognition and mood in humans (Psaltopoulou *et al.* 2013), effects that may be mediated by the host gut microbiome (e.g. Carlson *et al.* 2018). There is an increasing need for manipulative experiments in natural populations to understand the ecological and evolutionary significance of how diet affects microbiome and how microbiome affects

behaviours that have the potential to have direct impacts on food acquisition, such as foraging abilities.

Foraging innovations refer to instances where animals generate novel solutions to problems, or incorporate a novel food type into their diet (Reader & Laland 2003). Foraging generalists with wide ecological niches have larger brains and are more successful at adapting to changing habitats than specialist species because of their propensity to innovate (Sol *et al.* 2005). As a consequence, innovators may increase their access to a wide range of resources (Ducatez *et al.* 2015;Reader & MacDonald 2003; but see Overington *et al.* 2011). If innovators have a wider dietary breadth, then we predicted that they would have a more diverse gut microbiome than non-innovators (Davidson *et al.* 2018). Given that the microbiome can also affect behaviour directly, the direction of causality between innovative foraging behaviour and the gut microbiome can thus be in either direction.

We manipulated the diet of wild great tits (*Parus major*) temporarily brought into captivity to examine whether diet affected the gut microbiome and innovative problem solving performance. We also tested whether specific gut microbiome profiles correlated with innovative problem solving as further evidence for behaviour-gut microbiome associations. Great tits differ in dietary specialisations and preferences (Serrano-Davies *et al.* 2017;Pagani-Nunez *et al.* 2015) and are found in both rural and urban habitat types – factors that may influence diet. They are also opportunistic foragers that vary individually in their problem solving performance, which is a common measure of innovativeness (Cole *et al.* 2011). Individual problem solving performance has been reported to be consistent across time and tasks in this species (Cole *et al.* 2011), and while this suggests the potential for heritability, little or no genetic variation explained problem solving performance (Quinn *et al.* 2016). Instead, other ecological conditions during the nestling stage, including habitat characteristics that could be linked to diet, were a more important predictor of adult problem solving performance (Quinn et al. 2016). Therefore, the evidence from, at least one population, suggests that problem solving is primarily a plastic trait in response to environmental inputs. A prospective mechanistic explanation for plasticity in problem solving could include diet-induced changes to the gut microbiome that affect gut-brain axis communication. Here

METHODS

Subjects

Thirty six great tits were captured between January and March 2017 across four sites. Two sites were within Cork city (urban), 1.6 km apart, and two were in deciduous woodlands (rural) 23 km apart, and located at least 23km from the urban sites. All birds were banded with rings issued by the British Trust of Ornithology for individual identification. Upon capture, birds were transported to the aviary facilities at University College Cork and singly-housed in wire cages (45 × 50 × 60 cm) containing two wooden perches.

Faecal sampling

Faecal samples were collected within 1 hour of arrival into the aviary, and again on Day 12 of captivity. A clean sheet of brown paper was placed on the floor of each cage for faecal collection. Paper was used in order to soak liquid urea away from the faecal matter as urea can act as a downstream inhibitor to amplification (Khan *et al.* 1991). Using sterile inoculation loops, we

transferred the faecal matter into tubes containing 1ml of 100% ethanol and stored tubes at -20 degrees Celsius.

Dietary manipulation

From day 2-13 of captivity, birds were given one of two different dietary treatments designed to reflect real ecological variation seen in the wild, for example changes in the availability of seed or animal food sources (Perrins 1991;Vel'ky *et al.* 2011), or perhaps reflecting potential individual differences in dietary specialisations (Serrano-Davies *et al.* 2017): 1) seed and suet, n = 17; and 2) Insect diet, n = 19. The insect diet consisted of wax moth larvae (*Achroia grisella*) and mealworm larvae (*Tenebrio molitor*). Mealworms were provided ad libitum, and five wax worms were provided each morning and each evening (except during the problem solving task). The seed diet consisted of sunflower hearts, peanuts and suet. We provided birds in the seed diet five mealworms and one wax worm on day seven of captivity for welfare reasons to ensure the dietary treatment was not too extreme. Nutritional composition of each food item is provided in Supplementary Table 1. To limit more general nutritional deficiencies, all birds received vitamin powder mixed with their food and drops mixed in their water (AviMix®). Birds were assigned to treatment groups randomly, counterbalanced for age and sex.

Problem solving assay

To quantify individual foraging innovation, naïve birds were presented with a novel problem solving foraging task. This was derived from a similar foraging task, performance in which was consistent within individuals over their lifetimes, correlated with a range of environmental sources of variation, and was linked to behaviour and fitness-related traits (Dunn *et al.* 2011;Cole *et al.* 2012;Quinn *et al.* 2016) et al.; Cole et al.; Quinn et al. Phil Trans). The birds were given the task overnight from one hour before sunset to two hours after sunrise, once on Day 1 of captivity and once on Day 12 of captivity. Due to the length of the trial, birds were not food deprived for welfare reasons. During the first trial, all birds had access to mealworms, peanuts and sunflower hearts ad libitum. During the second trial, birds had access to their assigned diets ad libitum. During both trials, wax worms, a highly preferred food reward (Cole *et al.* 2011;O'Shea *et al.* 2017; G. Davidson personal observation), were placed inside a transparent Perspex tube 16cm (height) x 5cm (width). The worms could be accessed by solving at least one of three solutions: 1) by pulling a lever to drop a platform holding a worm; 2) by pushing a door to the side; and 3) by pulling a string attached to one of the worms from the top of the tube. By having multiple access possibilities, problem solving performance could be assessed without limiting solutions to one particular motor action that may be more feasible in some individuals over others. At the start of the problem solving assay, a freely available wax worm was placed outside, at the base of the problem solving device to measure birds' motivation to approach the apparatus and consume the wax worm. Wax worms were otherwise not provided when the problem solving task was presented. One bird died of unknown causes following 10 days of captivity and therefore only data from Day 1 were included for this bird.

Microbiome analysis

DNA extraction and amplification: Microbial DNA was extracted using the Qiagen QIAamp DNA Stool Kit, following the "Isolation of DNA from Stool for Pathogen Detection" protocol with modifications described in Zeale et al. (2011) to increase DNA yield and remove excess inhibitors expected to be

present in the uric acid of bird faeces (but see Crouch *et al.* 2019 where they found no evidence of uric acid in feacal matter from a subset of avian species). A 0.10 - 0.20 g aliquot of each faecal sample was added to the kit, alongside a negative control.

The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S metagenomic sequencing library protocol (Illumina) as described in Fouhy *et al* (2019). In the current study, each PCR reaction contained 23 µl DNA template, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) and 25 µl 2X Kapa HiFi Hotstart ready mix (Roche, Ireland), to a final volume of 50 µl. Two negative controls were run in parallel – one from the DNA extraction and one containing PCR water instead of DNA template. Of the bird samples, ten failed to amplify and were not pooled for sequencing (Day 1: n = 1 (seed), Day 13: n = 3 (seed), 6 (insect)). Successful PCR products were cleaned using AMPure XP magnetic bead based purification (Labplan, Dublin, Ireland). Samples were sequenced at the Teagasc Sequencing Centre on the MiSeq sequencing platform, using a 2 x 300 cycle kit, following standard Illumina sequencing protocols.

Three hundred base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME. Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123. Alpha diversities were generated in QIIME 4.

Statistical analyses

The QIIME files (Operational Taxonomic Unit table, taxonomic table, phylogenetic tree file) and metadata files were analysed using phyloseq (McMurdie & Holmes 2013) in R Statistical Software (R Development Core Team 2011). Sequences with less than 15,000 reads, singletons and taxa present at <0.005% were removed (Bokulich *et al.* 2013). Samples were CSS normalised for beta diversity analysis. We included all taxa in the dataset, most notably those that are often viewed as 'dietary contaminants' (i.e. microbes originating from ingested food, such as cyanobacteria present in plants). This is because not all cyanobacteria are dietary contaminants (Di Rienzi et al. 2013) and because our study specifically tested differences between diets. Thereby removal of one so-called 'dietary contaminant' specific to plants (i.e. cyanobacteria), but none specific to insects (e.g. insect microbiome) could systematically bias our results.

Linear Mixed Models (LMMs) and Generalized Linear Mixed Models (GLMMs) were run using lme4 (Bates *et al.* 2014), and where relevant, p-values were obtained using lmerTest (Kuznetsova *et al.* 2017) in R (R Development Core Team 2011). Terms with $p < 0.1$ were sequentially removed from the models, starting with interaction terms. Site ID and Bird ID were included as random effects. Response variables were transformed where necessary to meet assumptions of normality (Natural log (Ln) or square root transformed), and therefore all models were run with a Gaussian distribution unless stated otherwise.

Dietary effects on microbiome

The effect of diet on the relative abundance (i.e. percentage abundance relative to all other phyla) was tested for Firmicutes, Proteobacteria, Bacteroidetes, Tenericutes and Actinobacteria as these were the most abundant phyla. Proteobacteria was modelled as a proportion and run with a binomial distribution, as it could not be transformed to fit a normal distribution. Three different measures of alpha diversity were used as response variables to test the effect of diet on the microbiome community: Shannon's index, total observed species, and Chao1. The above gut microbiome metrics did not differ between pre-assigned dietary groups, therefore we used diet as a three level factor accounting for both experimental date as a fixed effect: Diet (pre-dietary assignment (day 1), insect post-diet (day 12) and seed post-diet (day12)). Habitat (rural, urban), age (juvenile, adult (Svensson 1992)) and sex were included as fixed effects. To avoid overparameterization of models, we tested the interaction between diet and habitat only, as we expected inherent differences in diets between birds from the two habitats. We also investigated the effect of diet on genus-level relative abundance with mixed regression models using the web-based software Calypso (version 8.72) (Zakrzewski *et al.* 2017). ID was included as a random term and significant taxa were adjusted by a false discovery rate (FDR) whereby p values of less than 0.05 were considered statistically significant.

Dietary effects on problem solving performance

Problem solving performance was modelled as a binomial distribution (solved vs not solved) with diet (seed, insect), experiment day (day 1, day 12), habitat (rural, urban), age (juvenile, adult) and sex included as fixed effects. We included interactions between diet and habitat type and diet and experiment day. To test whether diet affected motivation, we ran a binomial GLMM with consumption of the freely available wax worm (Yes/No) as the response variable, assuming that birds who took the waxworm were more motivated to solve than those who were not. The fixed and random effects were included as described for the problem solving analysis above.

Relationship between problem solving and microbiome

We tested whether natural variation in the gut microbiome correlated with problem solving performance on Day 1. We tested for associations between microbial community and problem solving as a binomial response variable (solved vs not solved) in GLMMs for each microbial community measurement (the top five phyla, and the three measures of alpha diversity), and included habitat, sex and age as fixed effects and site as a random effect. Beta Diversity was calculated in four ways: Bray-Curtis, Jaccard, weighted (accounting for relative abundance of taxa) and unweighted (presence/absence of taxa) unifrac distance matrices. Each matrix was analysed using permutational multivariate analysis of variance (ADONIS) with 1000 permutations. We also investigated the relationship between problem solving and relative abundance at the genus-level as described above.

To test whether gut microbiome alteration as a consequence of dietary manipulation caused a change in problem solving performance, we ran the same analyses described above, but included data from Day 12 and diet as a fixed effect with bird ID as a random term. We predicted that a change in problem solving performance should specifically be associated with the same metrics of the gut microbiome that were changed as a result of experimental diet manipulation.

Research and Animal Ethics: This study was conducted under licences from the Health Products Regulatory Authority (AE19130_P017), The National Parks and Wildlife Services (C11/2017) and the British Trust for Ornithology, and permission from Coillte Forestry and private landowners. The research project received ethical approval from the Animal Welfare Body at University College Cork, and was in accordance with the ASAB (Association for the Study of Animal Behaviour) Guidelines for the Treatment of Animals in Behavioural Research and Teaching.

RESULTS

Relative abundance, diet and microbial diversity

The most prominent phyla across all samples were as follows (mean percentage relative abundance ±SE): Proteobacteria (55.3%±4.0); Cyanobacteria (14.8%±2.6); Firmicutes (10.2%±2.1); Tenericutes (9.0%±2.3); Actinobacteria (4.0%±1) and Bacteroidetes (2.0%±0.5). Proteobacteria increased significantly over the course of captivity in the insect-diet group ($z = 2.02$, $p=0.04$), but not in the seed-diet group ($z = -0.28$, $p = 0.78$), and tended to be higher in adults than juveniles ($z = 1.84$, $p = 0.07$). Bacteroidetes (natural log (Ln)-transformed) increased in the insect group ($t = 0.22$, $p = 0.03$), but not the seed group ($t = 1.01$, $p = 0.32$), and tended to be higher in urban compared to rural habitats ($t = 1.80$, $p = 0.08$). Actinobacteria was significantly higher in birds from urban habitats ($t= 2.47$, $p =0.02$) There was no significant effect of diet, habitat, sex or age on Firmicutes (Ln-transformed) or Tenericutes (Ln-transformed). There were no significant interactions between diet and habitat for all models. Figures 1 and 2 display relative abundance across treatments and across individual samples. Full model outputs are provided in Table S2, supplementary.

Significant differences in genus-level abundance attributed to dietary treatments were found for 22 genera. Bird given the insect diet showed a decrease in *Devosia, Rickettsiella, Sphingomonas, Pantoea, Arthrobacter, Brevibacterium, Brachybacterium, Clostridium and Carnobacterium,* and an increase in *Candidatus, Methylobacterium.* Birds given the seed diet showed a decrease in *Cronobacter* and *Serratia*, and an increase in *Microbacterium.* Birds in both dietary groups showed a decrease in *Bradyrhizobium, Staphylococcus and Rahnella*, and an increase in *Lactobacillus, Bacillus, Ureaplasma, Delftia, Flavobacterium, Streptococcus and Rhodococcus* (Table S3, supplementary).

There was a significant decrease in Chao1 (square root transformed) in the insect diet group ($t = -2.51$, $p = 0.02$), but not in the seed group ($t = – 0.07$, $p = 0.94$) compared to the pre-diet assignment birds (Figure 3a). Shannon's index also decreased in the insect group ($t= -2.02$, $p = 0.06$) but not the seed group ($t = 0.01$, $p = 0.99$), but this was marginally non-significant (Figure 3b). Number of observed species (Ln-transformed) did not differ significantly across treatments (insect group $t = -1.74$, $p = 0.10$; seed group $t = 0.43$, $p=0.67$) (Figure 3c). There was no significant effect of sex, age, or habitat type on alpha diversity. There was no significant interaction between diet and habitat. (Table S2, supplementary).

Problem solving, diet and microbial diversity

The innovation task was solved 17 times, by 15 different individuals across both trial days. None of the birds from the insect group solved during the post-dietary treatment. Natural variation in beta diversity prior to dietary manipulation tended to be associated with problem solving performance (PSP) (unweighted unifrac distances $R = 0.05$, $p = 0.07$). All other metrics of natural variation in the gut microbiome were not significantly associated with problem solving (Table S4). However, PSP across both trials, when controlling for dietary treatment and repeated measures, was positively

correlated with alpha diversity (Shannon: z=2.22, p = 0.03; Chao1 z = 2.13, p = 0.04, observed species z =1.96; p = 0.06) (Figure 5a,b,c). Phylum-level and genus-level relative abundance was not associated with PSP.

Birds assigned to the seed group solved more than the birds assigned to the insect group (z = 2.22, p = 0.03), and birds tended to be more likely to solve on day 1 than day 12 (z = 1.93, p = 0.054), though this effect was likely driven by the post-diet insect group (Figure 4). There was a tendency for juveniles to solve more than adults (z = 1.94, p =0.053). Neither the interactions between habitat, nor between experiment day and diet were significant (Table S2, supplementary). Diet and experiment day did not influence whether birds consumed the freely available wax worm (diet z = 1.18, p 0.24, experiment day z= 0.85, p=0.40)

Beta diversity: ADONIS tests showed dietary treatment significantly influenced beta diversity across all four metrics. Post-hoc test with Bonferroni correction indicate that the differences were between day 1 and the post-diet insect group (R2=0.07, p<0.01), and between the two post-diet groups (R2=0.10, p=0.03). PSP and habitat were significantly different for some beta diversity metrics (Table S5). Differences in diet across experiment days, and differences in PSP can be visualised in Nonmetric Multidimensional Scaling (NMDS) ordination plots (Figure 6).

DISCUSSION

We demonstrate that an experimentally induced dietary change caused significant alterations to the gut microbiome diversity and phylum- and genus-level abundance in a wild bird species, which in turn may have led to reduced innovative behaviour. To our knowledge, this is the first study providing evidence that microbial communities in the gut may be in part determined by foraging innovations, and that there is an effect of gut gross microbiota composition on problem solving performance. We discuss these findings in the context of foraging ecology, the gut-brain axis, and environment-behaviour interactions.

Dietary manipulation in this experiment affected both the likelihood of solving and the gut microbiome. Specifically, those on the insect-only diet had reduced microbiome diversity and were less likely to solve, suggesting that the dietary induced reduction in the microbiome reduced innovative problem solving behaviour. Moreover, our results suggest that individual variation in problem solving performance was associated with natural variation in microbial beta diversity, although this result was marginally non-significant. This supports our hypothesis that innovators who are expected to have a more diverse diet should consequently also have a more diverse microbiome. This was nonetheless correlational, so the alternative causal direction cannot be discounted. Nevertheless, together our findings lend support to the hypothesis that the gut microbiome, innovation and diet are interlinked. We encourage further manipulative investigations to pinpoint causal directions of these relationships, in particular whether innovative behaviour leads to variation in microbial diversity through food access, or indeed whether innovation arises because of microbial diversity caused by some other mechanism.

Seasonal and geographic differences in gut microbial communities in wild mammals have been attributed to changes in food availability (Amato *et al.* 2015;Maurice *et al.* 2015;Hicks *et al.* 2018). In a population of wild birds temporarily taken into captivity, we show that changes in microbial community composition can be sensitive to dietary changes, independent of other factors that may

differ with seasonality and impact on gut microbiota, such as hormonal differences (Escallon *et al.* 2019), because the changes here were recorded under controlled conditions over a two week period. We show that phylum-level, genus-level and diversity changes to the gut microbiome are dependent on the food type. While birds in both the seed and insect diets showed both decreases and increases in genus-level abundance, only birds given the insect diet showed significant changes in diversity and phylum-level abundance. This could perhaps be explained because our birds had already been taking seeds at the feeders we used to lure them for capture, and because great tits consume a high proportion of plant-based foods in the winter (Vel'ky *et al.* 2011). The use of garden feeders in both urban and rural environments may also explain why there were no rural versus urban habitat differences in alpha diversity. Nevertheless, there was a significant difference in beta diversity as well as a higher proportion of Actinobacteria in urban birds compared to rural birds, similar metrics to those that have previously been shown to be related to urban environments in house sparrows (*Passer domesticus*) (Teyssier *et al.* 2018). While our study aimed to mimic variation in individual food consumption, or changes during the course of the winter, longitudinal sampling of individuals across seasons would be necessary to confirm whether similar microbial taxa changes would occur, particularly given that the invertebrate species accessible in the wild would differ from those provided in our experiment.

Problem solving performance dropped significantly in birds that were given an insect diet, which also caused alterations to gut microbiome profile, suggesting a potential causal link between microbiome and behaviour via the gut-brain axis. We acknowledge that motivation can influence problem solving performance (reviewed in Griffin & Guez 2014), and that an all-insect diet may have decreased motivation to engage in the problem solving task baited with an insect reward, even if of a preferred species, the waxmoth. However, the insect diet did not influence the birds' motivation to consume the freely available wax worm, and the same birds solved on day one when mealworms were freely available, indicating that wax worms are a highly-valued and preferred food reward, irrespective of dietary treatment during captivity. Our results showed that the indices of microbial community diversity that decreased as a consequence of diet (i.e. Chao1 index, beta diversity) were the same metrics that were associated with variation in problem solving performance. Proteobacteria and Bacteroidetes increased following an insect-diet, but these two phyla were not associated with problem solving performance, nor were the genus-level microbial taxa that were altered as a consequence of diet, suggesting that the microbial community structure as a whole may be important for regulating behaviour.

How the gut microbiome impacts behaviour via the gut-brain axis may be attributed to the metabolic functions of the microbial community (e.g. Stilling *et al.* 2016), derived from the diets of the host (e.g. reviewed in Roager & Dragsted 2019). Studies have attempted to disentangle nutritional and microbial effects on behaviour by depleting the original microbes of the hosts and re-introducing specific bacterial organisms, or transplanting gut microbiomes between hosts (e.g. Bruce-Keller *et al.* 2015; Mohle *et al.* 2016). However, the aim of our study was to test diet-microbiome-behaviour relationships within an ecologically relevant context that would translate to wild animals in their natural environment. To control for nutritional deficiencies that may have an impact on behaviour independent of microbiome, we provided vitamin supplements; however, other nutritional differences were present. Fat content and fibre content was five-fold and three-fold higher in the seed diet compared to the insect diet, respectively. Mice fed on high fat diets, or given microbiome transplantations from obese donors show poorer cognitive performance than

control mice (Magnusson *et al.* 2015;Bruce-Keller *et al.* 2015); whereas, our study showed that birds fed the lower-fat diet (i.e. insect) had poorer problem solving performance. Having a higher proportion of fibre present in the seed diet may have offset any negative effects of a high-fat diet. Non-digestible carbohydrates are fermented by gut microbes in the large intestines, promote the growth of microbial organisms and can have positive effects on cognition and behaviour in mammals (reviewed in Cryan *et al.* 2019). Metabolomics profiling would be an informative future endeavour to provide a functional assessment of microbial products such as short chain fatty acids involved in gut-brain axis communication (reviewed in Stilling *et al.* 2016;Heintz-Buschart & Wilmes 2018;Cryan *et al.* 2019).

CONCLUSIONS:

We have shown phenotypic plasticity in innovative behaviour as a consequence of diet-related changes to the gut microbiome, demonstrating an association with foraging behaviour in wild animals. Moreover, food consumption determined the gut microbiome, indicating that problem solving performance, diet and the gut microbiome are intercorrelated, and that problem solving performance is a trait that is largely influenced by environmental inputs. We have established a novel approach for investigating causes and consequences of innovative foraging, which provides the groundwork for further investigations into the ecological relevance of host-microbiome relationships in natural systems.

Authors contributions: GLD, ACC and JLQ designed the experiment with input from CS and RPR. GLD and ACC ran the experiment with assistance from MSR, IGK, IDH and JMSC. GLD, NW and CNJ carried out DNA extraction and library prep. FF and GLD analysed data. GLD wrote the manuscript with input from all authors. All authors gave final approval for publication.

Acknowledgements: James Nichols, Ally Phillimore and Sarah Knowles for helpful discussions on molecular methodology; Shane Somers for advice on statistics; Jennifer Coomes for assistance with fieldwork; Dr. Paul Cotter, Dr. Fiona Crispie and Ms. Laura Finnegan from the Teagasc Sequencing facility for their role in relation to the 16S rRNA sequencing. Funding for GLD, ACC, MSR, IGK, IDH and JMSC from the European Research Council under the European Union's Horizon 2020 Programme (FP7/2007-2013)/ERC Consolidator Grant "Evoecocog" Project No. 617509, awarded to JLQ. This work was funded in part by Science Foundation Ireland through APC Microbiome Ireland.

References

Amato K.R., Leigh S.R., Kent A., Mackie R.I., Yeoman C.J., Stumpf R.M., Wilson B.A., Nelson K.E., White B.A. & Garber P.A. (2015). The Gut Microbiota Appears to Compensate for Seasonal Diet Variation in the Wild Black Howler Monkey (Alouatta pigra). *Microbial Ecology,* 69, 434-443.

Bates D., Maechler M., Bolker B. & Walker S. (2014). lme4: Linear mixed-effects models using Eigen and S4. In.

Bokulich N.A., Subramanian S., Faith J.J., Gevers D., Gordon J.I., Knight R., Mills D.A. & Caporaso J.G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods,* 2012/12/02, 57-59.

Bruce-Keller A.J., Salbaum J.M., Luo M., Blanchard E., IV, Taylor C.M., Welsh D.A. & Berthoud H.R. (2015). Obese-type Gut Microbiota Induce Neurobehavioral Changes in the Absence of Obesity. *Biological Psychiatry,* 77, 607-615.

Carlson A.L., Xia K., Azcarate-Peril M.A., Goldman B.D., Ahn M., Styner M.A., Thompson A.L., Geng X., Gilmore J.H. & Knickmeyer R.C. (2018). Infant Gut Microbiome Associated With Cognitive-Development. *Biological Psychiatry,* 83, 148-159.

Clarke G., Grenham S., Scully P., Fitzgerald P., Moloney R.D., Shanahan F., Dinan T.G. & Cryan J.F. (2012). The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Molecular Psychiatry,* 18, 666.

Clarke S.F., Murphy E.F., Sullivan O., Lucey A.J., Humphreys M., Hogan A., Hayes P., Reilly M., Jeffery I.B., Wood-Martin R., Kerins D.M., Quigley E., Ross R.P., Toole P.W., Molloy M.G., Falvey E., Shanahan F. & Cotter P.D. (2014). Exercise and associated dietary extremes impact on gut microbial diversity. *Gut,* 63, 1913.

Cole E.F., Cram D.L. & Quinn J.L. (2011). Individual variation in spontaneous problem-solving performance among wild great tits. *Animal Behaviour,* 81, 491-498.

Cole E., Morand-Ferron J., Hinks A. & Quinn J. (2012). Cognitive Ability Influences Reproductive Life History Variation in the Wild. *Current Biology,* 22, 1808-1812.

Costa S., Lopes I., Proenca D.N., Ribeiro R. & Morais P.V. (2016). Diversity of cutaneous microbiome of Pelophylax perezi populations inhabiting different environments. *Science of The Total Environment,* 572, 995-1004.

Crouch N.M.A., Lynch V.M. & Clarke J.A. (2019). A re-evaluation of the chemical composition of avian urinary excreta. *Journal of Ornithology*.

Cryan J.F. & Dinan T.G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience,* 13, 701.

Cryan J.F., O'Riordan K.J., Cowan C.S.M., Sandhu K.V., Bastiaanssen T.F.S., Boehme M., Codagnone M.G., Cussotto S., Fulling C., Golubeva A.V., Guzzetta K.E., Jaggar M., Long-Smith C.M., Lyte J.M., Martin J.A., Molinero-Perez A., Moloney G., Morelli E., Morillas E., O'Connor R., Cruz-Pereira J.S., Peterson V.L., Rea K., Ritz N.L., Sherwin E., Spichak S., Teichman E.M., van de Wouw M., Ventura-Silva A.P., Wallace-Fitzsimons S.E., Hyland N., Clarke G. & Dinan T.G. (2019). The Microbiota-Gut-Brain Axis. *Physiological Reviews,* 99, 1877-2013.

David L.A., Maurice C.F., Carmody R.N., Gootenberg D.B., Button J.E., Wolfe B.E., Ling A.V., Devlin A.S., Varma Y., Fischbach M.A., Biddinger S.B., Dutton R.J. & Turnbaugh P.J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature,* 505.

Davidson G.L., Cooke A.C., Johnson C.N. & Quinn J.L. (2018). The gut microbiome as a driver of individual variation in cognition and functional behaviour. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 373, 20170286.

De Filippo C., Cavalieri D., Di Paola M., Ramazzotti M., Poullet J.B., Massart S., Collini S., Pieraccini G. & Lionetti P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci USA,* 107, 14691.

Desbonnet L., Clarke G., Shanahan F., Dinan T.G. & Cryan J.F. (2014). Microbiota is essential for social development in the mouse. *Molecular Psychiatry,* 19, 146-148.

Diaz Heijtz R., Wang S., Anuar F., Qian Y., Bjorkholm B., Samuelsson A., Hibberd M.L., Forssberg H. & Pettersson S. (2011). Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci USA,* 108, 3047-3052.

Ducatez S., Clavel J. & Lefebvre L. (2015). Ecological generalism and behavioural innovation in birds: technical intelligence or the simple incorporation of new foods? *J Anim Ecol,* 84, 79-89.

Dunn J.C., Cole E.F. & Quinn J.L. (2011). Personality and parasites: sex-dependent associations between avian malaria infection and multiple behavioural traits. *Behavioral Ecology and Sociobiology,* 65, 1459-1471.

Escallon C., Belden L.K. & Moore I.T. (2019). The Cloacal Microbiome Changes with the Breeding Season in a Wild Bird. *Integrative Organismal Biology,* 1.

Fava F., Gitau R., Griffin B.A., Gibson G.R., Tuohy K.M. & Lovegrove J.A. (2013). The type and quantity of dietary fat and carbohydrate alter faecal microbiome and short-chain fatty acid excretion in a metabolic syndrome ΓÇÿat-riskΓÇÖ population. *International Journal of Obesity,* 37, 216-223.

Foster J.A. & McVey Neufeld K.A. (2013). Gut-brain axis: how the microbiome influences anxiety and depression. *Trends in Neurosciences,* 36, 305-312.

Fouhy F., Watkins C., Hill C.J., OΓÇÖShea C.A., Nagle B., Dempsey E.M., OΓÇÖToole P.W., Ross R.P., Ryan C.A. & Stanton C. (2019). Perinatal factors affect the gut microbiota up to four years after birth. *Nature Communications,* 10, 1517.

Gareau M.G., Wine E., Rodrigues D.M., Cho J.H., Whary M.T., Philpott D.J., MacQueen G. & Sherman P.M. (2011). Bacterial infection causes stress-induced memory dysfunction in mice. *Gut,* 60, 307.

Gillingham M.A.F., Bechet A., Cezilly F., Wilhelm K., Rendon-Martos M., Borghesi F., Nissardi S., Baccetti N., Azafzaf H., Menke S., Kayser Y. & Sommer S. (2019). Offspring Microbiomes Differ Across Breeding Sites in a Panmictic Species. *Frontiers in Microbiology,* 10, 35.

Griffin A.S. & Guez D. (2014). Innovation and problem solving: A review of common mechanisms. *Behavioural Processes,* 109, 121-134.

Heintz-Buschart A. & Wilmes P. (2018). Human Gut Microbiome: Function Matters. *Trends in Microbiology,* 26, 563-574.

Hicks A.L., Lee K.J., Couto-Rodriguez M., Patel J., Sinha R., Guo C., Olson S.H., Seimon A., Seimon T.A., Ondzie A.U., Karesh W.B., Reed P., Cameron K.N., Lipkin W.I. & Williams B.L. (2018). Gut microbiomes of wild great apes fluctuate seasonally in response to diet. *Nature Communications,* 9, 1786.

Hird S.M., Sanchez C., Carstens B.C. & Brumfield R.T. (2015). Comparative Gut Microbiota of 59 Neotropical Bird Species. *Front Microbiol,* 6, 1403.

Hoban A.E., Stilling R.M., Moloney G., Shanahan F., Dinan T.G., Clarke G. & Cryan J.F. (2017). The microbiome regulates amygdala-dependent fear recall. *Molecular Psychiatry*.

Khan G., Kangro H.O., Coates P.J. & Heath R.B. (1991). Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. *J Clin Pathol,* 44, 360-365.

Knutie S.A., Chaves J.A. & Gotanda K.M. (2019). Human activity can influence the gut microbiota of Darwin's finches in the Galapagos Islands. *Mol Ecol,* 28, 2441-2450.

Kropackova L., Tesicky M., Albrecht T., Kubovciak J., Cizkova D., Tomasek O., Martin J., Bobek L., Kreisinger T., Prochazka P. & Kreisinger J. (2017). Codiversification of gastrointestinal microbiota and phylogeny in passerines is not explained by ecological divergence. *Mol Ecol,* 26, 5292-5304.

Kuznetsova A., Brockhoff P.B. & Christensen R.H.B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software; Vol 1, Issue 13 (2017)*.

Ley R.E., Hamady M., Lozupone C., Turnbaugh P.J., Ramey R.R., Bircher J.S., Schlegel M.L., Tucker T.A., Schrenzel M.D., Knight R. & Gordon J.I. (2008). Evolution of Mammals and Their Gut Microbes. *Science,* 320, 1647.

Li W., Dowd S.E., Scurlock B., Acosta-Martinez V. & Lyte M. (2009). Memory and learning behavior in mice is temporally associated with diet-induced alterations in gut bacteria. *Physiology & Behavior,* 96, 557-567.

Lozupone C.A., Stombaugh J.I., Gordon J.I., Jansson J.K. & Knight R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature,* 489, 220.

Magnusson K.R., Hauck L., Jeffrey B.M., Elias V., Humphrey A., Nath R., Perrone A. & Bermudez L.E. (2015). Relationships between diet-related changes in the gut microbiome and cognitive flexibility. *Neuroscience,* 300, 128-140.

Maurice C.F., CL Knowles S., Ladau J., Pollard K.S., Fenton A., Pedersen A.B. & Turnbaugh P.J. (2015). Marked seasonal variation in the wild mouse gut microbiota. *The Isme Journal,* 9, 2423.

Mohle L., Mattei D., Heimesaat M., Bereswill S., Fischer A., Alutis M., French T., Hambardzumyan D., Matzinger P., Dunay I. & Wolf S. (2016). Ly6C-hi- Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult Hippocampal Neurogenesis. *Cell Reports,* 15, 1945-1956.

Morand-Ferron J., Cole E.F. & Quinn J.L. (2016). Studying the evolutionary ecology of cognition in the wild: a review of practical and conceptual challenges. *Biol Rev,* 91, 367-389.

Muegge B.D., Kuczynski J., Knights D., Clemente J.C., Gonz+ílez A., Fontana L., Henrissat B., Knight R. & Gordon J.I. (2011). Diet Drives Convergence in Gut Microbiome Functions Across Mammalian Phylogeny and Within Humans. *Science,* 332, 970.

O'Shea W., Serrano-Davies E., Quinn J.L. & Pruitt J. (2017). Do personality and innovativeness influence competitive ability? An experimental test in the great tit. *Behavioral Ecology,* 28, 1435-1444.

Ogbonnaya E.S., Clarke G., Shanahan F., Dinan T.G., Cryan J.F. & O'Leary O.F. (2015). Adult Hippocampal Neurogenesis Is Regulated by the Microbiome. *Biological Psychiatry,* 78, e7-e9.

522 Overington S.E., Griffin A.S., Sol D. & Lefebvre L. (2011). Are innovative species ecological
523 generalists? A test in North American birds. *Behavioral Ecology,* 22, 1286-1293.

524 Pagani-Nunez E., Valls M. & Senar J.C. (2015). Diet specialization in a generalist population: the case
525 of breeding great tits Parus major in the Mediterranean area. *Oecologia,* 179, 629-640.

526 Pan D. & Yu Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. *Gut*
527 *Microbes,* 5, 108-119.

528 Perrins C. (1991). Tits and their caterpillar food supply. *Ibis,* 133, 49-54.

529 Psaltopoulou T., Sergentanis T.N., Panagiotakos D.B., Sergentanis I.N., Kosti R. & Scarmeas N. (2013).
530 Mediterranean diet, stroke, cognitive impairment, and depression: A meta-analysis. *Ann Neurol.,* 74,
531 580-591.

532 Quinn J.L., Cole E.F., Reed T.E. & Morand-Ferron J. (2016). Environmental and genetic determinants
533 of innovativeness in a natural population of birds. *Philos Trans R Soc Lond B Biol Sci,* 371, 20150184.

534 R Development Core Team (2011). R: a language and environment for statistical computing. In: R
535 Foundation for Statistical Computing, Vienna, Austria.

536 Reader S.M. & Laland K.N. (2003). Animal innovation: An introduction. In: *Animal innovation* Oxford
537 University Press, New York, NY, US, pp. 3-35.

538 Reader S.M. & MacDonald K. (2003). Environmental variability and primate behavioural flexibility. In:
539 *Animal innovation* Oxford University Press, New York, NY, US, pp. 83-116.

540 Roager H.M. & Dragsted L.O. (2019). Diet-derived microbial metabolites in health and disease. *Nutr*
541 *Bull,* 44, 216-227.

542 Serrano-Davies E., O'Shea W. & Quinn J.L. (2017). Individual foraging preferences are linked to
543 innovativeness and personality in the great tit. *Behavioral Ecology and Sociobiology,* 71, 161.

544 Sherwin E., Bordenstein S.R., Quinn J.L., Dinan T.G. & Cryan J.F. (2019). Microbiota and the social
545 brain. *Science,* 366, eaar2016.

546 Singh R.K., Chang H.W., Yan D., Lee K.M., Ucmak D., Wong K., Abrouk M., Farahnik B., Nakamura M.,
547 Zhu T.H., Bhutani T. & Liao W. (2017). Influence of diet on the gut microbiome and implications for
548 human health. *Journal of Translational Medicine,* 15, 73.

549 Sol D., Duncan R.P., Blackburn T.M., Cassey P. & Lefebvre L. (2005). Big brains, enhanced cognition,
550 and response of birds to novel environments. *Proc Natl Acad Sci U S A,* 102, 5460.

551 Stephens D.W. & Krebs J.K. (1986). *Foraging Theory*. Princeton University Press, Princeton, New
552 Jersey.

553 Stilling R.M., van de Wouw M., Clarke G., Stanton C., Dinan T.G. & Cryan J.F. (2016). The
554 neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis?
555 *Neurochemistry International,* 99, 110-132.

556 Sullam K.E., Essinger S.D., Lozupone C.A., O'Connor M.P., Rosen G.L., Knight R., Kilham S.S. & Russell
557 J.A. (2012). Environmental and ecological factors that shape the gut bacterial communities of fish: a
558 meta-analysis. *Mol Ecol,* 2012/04/04, 3363-3378.

559 Svensson L. (1992). *Identification guide to European passerines*. British Trust for Ornithology,
560 Thetford, United Kingdom.

561 Teyssier A., Rouffaer L.O., Saleh Hudin N., Strubbe D., Matthysen E., Lens L. & White J.l. (2018).
562 Inside the guts of the city: Urban-induced alterations of the gut microbiota in a wild passerine.
563 *Science of The Total Environment,* 612, 1276-1286.

564 Vel'ky M., Kanuch P. & Kristin A. (2011). Food composition of wintering great tits (Parus major):
565 habitat and seasonal aspects. *2011,* 60, 228-236.

566 Wu G.D., Compher C., Chen E.Z., Smith S.A., Shah R.D., Bittinger K., Chehoud C., Albenberg L.G.,
567 Nessel L., Gilroy E., Star J., Weljie A.M., Flint H.J., Metz D.C., Bennett M.J., Li H., Bushman F.D. &
568 Lewis J.D. (2016). Comparative metabolomics in vegans and omnivores reveal constraints on diet-
569 dependent gut microbiota metabolite production. *Gut,* 65, 63.

570 Youngblut N.D., Reischer G.H., Walters W., Schuster N., Walzer C., Stalder G., Ley R.E. & Farnleitner
571 A.H. (2019). Host diet and evolutionary history explain different aspects of gut microbiome diversity
572 among vertebrate clades. *Nature Communications,* 10, 2200.

573 Zakrzewski M., Proietti C., Ellis J.J., Hasan S., Brion M.J., Berger B. & Krause L. (2017). Calypso: a user-
574 friendly web-server for mining and visualizing microbiome-environment interactions. *Bioinformatics,*
575 33, 782-783.

576 Zeale M.R.K., Butlin R.K., Barker G.L.A., Lees D.C. & JONES G.A.R.E. (2011). Taxon-specific PCR for
577 DNA barcoding arthropod prey in bat faeces. *Mol Ecol Resour,* 11, 236-244.

578 Zimmer J., Lange B., Frick J.S., Sauer H., Zimmermann K., Schwiertz A., Rusch K., Klosterhalfen S. &
579 Enck P. (2012). A vegan or vegetarian diet substantially alters the human colonic faecal microbiota.
580 *European Journal of Clinical Nutrition,* 66, 53-60.
581
582
583
584
585
586
587
588
589
590
591
592
593

FIGURES

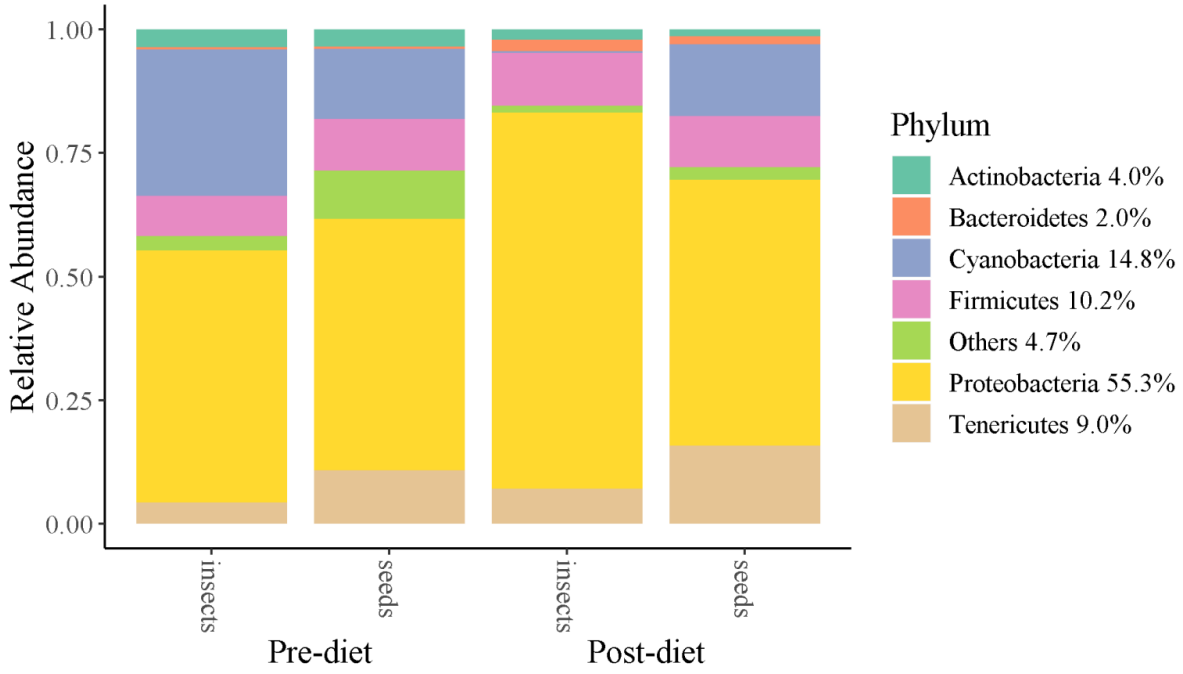


Figure 1. Relative abundance of top seven phyla across dietary treatments. Percentages reflect overall abundance, independent of treatment groups.

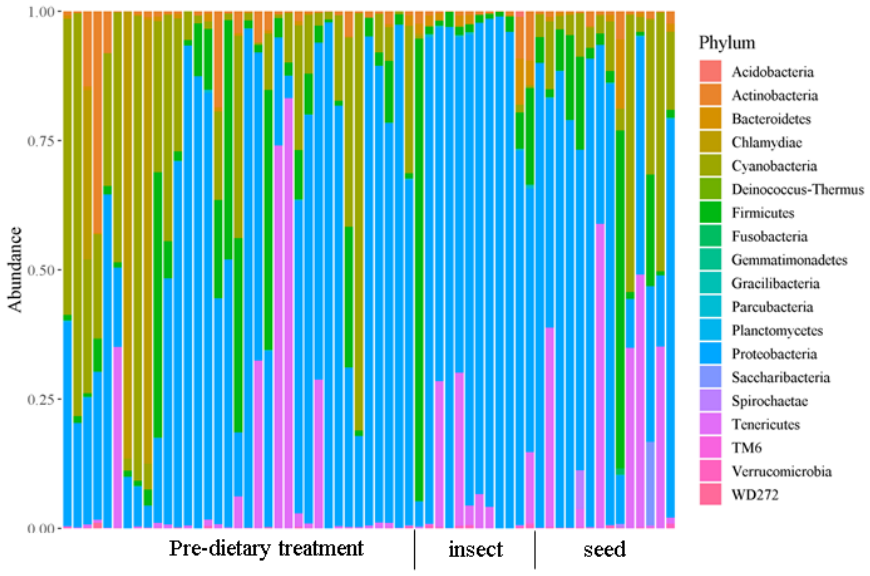
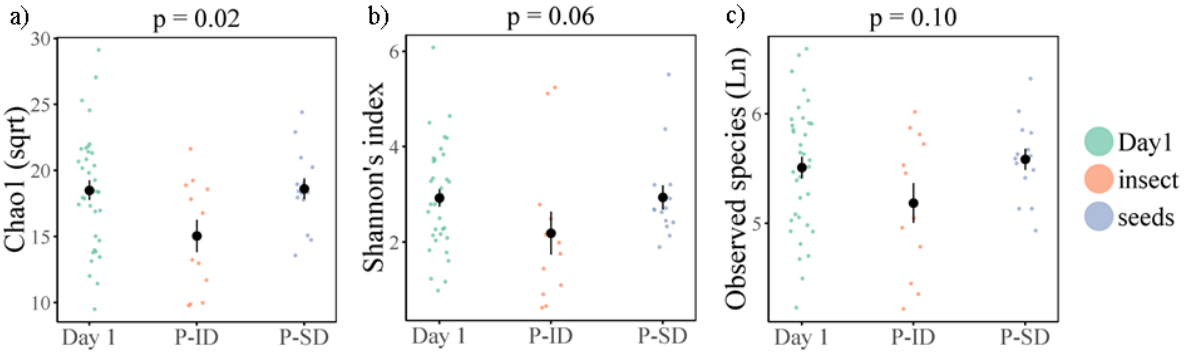
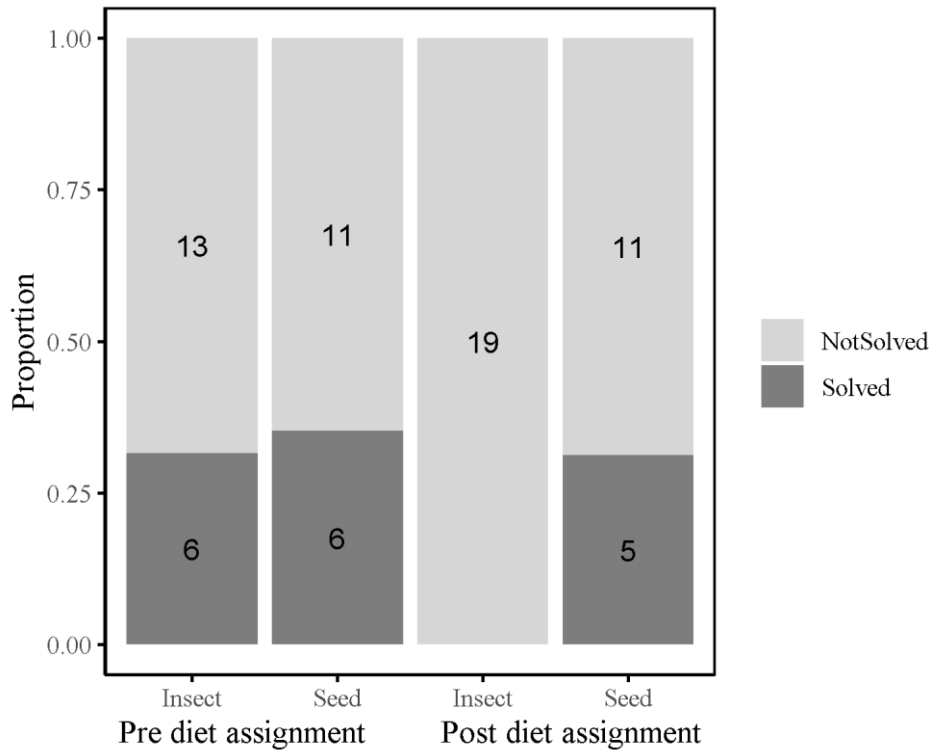


Figure 2. Differential abundance of all phyla per individual sample pre and post-insect and post-seed dietary treatments.



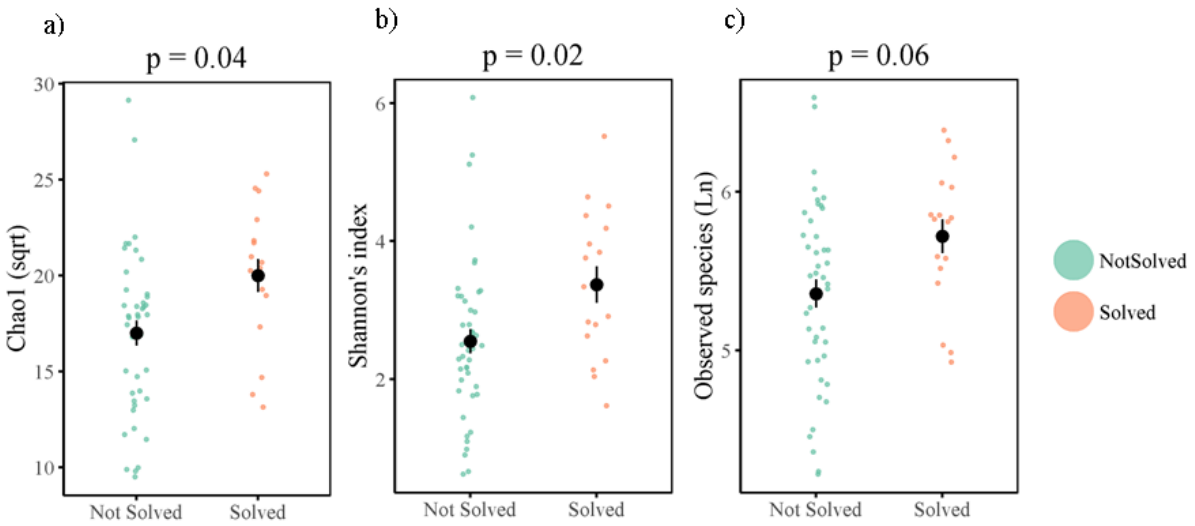
601

602 Figure 3. Alpha diversity for birds pre-dietary assignment (Day 1), post-insect diet and post-seed diet
603 for a) Chao1 (sqrt), b) Shannon's index, c) Observed species (Ln). Coloured points denote individual
604 data points, black points and line denote mean and ± SE. p values represent the comparison
605 between post-insect diet group and day 1.



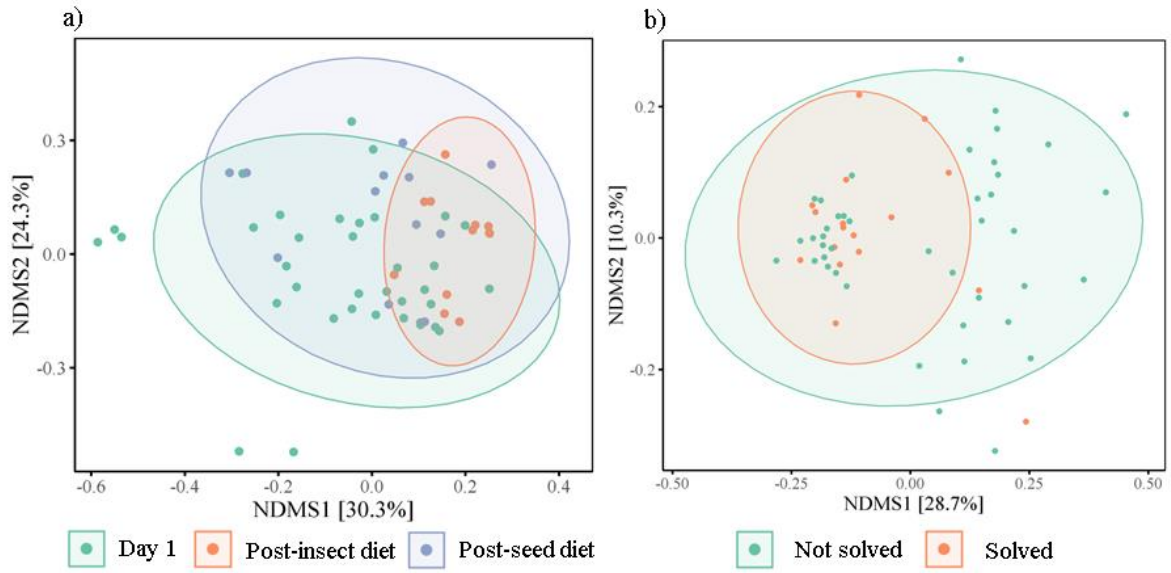
606

607 Figure 4. Problem solving performance as a measure of innovation. Number of individuals that
608 solved (dark grey) and number of birds that did not solve (light grey) pre- and post- dietary
609 treatments.



610

611 Figure 5. Problem solving-alpha diversity relationships in a) Chao1 (sqrt), b) Shannon's index, c)
612 Observed species (Ln) including data points from both day 1 and day 12. Coloured points denote
613 individual data points, black points and line denote mean and ± SE.



614

615 Figure 6. Nonmetric Multidimensional Scaling (NMDS) ordination plots based on (a) weighted unifrac
616 distances of diet and experiment day, and (b) unweighted unifrac distances of problem solving
617 performance. Ellipses represent standard deviations around the centroids of the groups. Numbers in
618 brackets refer to the variance explained by NDMS axes.

619

620

621

622