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Animal behaviour

Evidence supporting the microbiota–gut–brain axis in a songbird

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Recent research in mammals supports a link between cognitive ability and the gut microbiome, but little is known about this relationship in other taxa. In a captive population of 38 zebra finches (*Taeniopygia guttata*), we quantified performance on cognitive tasks measuring learning and memory. We sampled the gut microbiome via cloacal swab and quantified bacterial alpha and beta diversity. Performance on cognitive tasks related to beta diversity but not alpha diversity. We then identified differentially abundant genera influential in the beta diversity differences among cognitive performance categories. Though correlational, this study provides some of the first evidence of an avian microbiota–gut–brain axis, building foundations for future microbiome research in wild populations and during host development.

1. Background

An animal's gut microbiome can have wide-ranging effects on health [1–3], cognitive performance [4,5] and behaviour [6], coining the conceptual framework 'microbiota–gut–brain axis.' The gut microbiome can affect the brain directly by releasing neurotransmitters and precursors that stimulate the vagus nerve [7–10] and indirectly by influencing the immune system [1,11]. By disrupting the microbiome through immune challenges [6,12], inducing stress responses [1,12–16], or using observational studies and germ-free models, gut microbiome characteristics have been linked in rodents and humans to learning and memory [12] and mental health [6,17–20].

Despite extensive support for a microbiota–gut–brain axis in humans and rodents, little is known about these relationships in other taxa. Differences among avian, reptile and rodent brains may translate to differences in their microbiota–gut–brain relationships. Furthermore, few studies describe the core microbiome for most taxa [4,21], especially for wild populations [22]. To address these knowledge gaps, we studied the relationship between cognition and the gut microbiome of captive zebra finches (*Taeniopygia guttata*). Songbirds provide an opportunity to test for a microbiota–gut–brain axis because of recent advances in understanding avian cognition [23–25]. Gut microbiome characteristics are expected to associate with cognitive ability because the gut microbiome is tied to development and maintenance of brain function [5,10,17], as repeatedly demonstrated in model organisms [4]. Therefore, we hypothesized a correlation between a bird's cognitive ability and gut microbiome characteristics, assessed by bacterial alpha and beta diversity. We predicted that birds with a more diverse microbiome with fewer opportunistic taxa [26,27] would perform better on cognitive tasks than birds with a less diverse microbiome with more opportunistic taxa. This study provides one of the first looks at how the avian gut microbiome can covary with cognitive performance, building a foundation for experimental

microbiome manipulation during development [28] and understanding the microbiome's role in wild population health [29].

2. Methods

To assess cognitive performance, we tested 2–3-year-old zebra finches (bred at Kent State University and obtained October 2018) using three tasks measuring learning and memory: novel foraging, colour association and colour reversal [30–32]. Birds were first presented with a neophobia test (latency to approach the testing apparatus; see electronic supplementary material S1 for detailed methods and colony information). The novel foraging task employs operant conditioning and stepwise shaping to teach birds to prise opaque blue and white lids from wells to obtain a seed reward (same as regular diet). The performance measure was the number of trials required to learn to remove lids to obtain the reward. Once birds mastered this foraging technique, they were presented with blue and white lids but only one colour was rewarded. This colour association task tests associative learning: the ability to mentally connect multiple stimuli [33]. The performance measure was the number of trials required to learn to remove all rewarded lids before any unrewarded lids. Finally, the colour reversal task (rewarded colour is switched) tests for associative learning and behavioural flexibility [34,35]. Performance was measured as the number of trials required to remove all of the newly rewarded colour before removing any lids of the formerly rewarded colour. For all tasks, cognitive performance (hereafter performance) is an inverted variable: passing in fewer trials signifies higher performance.

Subjects were tested after 4 h of food restriction, ensuring motivation to obtain food rewards. Each bird was tested individually (visually but not acoustically isolated from other subjects) for 4 h each day, consisting of eight 2 min trials separated by 20 min. We viewed and scored trials remotely via video. Continued motivation to eat was confirmed at the end of each test day by returning the normal seed dish and noting the bird's latency to eat.

To assess gut microbiome community characteristics, we swabbed each bird's cloaca (Puritan 25-8001PD sterile swab, USA) 2 days before testing, then resampled each bird less than or equal to 1 week after testing to identify any microbiome changes during testing. The zebra finch cloacal microbiome is representative of that of its large intestine [36]. We stored samples in RNAlater (Qiagen, Germany) at −80°C until DNA extraction using PowerSoil DNA Isolation Kits (Qiagen, Germany) following slightly modified manufacturer's instructions [36,37] and verified quality using a Nanodrop 2000 (Oxford Technologies, UK). We amplified the V4 region of the 16S rRNA gene using modified primers 515F/806R with Illumina adaptors following the Earth Microbiome Protocol for PCR [38]. We submitted final pooled PCR products to Cornell's Biotechnology Resource Center for quantification, normalization, library preparation and sequencing. In total, we sequenced 72 cloacal swab samples and 14 negative controls in one Illumina MiSeq paired-end 2 × 250 bp run.

Using Quantitative Insights into Microbial Ecology 2 (QIIME2) [39], raw sequences were trimmed of their primers [40,41], joined [42], per-nucleotide-quality-filtered [43] and denoised [44]. Amplicon sequence variants (ASVs) were annotated using the Scikit-learn system and the SILVA 132 database [45,46]; mitochondria, chloroplasts and unassigned sequences were removed. ASVs were aligned using MAFFT [47] and masked [48,49] to make a midpoint-rooted phylogenetic tree using FastTree [50]. We decontaminated samples with package decontam in R [51] using negative controls and DNA yield. ASVs with < 10 sequences across all samples were removed [49,52]. Mean sequencing depth was 18 758 ± 49 reads before decontamination and filtering and 17 711 ± 1331 afterwards. We applied a zeroed variance stabilizing transformation (package DESeq2 [53]; electronic supplementary material S2, figure S1) using a negative binomial mixed model to account for library size differences across samples. This uses all available data and is therefore preferable to rarefying approaches [54]. Raw sequences were submitted to NCBI's Sequence Read Archive (BioProject PRJNA636961). Snakemake files (pre-configured coding loops) used in QIIME2 [55] and R scripts for statistical analysis are available on GitHub: (https://github.com/djbradshaw2/General_16S_Amplicon_Sequencing_Analysis [56]).

We tested for differences between pre- and post-trial sample alpha diversity (within individuals) using paired *t*-tests, and beta diversity (among performance groups) using permutational multivariate analyses of variances (PERMANOVAs) in PRIMER7 [57]. To evaluate the relationship between alpha diversity (Shannon, observed ASVs and Faith's phylogenetic) and performance, we built linear models in R [58] for each cognitive task. To assess beta diversity, we built dissimilarity matrices for Morisita–Horn, unweighted UniFrac, and weighted UniFrac distances and used quantiles for each task to categorize performance into poor- (greater than 3rd quantile), medium- (1st–3rd quantile) and high-performance (less than 1st quantile) categories. PERMANOVAs (package vegan [59]) compared dissimilarity matrix distances among performance categories. All *p*-values were adjusted with Benjamini–Hochberg multiple test corrections to reduce Type 1 error. However, permutation tests are widely perceived as being less susceptible to these errors [60]; therefore, we present raw and adjusted *p*-values for beta diversity analyses so as not to overlook true rejections of the null hypotheses. We estimated taxa-specific differential abundances among performance categories from non-normalized data by building beta-binomial regression models using package corncob (Wald test; false discovery rate cut-off = 0.05 [61,62]). This determined which microbial taxa accounted for relative abundance differences with respect to performance. Data are accessible on Dryad [63].

Table 1. Cognitive testing summary statistics for 38 captive zebra finches.

assay	mean performance ± s.e.	*n* trials
novel foraging	15.54 ± 1.54 trials	584
colour association	9.61 ± 0.87 trials	336
colour reversal	15.89 ± 1.00 trials	605
neophobia	721.63 ± 226.54 s	38
motivation check	12.00 ± 0.002 s	242

3. Results

We sampled the gut microbiome and scored performance for 21 male and 17 female zebra finches (table 1). Alpha diversity (electronic supplementary material S2, table S1) did not differ between sexes for pre-trial (Shannon: $\chi^2 = 0.03$, $p = 0.87$; observed ASVs: $\chi^2 = 0.2$, $p = 0.82$; Faith's: $\chi^2 = 0.6$, $p = 0.72$) or post-trial samples (Shannon: $\chi^2 = 0.7$, $p = 0.73$; observed ASVs: $\chi^2 = 0.7$, $p = 0.73$; Faith's: $\chi^2 = 0.5$, $p = 0.73$; electronic supplementary material S2, figure S2); however, beta diversity differed for pre-trial Morisita–Horn (pseudo-$F_{1,36} = 3.4$, $p < 0.001$) and weighted UniFrac (pseudo-$F_{1,36} = 5.1$, $p < 0.001$; electronic supplementary material S2, figure S3), but not Morisita–Horn post-trial (pseudo-$F_{1,32} = 1.9$, unadjusted $p = 0.05$, corrected $p = 0.14$), unweighted UniFrac (pre-trial: pseudo-$F_{1,36} = 1.4$, $p = 0.16$; post-trial: pseudo-$F_{1,32} = 1.5$, unadjusted $p = 0.10$, corrected $p = 0.14$), or weighted Unifrac post-trial

Table 2. Model summaries for how zebra finch cognitive performance relates to alpha diversity ($t/\beta \pm$ s.e.) and beta diversity (pseudo-F_{df}/r^2).

				cognitive task		
measure	diversity metric	time point	sex	novel foraging	colour association	colour reversal
alpha diversity	Shannon diversity	pre-trial	both	0.8/0.01 ± 0.01	0.1/−0.001 ± 0.02	−0.5/−0.01 ± 0.01
		post-trial	both	0.6/0.01 ± 0.01	0.6/0.01 ± 0.01	−0.4/−0.01 ± 0.02
	observed ASVs	pre-trial	both	1.0/0.20 ± 0.19	−0.1/−0.03 ± 0.37	−0.6/−0.21 ± 0.32
		post-trial	both	0.5/0.10 ± 0.18	−0.2/−0.08 ± 0.35	0.1/0.02 ± 0.31
	Faith's phylogenetic diversity	pre-trial	both	0.3/0.005 ± 0.02	0.2/0.01 ± 0.03	−0.7/−0.02 ± 0.03
		post-trial	both	0.3/0.01 ± 0.02	−0.3/−0.01 ± 0.04	0.1/0.005 ± 0.03
beta diversity	Morisita–Horn distance	pre-trial	M	$1.0_{2,18}$/0.10	$1.0_{2,18}$/0.10	$1.1_{2,18}$/0.11
			F	$1.3_{2,14}$/0.16	$1.4_{2,14}$/0.17	$1.0_{2,14}$/0.12
		post-trial	M	$1.0_{2,17}$/0.10	$0.4_{2,17}$/0.05	$1.2_{2,17}$/0.12
			F	$1.8_{2,11}$/0.25[a]	$1.0_{2,11}$/0.16	$0.8_{2,11}$/0.13
	UniFrac distance	pre-trial	M	$0.9_{2,18}$/0.09	$1.4_{2,18}$/0.14	$0.7_{2,18}$/0.07
			F	$0.8_{2,14}$/0.11	$0.8_{2,14}$/0.10	$1.3_{2,14}$/0.15
		post-trial	M	$1.0_{2,17}$/0.11	$0.7_{2,17}$/0.08	$0.9_{2,17}$/0.09
			F	$1.2_{2,11}$/0.17	$1.3_{2,11}$/0.19	$0.9_{2,11}$/0.14
	weighted UniFrac distance	pre-trial	M	$0.9_{2,18}$/0.09	$1.7_{2,18}$/0.16[a]	$0.9_{2,18}$/0.09
			F	$1.6_{2,14}$/0.19[a]	$0.5_{2,14}$/0.06	$1.0_{2,14}$/0.13
		post-trial	M	$1.6_{2,17}$/0.16[a]	$0.4_{2,17}$/0.05	$0.7_{2,17}$/0.07
			F	$2.0_{2,11}$/0.26[b]	$1.1_{2,11}$/0.17	$0.8_{2,11}$/0.13

[a]Denotes a relationship approaching significance ($\alpha < 0.10$).
[b]Denotes a significant relationship ($\alpha < 0.05$).

samples (pseudo-$F_{1,36} = 1.5$, $p = 0.14$). Therefore, we tested relationships between performance and alpha diversity with sexes pooled, but separately by sex for beta diversity. Neither alpha (Shannon: $t_{33} = 1.2$, $p = 0.38$; observed ASVs: $t_{33} = 1.2$, $p = 0.38$; Faith's: $t_{33} = 0.01$, $p = 0.99$) nor beta diversity (Morisita–Horn: pseudo-$F_{7,60} = 1.1$, $p = 0.57$; unweighted UniFrac: pseudo-$F_{7,60} = 0.94$, $p = 0.63$; weighted UniFrac: pseudo-$F_{7,60} = 1.0$, $p = 0.57$) differed significantly between pre- and post-test microbiome samples.

Alpha diversity (both sexes) showed no relationship with performance on any cognitive task (table 2, electronic supplementary material S2, figure S4). Beta diversity differed among performance categories depending on cognitive task, sex, sample timepoint and distance metric (table 2; electronic supplementary material S2, figure S5). Specifically, before multiple test correction, novel foraging performance related to male and female post-trial weighted UniFrac distance (male: unadjusted $p = 0.09$, corrected $p = 0.28$, $r^2 = 0.16$; female: unadjusted $p = 0.05$, corrected $p = 0.11$, $r^2 = 0.26$), female pre-trial weighted UniFrac distance (unadjusted $p = 0.10$, corrected $p = 0.28$, $r^2 = 0.19$) and female post-trial Morisita–Horn distance (unadjusted $p = 0.07$, corrected $p = 0.11$, $r^2 = 0.25$), while colour association performance related to male pre-trial weighted UniFrac distance (unadjusted $p = 0.07$, corrected $p = 0.15$, $r^2 = 0.16$; figure 1).

Differentially abundant genera among male pre-trial colour association performance categories included *Pseudomonas* ($p = 0.0001$), an uncultured Pasteurellaceae species ($p = 0.005$), *Gallibacterium* ($p < 0.001$), *Stenotrophomonas* ($p = 0.01$), *Helicobacter* ($p = 0.03$) and *Enterococcus* ($p = 0.02$, figure 2*a*). *Enterococcus* ($p = 0.01$, figure 2*b*) was differentially abundant among male post-trial novel foraging categories. *Gallibacterium* ($p < 0.001$), *Catellicoccus* ($p = 0.03$) and *Rothia* ($p = 0.05$) were differentially abundant among both sample timepoints' female novel foraging performance categories (figure 2*c*). Many of the above taxa, for example, *Stenotrophomonas* and *Rothia*, had very low relative abundances (less than 1%) regardless of how abundance compared among performance categories (electronic supplementary material S2, table S2). However, others were relatively very abundant, e.g. *Helicobacter* for males that performed poorly on colour association (44.62%) versus medium- (29.30%) and high-performance categories (10.63%). The same relationship was found for *Gallibacterium* among female novel foraging categories (poor: 3.1%, medium: 1.1%, high: 0.02%).

4. Discussion

We found that beta diversity of a bird's gut microbiome, but not alpha diversity, correlated with performance on learning and memory tests, partially supporting our hypothesized relationship between cognitive performance and gut microbiome characteristics. Although correlational, these findings provide some of the first evidence supporting a microbiota–gut–brain axis in an avian model and provide an important foundation for future experimentation.

The lack of a relationship between alpha diversity and performance does not support our predictions, contrasting with previous work detailing the benefits of a diverse gut microbiome. High alpha diversity is generally associated with good health in humans [64], improving microbial

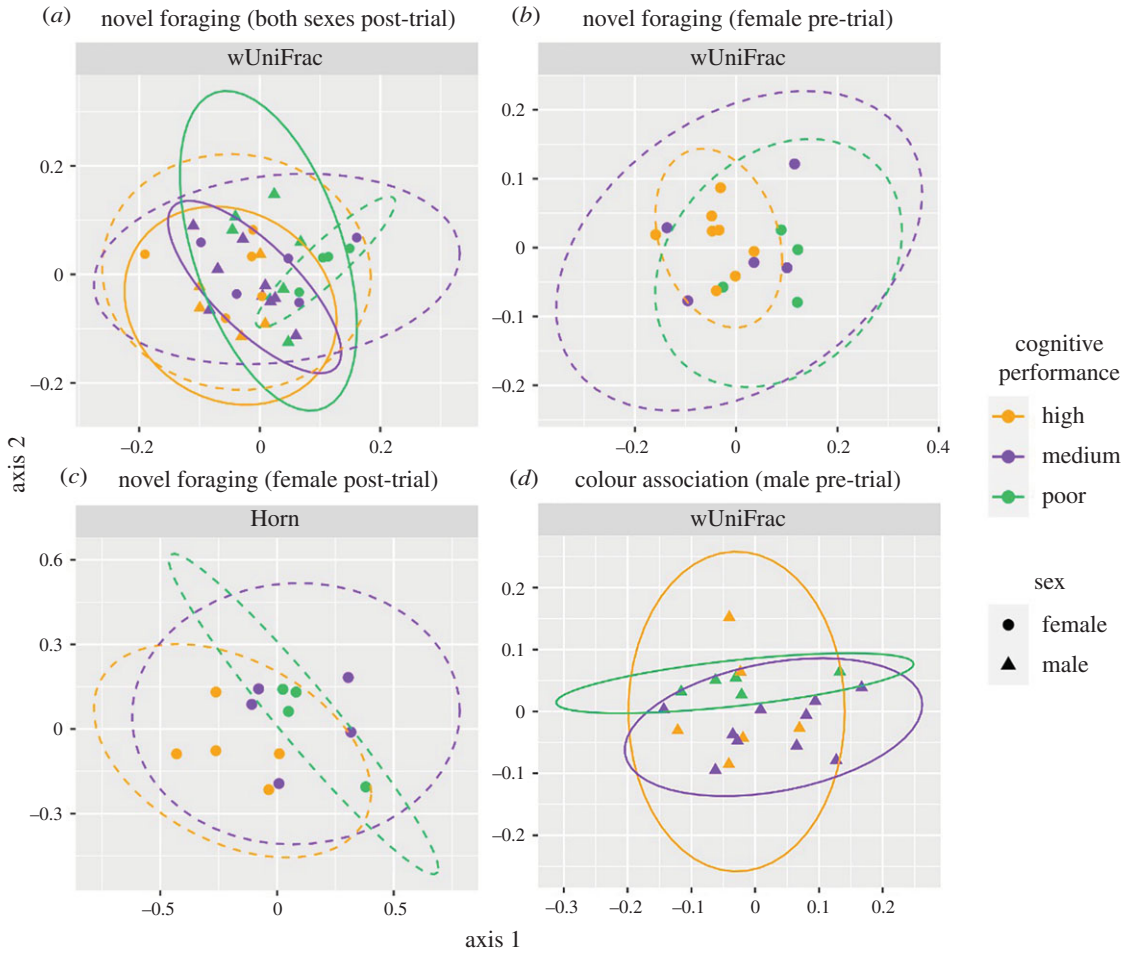

Figure 1. Zebra finch gut microbiome beta diversity distances related to cognitive performance. Novel foraging performance related to (*a*) male and female post-trial weighted UniFrac, (*b*) female pre-trial weighted UniFrac, and (*c*) female post-trial Morisita–Horn, and (*d*) male pre-trial weighted UniFrac related colour association performance. Ellipses indicate 95% confidence intervals (solid: male, dashed: female).

community stability during perturbation [26], while low alpha diversity may predict poor intestinal health or obesity [65,66]. However, whether host health and bacterial diversity translate into improved cognitive function remains unknown. Germ-free rodents raised sterile with quintessentially low alpha diversity show cognitive health deficits [12,17,20] compared with rodents with diverse gut microbiomes. Perhaps we did not find this relationship because low-DNA-yield microbiome samples from swabbing are not comparable with rodent studies employing faecal or sacrificial sampling. Studies testing for relationships between alpha diversity and cognitive performance in birds could employ multiple sampling techniques varying in DNA yield.

Several beta diversity metrics related to cognitive performance, supporting our hypothesized microbiota–gut–brain axis. However, while there is general agreement that robust microbiome communities are pathogen-free and enriched in beneficial bacteria, it is less clear how specific taxa relate to cognitive function. Certain taxa regulate neurotransmitter release or produce short-chain fatty acids critical for neurotransmitter production [67] and other physiological processes [11,68]. In our differential abundance modelling, many of the genera had low relative abundance, were poorly described genera, or were too diverse to make assumptions about functionality without species-level sequencing. But noteworthy among these were two largely pathogenic genera. *Helicobacter*, responsible for many intestinal diseases [69,70], and *Gallibacterium*, with many haemolytic species found in birds [71,72], were generally more abundant in poor-performance birds (figure 2*b*,*c*). While we did not identify beneficial taxa responsible for differences among performance categories, we suggest *Helicobacter* and *Gallibacterium* may signal microbiome dysbiosis in poor-performance birds. This raises the questions: Do specific taxa influence cognitive performance? Or, is a songbird's gut microbiome simply indicative of host quality and thus correlated with cognitive ability? Research could address these questions by describing the functionality of the core microbiome members for more bird species [21] and testing how specific pre- and probiotic treatments affect cognitive ability (e.g. [73,74]).

Notably, beta diversity related to performance on only two out of three tasks, varying by sex and distance metric, with most of the relationships appearing for weighted UniFrac distance in females. While unweighted UniFrac distance calculates relative relatedness in a qualitative manner, weighted UniFrac incorporates relative abundances to better characterize community structure. We conclude relative abundance was influential in revealing differences among performance categories and we suggest weighted UniFrac be integrated into future microbiota–gut–brain axis studies. But microbiome dysbiosis may impact each sex differently [20], or not at all, depending on the cognitive process studied. Another intriguing possibility is that microbiome characteristics impact some cognitive processes more than others, depending on

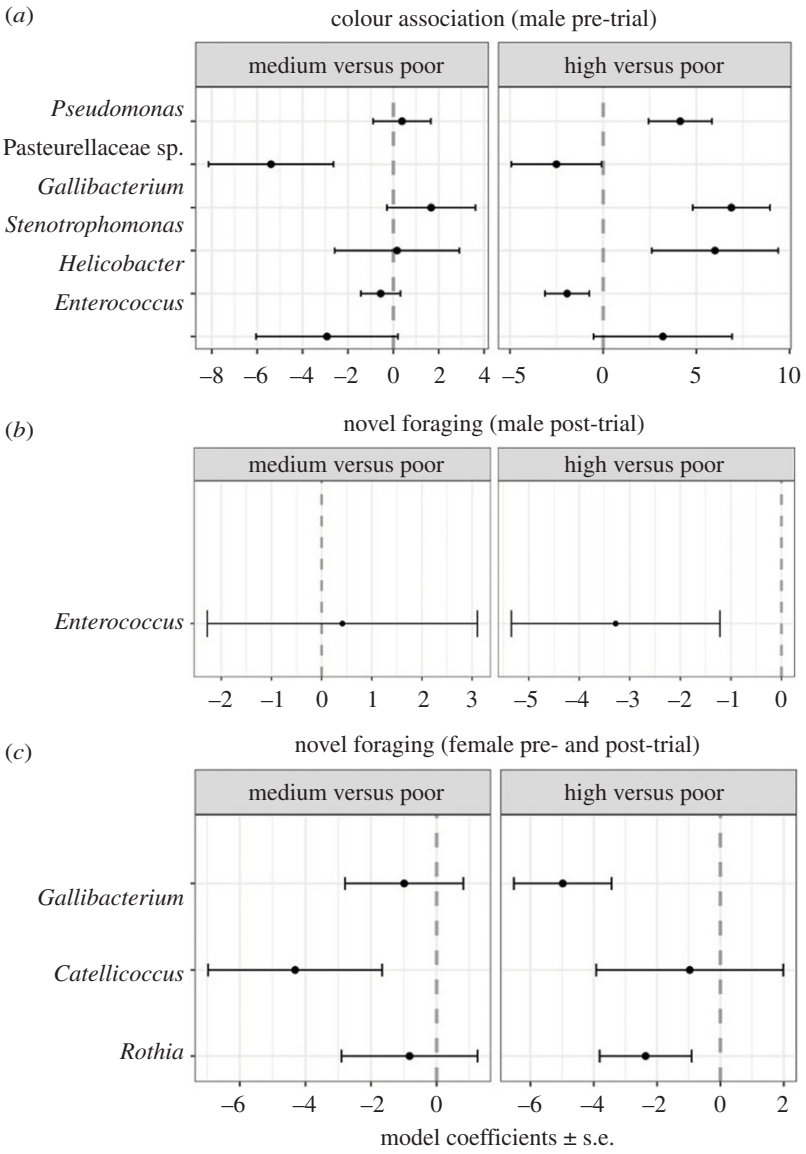

Figure 2. Bacterial genera in zebra finch gut microbiome samples were differentially abundant among cognitive performance groups depending on sex and cognitive task. Positive model covariate values indicate greater relative abundance. The left column compares relative abundances in medium performing birds to poor performing birds, and the right column compares high performance to poor performance.

sex, such as motor learning and short-term memory (novel foraging), compared with longer-term associative memory (colour association) and flexibility (colour reversal).

Despite the potential for identifying the songbird gut microbiome as a determinant of individual variation in cognitive ability, we must treat these correlational results with caution. Experimental microbiome manipulations are needed to understand causal mechanisms linking cognition to the gut. One consideration for distinguishing between causation and correlation is understanding how the host's current and past physiological state interweave with its microbiome and behaviour. Downstream effects of developmental stress are well documented in birds [28,75,76], but little is known about avian microbiome development, and how abnormal microbiome development affects adult cognition. Despite these knowledge gaps, rodent models suggest developmental stress severely alters adult microbiome characteristics, cognitive processes and health [6,17,73,77]. It is possible that the unknown developmental conditions of our birds influenced microbiome dysbiosis and performance deficits. Future research can use repeated measures experiments assessing avian cognitive performance before and after gut microbiome manipulation by diet, disrupting the microbiome with antibiotics, administering probiotics, or inducing stress, especially during critical developmental stages. These studies will be crucial to understanding how the microbiome affects the brain and overall health of wild and captive animals.

Ethics. All animals were cared for in accordance with protocols approved by the Institutional Animal Care and Use Committee of Florida Atlantic University, permit no. A18-35.

Data accessibility. Raw sequences have been submitted to the Sequence Read Archive at the National Center for Biotechnology Information under BioProject PRJNA636961 and are accessible at https://dataview.ncbi.nlm.nih.gov/object/PRJNA636961. Snakemake files used for sequence analysis and R scripts for statistical analysis are available on GitHub (https://github.com/djbradshaw2/General_16S_Amplicon_Sequencing_Analysis) and viewable in a preprint at https://www.biorxiv.org/content/10.1101/2020.07.07.191254v1. All data have been submitted to Dryad and are accessible at https://dx.doi.org/10.5061/dryad.8gtht76mc [63].

Authors' contributions. M.C.S. designed the experiment, performed the cognitive testing, microbiome sampling, DNA extraction, and data analysis, and drafted and significantly revised the manuscript.

J.L.H. performed the PCR, drafted a portion of the manuscript and helped significantly revise the manuscript. D.J.B. contributed original code for data filtering and analysis, drafted a portion of the manuscript and helped significantly revise the manuscript. R.C.A. designed the experiment, contributed start-up funding and helped significantly revise the manuscript. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

Competing interests. We declare we have no competing interests.

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