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Assessing longitudinal gut microbiome dynamics in relation to age and senescence in a wild animal population

Sarah F. Worsley (s.worsley@uea.ac.uk)
University of East Anglia
Charli S. Davies
University of East Anglia
Chuen Zhang Lee
University of East Anglia
Maria-Elena Mannarelli
University of East Anglia
Terry Burke
University of Sheffield
Jan Komdeur
University of Groningen
Hannah L. Dugdale
University of Groningen
David S. Richardson
University of East Anglia

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Abstract

Background

In humans, gut microbiome (GM) differences are often correlated with, and sometimes causally implicated in, ageing. However, it is unclear how these findings translate in wild animal populations. Furthermore, studies that investigate how GM dynamics change within individuals (as opposed to among individuals), and with declines in physiological condition, are needed to fully understand links between chronological age, senescence, and the GM, but have rarely been done. Here, we use longitudinal data collected from a closed population of Seychelles warblers (*Acrocephalus sechellensis*) to investigate how bacterial GM alpha diversity, composition, and stability are associated with chronological age and the year leading up to death. We hypothesise that GM diversity and composition will differ, and variability will increase, in older adults, particularly in the terminal year prior to death, as it becomes dysregulated due to host senescence.

Results

GM alpha diversity and composition remained largely invariable with respect to adult age and did not differ in an individual's terminal year. Furthermore, there was no evidence that the GM became more heterogenous in senescent age groups (individuals older than 6 years), or in the terminal year. Instead, environmental variables such as season, territory quality, and time of day, were the strongest predictors of GM variation in adult Seychelles warblers.

Conclusion

We found no evidence to suggest that host senescence is associated with GM restructuring within a natural population. This contrasts with studies on humans, captive animal populations, and some (but not all) studies on non-human primates, suggesting that GM deterioration may not be a universal hallmark of senescence in wild animal species. Further work is needed to disentangle the factors driving variation in GM-senescence relationships across different host taxa.

Introduction

Senescence - the decline in function with age - occurs in most organisms and results in substantial reductions to health and fitness [1]. Even within a single population of a species, considerable variation may exist in the age at which individuals begin to senesce and the rate at which senescence occurs [1, 2]. Determining the biological processes that contribute to this individual variation may have implications for extending the health span of individuals living in ageing populations and could improve our understanding of the evolution of senescence.

One factor that has received increasing interest for its possible role in senescence is the vertebrate gut microbiome (GM). The GM is a highly diverse microbial ecosystem that plays a significant role in many aspects of host physiology, including digestion, cognition, and immunity [3–5]. However, it is also tightly regulated by the host, for example via gut epithelial cell function and the host immune system [6–8]. As host systems deteriorate with age, due to the accumulation of molecular and cellular damage, the GM may become increasingly dysregulated; this could, in turn, have negative consequences for host health and exacerbate further functional declines in other host systems [9–11]. Thus, the association between host senescence and the GM could involve complex interactions that make it difficult to disentangle cause from consequence [10, 11].

Corresponding with this, a growing body of literature has identified age-related changes in the GM, particularly in human and captive animal populations. For example, several studies have reported a loss of bacterial diversity and a corresponding increase in the abundance of proinflammatory bacterial taxa in the GM of older individuals [e.g. 12–15]. These changes have been correlated with chronic inflammation, impaired intestinal integrity, and increased mortality risk [16–18]. Furthermore, experiments on captive killifish (*Nothobranchius furzeri*) have shown that recolonising the GM of older individuals with bacteria from young donors can reverse functional declines and extend their lifespan, suggesting that the GM can play a causal role in host ageing [14]. However, the extent to which these findings can be generalised to wild animals remains unclear given a range of confounding factors related to age and captivity [19].

In humans, a variety of lifestyle factors can correlate with age (and thus apparently senescence) whilst also impacting the GM. For example, elderly individuals are more likely to take medication, experience malnutrition, and enter residential care, all of which can directly alter GM composition via processes that are independent of senescence [19, 20]. Extrapolating from studies on laboratory animals is also difficult because they often live in highly controlled environments and have very low genetic diversity. As such, they frequently harbour low diversity GM communities that differ radically in composition compared to their wild counterparts [21–23]. Furthermore, captive lines often exhibit significant differences in longevity compared to wild animals [24]. Given these discrepancies, an assessment of the extent to which GM imbalances are associated with age and senescence in wild hosts is warranted.

Whilst the majority of research on wild animal GMs has focussed on early-life development [e.g. 25–27], several recent studies on mammalian species have sampled the GM of older, post-prime individuals. However, no clear consensus has emerged from this research. Whilst some studies have reported shifts in GM composition [28, 29] and greater GM heterogeneity with increasing age [30], others have found that GM dynamics and overall composition remain largely invariable throughout adulthood [31–33].

One limitation of the studies done to date is that they have all focussed on changes associated with chronological age. To our knowledge, none have also included information about the biological condition of individuals. Individuals vary in the rate at which they undergo physiological deterioration in later life but die when this damage reaches a particular threshold [34]. This means that, although senescence can be correlated with chronological age, it may depend more strongly on the rate of damage accumulation.

Thus, incorporating a measure of biological condition, for example 'time to death' (i.e. assessing time backwards from death instead of forwards from birth), could be more informative than chronological age when considering variation in senescent declines across individuals [34, 35].

Most wild studies also take a cross-sectional approach to examining GM changes with respect to age [but see 30, 32], by comparing samples from older adults to those from different, younger individuals [28, 29, 31]. However, as senescence is a process that occurs within individuals, repeated measures from the same individual are needed, alongside accurate death dates, to properly ascertain the extent to which the GM changes in association with host senescence in the run up to death [2, 36]. This is because cross-sectional analyses can be confounded by the selective disappearance of individuals with particular traits which can mask patterns of change occurring at the within-individual level [2, 37]. Furthermore, since all studies on the late-life GM in wild animals have been conducted using mammalian systems (the majority on primates) investigations of other taxonomic groups are needed to assess whether patterns are consistent across host species.

Using the long-term study of the Seychelles warbler (*Acrocephalus sechellensis*), we expand on previous research by investigating the extent to which age and senescence predict changes in the GM of a wild, non-mammalian host. The Seychelles warbler population on Cousin Island is an excellent system to study senescence as the majority of individuals in this closed population are colour ringed (> 96% since 1997), enabling longitudinal monitoring across their lives [2, 38]. In many natural populations, most animals disperse or die before senescence can be measured, making it challenging to study this in wild animals [2]. However, there is virtually no migration into or out of the Cousin Island population [39] meaning that repeated sampling and accurate measures of survival can be achieved. Due to a lack of natural predators, a benign climate, and limited human disturbance, there is also very little extrinsic mortality within the Cousin Island population [2, 40]. As such, Seychelles warblers can reach a remarkably old age for a passerine species (a maximum lifespan of 19 years), although substantial variation in longevity exits between individuals, with the median lifespan at fledging being 5.5 years [41, 42]. Previous research on this population has identified senescent declines in fitness components, with individuals older than six years demonstrating a gradual decline in survival probability [43] and reproductive success [44].

Here, we use faecal samples collected across six consecutive years to investigate whether the Seychelles warbler GM shows signatures of change with age and senescent declines. The Seychelles warbler GM varies between juvenile and adult individuals [6, 45]. However, in these previous studies, all adults (ages l-17 years) were grouped together into one age class as there were not enough longitudinal samples to investigate within individual dynamics in later life. We now have more samples (including within individual repeat samples) enabling a thorough investigation of how GM dynamics change with age, and in the time leading up to death.

We first tested whether GM alpha diversity and overall GM composition (beta diversity) change with increasing chronological age. We hypothesised that GM alpha diversity- the number and evenness of

bacterial taxa within the GM - will be lower in older adults, consistent with previous studies on humans and captive animals [12, 14, 15]. We also hypothesised that GM composition will differ between senescent individuals and younger adults, with a decrease in core bacterial taxa and an increase in the abundance of potentially proinflammatory groups identified in other systems [11]. Furthermore, we predicted that these differences will be particularly pronounced in an individual's terminal year (the year before death) as host condition deteriorates more rapidly due to senescence [34]. However, convergence on a typical "old" GM composition may be unlikely if senescence results in dysregulation and GM instability; instead, individual GMs may follow divergent trajectories. Thus, we also tested for greater intra- and inter-individual variation amongst samples taken in older age groups. Such heterogeneity has previously been identified in humans [15, 46] and wild macaques (*Macaca assamensis)* [30]. We extended this research by also testing whether GM variation is greatest during the terminal year due to greater instability.

Methods

Study species and sample collection

Samples were collected from a population of Seychelles warblers on Cousin Island (29 ha; 04° 20' S, 55° 40' E) which consists of *ca* 320 adult individuals distributed across *ca* 115 territories [41, 47]. Virtually all individuals have been marked with a unique combination of a British Trust for Ornithology (BTO) metal ring and three plastic colour rings enabling longitudinal monitoring throughout their lives [48]. Population monitoring is carried out in the minor (January-March) and major (June-September) breeding seasons of each year [42, 49]. The annual resighting probability for adult individuals is very high (0.98 ± 0.01) [50] and dispersal from the island is virtually absent [39]. Thus, if an individual is not seen in a particular breeding season it is assumed to be dead rather than having dispersed from the island, providing accurate survival data. Average annual survival probability is exceptionally high in adults (0.84 ± 0.04 SE) and juveniles (0.61 ± 0.09) compared to other passerine species [40]. Survival was assessed each breeding season (until the end of the major season of 2023) and, for individuals that died during the study, death date was allocated as the final day of the breeding season in which the bird was last seen.

Faecal sampling took place over ten breeding seasons (six major, and four minor seasons) from 2017–2022. Individuals were caught in mist nets and placed into a disposable, flat-bottomed paper bag containing a sterilised weigh boat protected by a metal grate. This established protocol [6, 51] allows faecal matter to fall into the tray and reduces chances of contact with the bird's surface. Birds were removed from the bag after defecation or after 30 mins. Faecal samples were collected using a sterile flocked swab and placed into a microcentrifuge tube containing 1 ml of absolute ethanol. Control swabs taken from fieldworker hands and collection bags were also collected at time of sampling. All samples were stored at 4°C for the remainder of the field season before being transferred to -80°C for long-term storage.

Prior to release, a blood sample was taken from the bird via brachial venipuncture and stored in absolute ethanol at 4°C. DNA was subsequently extracted from blood samples using the DNeasy Blood and Tissue kit (Qiagen, Crawley, UK); this was used for molecular sexing via a PCR-based method [42, 52]. Age was calculated based on a combination of lay, hatch, or fledge date at point of first capture for most birds. If these dates were unknown, age was estimated based on eye colour which changes from grey in fledglings to red-brown in adults [53].

Every breeding season, an index of quality was calculated for each territory on the island. As Seychelles warblers are insectivorous, this is based on the number of insect prey available, the territory size, and the foliage cover during that breeding season [see 53]. For territories with missing scores in a season, quality was calculated as the mean of the scores for that territory in the preceding and following sampling period of the same season type.

Microbiome extraction and sequencing

Total genomic DNA was extracted from all faecal and control samples using the DNeasy PowerSoil kit (Qiagen) according to a modified version of the manufacturer's instructions [see 6]. Samples were randomised across extractions. Extracted DNA was submitted for 16S rRNA gene amplicon sequencing at the NEOF Centre for Genomic Research (Liverpool, UK). In total, 1015 samples were submitted for sequencing of which 969 were derived from faecal samples. There were also 21 collection controls (from hands and sample bags), 15 negative extraction blanks (approximately two per extraction kit), and 10 positive controls (at least one per sequencing run). Positive controls consisted of DNA extracted from a ZymoBIOMICS Microbial Community Standard (D6300). Amplicon sequencing libraries were generated using the V4 primers 515F and 806R [see 6 for further information regarding the sequencing protocol]. Libraries underwent 2x 250 bp, paired-end sequencing on an Illumina MiSeq platform. Samples were sequenced across seven runs. To check for batch effects, 69 samples were sequenced twice either within the same, or across different, runs. Additionally, 10 samples were extracted (and sequenced) twice within the same run to check that the extraction protocol was repeatable [previously confirmed in 45].

Bioinformatic processing of sequencing data

All sequencing reads were processed using QIIME2 2019.10 [54]. Forward and reverse reads were truncated at 240 bp, and low quality base calls were trimmed from the 5' end using the DADA2 plugin [55]. Amplicon Sequencing Variants (ASVs) were then inferred for each sample, followed by dereplication and pair-end joining. Putative chimeras and singleton reads were also removed at this stage. Following processing in DADA2, files from the seven separate sequencing runs were merged. ASVs were taxonomically classified by training a naïve-Bayes classifier on the SILVA 132 reference database for 16S rRNA gene sequences. ASVs classified as chloroplast or mitochondria were removed. A mid-point rooted phylogeny was then constructed using MAFFT [56] and the Fast Tree [57] approach. The final ASV, taxonomy, and tree files were exported from QIIME2 into R 4.2.2 [58] using *phyloseq* 1.42.0 [59].

Once imported, ASVs were filtered to remove non-bacterial sequences and those unassigned at phylum level. Eight bacterial taxa were present in each of the positive controls; their identity matched those listed

in the commercial mock community. Potential contaminants were identified and removed from faecal samples using the prevalence method in *decontam* 1.18.0 [60]. First, contaminants introduced from laboratory steps were identified using extraction blanks as a reference; 32 ASVs were identified and removed. Second, controls collected from fieldworker hands and collection bags were used as a reference to identify putative contaminants introduced at the sampling stage. A total of 5,983 ASVs were conservatively filtered as possible contaminants. Following filtering, 51,360 ASVs remained across the 969 faecal samples. Faecal samples with fewer than 8,000 reads (27 samples) were subsequently removed following an assessment of sample completeness and rarefaction curves generated using *iNEXT* 3.0.0 [61]. These samples all had very low DNA concentrations. As a final filtering step, ASVs with fewer than 50 reads in total across all samples were removed prior to downstream analysis as these may represent possible sequencing errors. A total of 23,151 ASVs remained across 942 faecal samples (mean ASVs per sample = 225.62 ± 5.29 SE). Despite the loss of many ASVs only 1% of sequencing reads were removed by the abundance filtering step.

Statistical analyses

GM alpha diversity analysis

Faecal samples were rarefied to a depth of 8,000 reads prior to alpha diversity analysis to control for variation in library size across samples. The observed ASV richness (the number of ASVs) and Shannon diversity index (which accounts for the evenness of ASV abundances) were calculated for each sample using *phyloseq* 1.42.0 [59]. To check for batch effects, pairwise Euclidean distances were calculated between samples that had been sequenced/extracted twice, based on their Shannon diversity index. Shannon diversity was consistent for samples sequenced twice within and across sequencing runs and for duplicate extractions of the same samples included in the same run (Additional file 1: Figure S1a). As such, duplicates of the same faecal sample were filtered to retain the sample with the highest read count for downstream analyses. Where multiple samples had been taken from the same individual during the same catch, only a single sample was retained; samples were prioritised if they had been taken from the sterile sampling tray instead of the bag or, if both samples were taken from the same location, the one with highest read count was retained (58 samples removed). Samples from “floater” individuals were also removed as these individuals have no assigned territory and territory quality was controlled for in downstream analyses (12 samples removed). Finally, as we were interested in the association between the GM and senescence, only samples from adults (individuals > 1 year of age) were retained for this analysis. A total of 16,578 ASVs (mean number of ASVs per sample = 161.58 ± 5.14 SE) remained across 462 samples from 273 adult individuals in the final rarefied dataset. Each adult individual has between one and six sequenced samples; 129 individuals (47%) have > 1 sample, and 43 individuals have > 2 samples (16%) (Additional file 1: Figure S2).

To establish whether alpha diversity varied according to host age in adulthood, Generalised Additive Mixed Models (GAMMs) with a Gaussian (for Shannon diversity) or negative binomial (for observed ASV richness) distribution were constructed using *mgcv* 1.8.42 [62]. This enabled evaluation of possible non-

linear relationships between age and alpha diversity metrics which may be likely in the case of senescence. Age at sampling was included as a continuous smoothed term in the model. Time of sampling (minutes since sunrise at 06:00 am) and the number of days samples were stored at 4°C in the field were also included as smoothed terms in the model, since the GM can demonstrate circadian dynamics [33] and alpha diversity can be influenced by sample storage methods [63, 64]. Smoothed terms were included by fitting cubic regression splines; these are appropriate as they incorporate information on data density when calculating smoothing knots, meaning that periods of missing data don't generate unreliable trends. Sex (male or female), season (major or minor) and two metrics of territory quality were included as linear parametric terms in the model. The two metrics of territory quality were i) the mean territory quality (across all territories) for the sampling period and ii) deviation of each territory score from the overall mean in that sampling period (mean-centred territory quality). This method enabled us to test whether alpha diversity varied according to differences in overall territory quality across years, and/or variation in territory quality across the island within a particular sampling period [37]. To further test whether changes in alpha diversity were associated with host senescent declines, we included a term denoting whether a sample was taken in the bird's terminal year (yes or no) as an additional parametric fixed term. A total of 106 samples (out of 462) were taken in a bird's terminal year. Bird ID was included as a random effect in the model to control for repeated sampling of individuals. We tested for age dependent effects of sex and terminal year by including interaction terms in the model, however, for all interactions described herein, these terms were removed sequentially if not significant (in order of least significance) to enable interpretation of the main effects. Variance Inflation Factors (VIF) were < 3 for all fixed effects and terminal year samples were not restricted to the oldest individuals (Additional file 1: Figure S2) suggesting collinearity was not an issue in the model.

Compositional (beta diversity) analysis

Unrarefied reads were used, and ASVs were filtered to remove rare taxa that occurred in < 5% of samples as these can disproportionately influence beta diversity metrics. ASV abundances were then transformed using the Centred Log Ratio (CLR) transform function in *microbiome* 1.20.0 [65] which controls for differences in library size and is appropriate for compositional datasets [66]. Batch effects were checked in the same way as for alpha diversity but using a matrix of pairwise sample Aitchison distances calculated using the CLR transformed ASV abundances in *vegan* 2.6.4 [67]. Beta diversity was consistent for samples sequenced twice within and across sequencing runs and for duplicate extractions of the same sample (Additional file 1: Figure S1b). As such, samples were filtered as described above. A total of 674 ASVs were retained across the remaining 462 samples from 273 adult individuals.

To quantify whether overall GM composition varied in association with host age, a marginal permutational analysis of variance (PERMANOVA) was performed on pairwise Aitchison distances using the *adonis2()* function within *vegan* 2.6.4 [67], with 9,999 permutations. As with alpha diversity analyses, host age, time of day, days stored at 4°C, sex, season, mean territory quality for a sampling period, mean-centred territory quality, and a terminal year term were included as variables in the model. BirdID was included as a blocking factor to control for repeated sampling. We also tested age*sex and age*terminal

year interactions as described above. Differences in GM composition were visualised using Principal Components Analysis (PCA).

Changes in the abundance of core taxa with age

To understand whether abundances of individual bacterial taxa changed during adulthood, we modelled the abundances of 54 core bacterial genera (defined as those found in at least 50% of adult samples and at a minimum relative abundance of 0.01%) which represented 63% of adult sequencing reads. A Generalised Linear Latent Variable Model (GLLVM) was applied to CLR-transformed taxon abundances using *gllvm* 1.4.1 [68]. The model was fitted with a Gaussian distribution and two latent variables (default). GLLVMs are a form of joint species distribution model which model the response of species to explanatory variables whilst accounting for correlations between the different species' abundances [68]. The same predictor variables were included as for beta diversity analysis. Bird ID was included as a random effect. As GLLVMs only model linear effects we also ran GAMM analyses with the same parameters to visualise any non-linear trends for taxon abundances in association with host age.

GM personalisation and stability

Pairwise Aitchison distances between samples were scaled to similarity values that ranged between zero and one using the following formula: similarity = 1- (distance/maximum distance). A value of one would indicate that samples are identical in terms of GM composition. We then modelled these pairwise GM similarities using dyadic Bayesian regression models in *brms* 2.19.0 following methods described in [69]. Models were run on a High-Performance Computing Cluster at the University of East Anglia using a different version of R (R *3.6.2*) because of memory constraints.

To assess if inter-individual differences in GM composition increase in older age groups (i.e. the GM becomes more personalised), we assigned samples taken from adults to different age classes: young adult (Y, 1–3 years), middle-aged adult (M, 3–6 years), or old adult (O, > 6 years). We then modelled pairwise Aitchison similarities calculated between each individual and other members of its own age group by including a dyadic age comparison term in the model (YY, MM, or OO). Lower GM similarity in the OO comparison group (pairwise comparisons between old adults) would indicate increased GM personalisation with age. To reduce the complexity of the model, we only included one sample taken at random per individual and, between individuals, samples that were taken no more than one year apart. This left 4867 pairwise comparisons (2349 = YY; 1883 = MM; 635 = OO). The number of samples taken in the same (major-major or minor-minor) or different (major-minor) seasons were approximately equal within the same age group comparison, but the number of days between sampling points (temporal distance) was included in the model as a control variable. We also included a three-level factor in the model indicating whether each pairwise comparison was made between two terminal year samples (TT, N = 385 pairwise comparisons), between two non-terminal samples (NN, N = 2570), or between a terminal year and non-terminal year sample (NT, N = 1912). We expect GM similarity to be lowest in the NT and TT comparisons if the GM becomes more personalised/unstable in the last year of life due to senescence or pathology. Finally, a sex comparison term (0 = different sex, 1 = same sex) was also included as a

covariate in the model. All numerical covariates were scaled between zero and one to make model estimates comparable. To control for the non-independence of datapoints, whereby each sample occurs in multiple comparisons, we fitted a multi-membership random intercept [following 69] which captures the samples included in each dyad (SampleID_A + SampleID_B). Models were run using a beta error distribution and a logit link function. To penalize extreme estimates, regularising priors were assigned as follows: $\beta \sim$ normal(0, 1) for fixed effects; $\Phi \sim$ gamma(1, 0.1) for the dispersion parameter; student-t(3, 0, 2.5) for intercept terms. Prior choice did not impact results but ensured model convergence. We ran 8000 iterations, with 2000 warmup iterations, on 4 chains. The thinning parameter was set to two. Convergence was assessed by inspection of caterpillar plots and Rhat values ≤ 1.01.

To investigate whether GM composition becomes less stable *within* individuals with increasing age we ran the same analysis as above, but only included pairwise comparisons of samples collected from the same individual within each age group. There were 155 pairwise comparisons in this analysis (56 = YY; 58 = MM group; 41 = OO). The model included the dyadic age comparison term and temporal distance as fixed effects and the sample level multi-membership random effect. A terminal comparison term was not included as individuals were not sampled densely enough for there to be enough terminal comparisons (there were only 15 TT comparisons, and most were in young adults). Priors and model conditions were the same as above except that only 4000 iterations (with 2000 warmup iterations) were run with no thinning due to fewer samples.

Results

GM alpha diversity does not vary with age or in the terminal year

A total of 462 GM samples were collected from 273 adult individuals over the sampling period (Additional file 1: Figure S2); 211 of these samples were collected from females (N = 128 individuals), 251 from males (N = 145 individuals). Age at sampling ranged between 1–15.6 years for females (mean age = 4.2 ± 0.2 SE) and 1–17.2 years for males (mean = 4.2 ± 0.2 SE), respectively. A total of 98 samples (N = 43 female, N = 55 male) were collected from putatively senescent individuals > 6 years of age. Furthermore, there were 106 samples (44 from females, 62 from males) taken in an individual's terminal year prior to death (Additional file 1: Figure S2). Terminal samples were from individuals of different ages (Additional file 1: Figure S2), ranging from 1–15.6 years in females (mean age = 5.1 ± 0.6 SE) and 1–17.2 (mean 4.3 ± 0.4 SE) in males, respectively. As such, there was substantial variation in the dataset with which to conduct a powerful analysis of the effects of chronological age and senescence on the GM.

There was no relationship between chronological age and observed ASV richness (Table 1) or Shannon diversity (Additional file 1: Table S1) in adult Seychelles warblers. There was also no evidence that alpha diversity differed between samples taken in the terminal year of life and those taken in a non-terminal year during adulthood (Tables 1 & Additional file 1: Table S1). Also, the interaction term between chronological age and terminal year was not significant in either model (Additional file 1: Tables S2 &

S3). This suggests that GM alpha diversity did not vary according to age, or senescence, in adult Seychelles warblers.

Observed ASV richness was significantly lower in males than females (Table 1). A similar trend was identified between sex and Shannon diversity, but this was not statistically significant (Additional file 1: Table S1). Since only Shannon diversity is weighted by ASV abundances, this suggests that the lower ASV richness observed in males compared to females was largely driven by the loss of rare taxa. There was a negative association between Shannon diversity and time stored at 4°C in the field (Additional file 1: Table S1). None of the other predictors were associated with changes in GM alpha diversity (Tables 1 & Additional file 1: Tables S1-S3).

Table 1
A Generalised Additive Mixed Model (GAMM) investigating the relationship between age, terminal year and observed ASV richness in the gut microbiome of adult Seychelles warblers (N = 462 sample, 273 individuals). Significant (*P* < 0.05) predictors are shown in bold. Reference categories for categorical variables were as follows: female (sex), major (season), and yes (terminal year). Adjusted R^2 = 0.04.

Predictor	**Estimate**	**SE**	***z***	***P***
Intercept	5.193	0.044	118.255	< 0.001
Sex (male)	**-0.107**	**0.046**	**-2.313**	**0.021**
Season (minor)	0.040	0.070	0.570	0.569
Mean territory quality	-0.122	0.064	-1.904	0.057
Mean-centered territory quality	-0.079	0.064	-1.226	0.220
Terminal year (no)	0.031	0.054	0.574	0.566
Smoothed terms	**edf**	χ^2	**P**	
Age	1.000	1.566	0.211	
Time of day	1.659	2.140	0.355	
Time at 4°C	1.002	2.380	0.123	
Random	**462 observations**		**variance**	
Bird ID	273 individuals		0.120	

GM composition does not vary strongly with age or in the terminal year

We next explored whether overall GM composition differed with age, and between terminal year and non-terminal year samples collected during adulthood. A PERMANOVA analysis of CLR-transformed ASV abundances showed that there was a very weak, marginally significant association between age and GM

composition (Table 2), however, age only explained an extremely low proportion of the overall variance in GM composition (R^2 = 0.002, Table 2). Consistent with this, sample points showed very little clustering according to age on a PCA ordination plot (Fig. 1a). Furthermore, samples collected from adults in their terminal year versus those from a non-terminal year did not differ in terms of their GM composition (Table 2) indicating no association between senescence and changes in GM structure. An interaction term between adult age and terminal year was also not significant (Additional file 1: Table S4).

Table 2
A PERMANOVA analysis of associations between age, terminal year, and gut microbiome composition in adult Seychelles warblers. The analysis was performed using Aitchison distances calculated using centred log ratio (CLR)-transformed amplicon sequencing variant (ASV) abundances. Significant predictors (*P* < 0.05) are shown in bold. N = 462 samples from 273 individuals. Bird ID was included as blocking factor to control for repeated measures.

Predictor	Df	R^2	*F*	*P*
Age	**1**	**0.002**	**1.134**	**0.046**
Sex	1	0.002	1.106	0.619
Mean centred territory quality	1	0.003	1.409	0.574
Mean territory quality	**1**	**0.006**	**2.722**	**< 0.001**
Season	**1**	**0.009**	**4.069**	**< 0.001**
Time of day	**1**	**0.008**	**3.685**	**< 0.001**
Time at 4°C	**1**	**0.007**	**3.158**	**0.005**
Terminal year	1	0.002	0.906	0.988

Host sex was not significantly associated with differences in GM composition (Table 2) despite an association between sex and GM richness (Table 1). Instead, environmental variables were the strongest predictors of GM composition in adults; GM composition varied significantly according to differences in territory quality between field periods (Table 2, Fig. 1b), season (Table 2, Fig. 1c), and the time of day at which an individual was sampled (Table 2, Fig. 1d). Clustering along the PC1 and PC2 axes of a PCA ordination plot was primarily associated with seasonal differences (Fig. 1c) and variation in territory quality (Fig. 1b), respectively, whilst clustering along the PC3 axis was associated with the time of day at which samples were collected (Fig. 1d). The number of days each sample was stored at 4°C in the field was also a significant predictor of GM composition (Table 2); points clustered along the PC1 and PC2 axis of a PCA ordination according to whether samples were stored for less, or more than, 30 days at 4°C (Additional file 1: Figure S3).

Changes in taxon abundance

To identify whether the abundance of specific microbial genera (rather than overall GM composition) changed with age in adult Seychelles warblers, we applied a GLLVM model to 54 core genera (those present in at least 50% of adults). Only six core genera showed a significant association with host age; the genera *Kineococcus*, *Pseudonocardia*, *Quadrisphaera* and one genus in the family *Micromonosporaceae* showed an increase in abundance with age, whereas the genus *Gordonia* and one genus in the family *Ruminococcaceae* decreased with age in adult Seychelles warblers (Additional file 1: Figure S4). These findings were consistent with the output of GAMM models which identified the same linear trends for each of these taxa, apart from *Ruminococcaceae* which showed a significant, weakly non-linear, decrease in abundance with age (Fig. 2, Additional file 1: Table S5). Significant taxa were present at relatively low abundances within samples: *Kineococcus* (0.24% ± 1.05%, mean relative abundance across samples ± SD); *Pseudonocardia* (0.89% ± 1.94%); *Quadrisphaera* (0.30% ± 0.62%), *Gordonia* (0.54% ± 1.05%); *Micromonosporaceae* genus (0.30% ± 1.02%); and the *Ruminococcaceae* genus (1.07% ± 1.89%).

Only two genera were differentially abundant in the terminal year of life in adults in the GLLVM model (Additional file 1: Figure S4); *Friedmanniella* and *Microbacterium* were both present at greater abundance in terminal year samples. Many more genera were associated with environmental variables (Additional file 1: Figure S5). For example, 20 core genera demonstrated a significant change in abundance according to season, whilst 11 core genera changed in association with the time of day and mean territory quality terms, respectively (Additional file 1: Figure S5).

GM personalisation and stability

The GM may not converge on a typical “old” composition if it becomes increasingly dysregulated with age. Instead, inter-individual variation may increase with age as the GM follows different, more unstable, trajectories. We found no evidence that GM samples taken from different individuals were compositionally less similar when pairwise comparisons were made between two old adults, versus two middle aged or two young adult individuals (Fig. 3a, Additional file 1: Table S6). This indicates that the Seychelles warbler GM does not become more personalised in older individuals. Furthermore, there was no difference in GM similarity between comparisons involving samples taken from birds in their terminal year, versus those taken in a non-terminal year (Fig. 3a, Additional file 1: Table S6). Thus, GM personalisation did not increase the year before death. Only the time interval (in days) between samples was significantly negatively associated with GM similarity (Fig. 3a, Additional file 1: Table S6).

Relationships observed between individuals may not reflect patterns of change within individuals [37] and can be confounded, for example by the selective disappearance of individuals with extreme GM communities. However, an analysis of within individual GM similarities revealed very similar patterns to the between individual analysis. GM similarity in middle-aged sample comparisons (MM posterior mean − 0.025, CI= -0.228−0.173) and young adult sample pairs (YY posterior mean − 0.080, CI -0.286−0.124), did not differ statistically from that of sample pairs taken when an individual was in the old adult age group (Fig. 3b, Additional file 1: Table S7). This indicates that, within individuals, the warbler GM does not become more unstable with increasing host age. As with between individual comparisons, the time

interval between samples was negatively associated with GM similarity within the same individual (Fig. 3b, Additional file 1: Table S7).

Discussion

We used longitudinal data collected from Seychelles warblers to investigate the association between host age, senescence, and GM characteristics. We found no evidence of senescent declines in the GM; both bacterial alpha diversity and composition remained largely invariable with respect to age in adults and did not differ in an individual's terminal year. Instead, environmental factors, including season and variation in mean territory quality across the study period, appeared to have the greatest impact on the GM during adulthood. Within individuals, we also found no evidence of increased GM personalisation or instability in older age groups, even in the terminal year. This is despite including some relatively very old individuals in the dataset (the oldest individual was c.a. 17 years of age, Additional file 1: Figure S2).

Seychelles warblers have a median lifespan at fledging of 5.5 years [41, 42] and, at the population level, there is evidence of reproductive and survival senescence from approximately six years of age [43, 44]. In our analysis, we included samples from 66 individuals older than six years of age. Of these, 37 individuals had at least two samples taken longitudinally, including 19 individuals with samples taken before and after 6 years of age. There was a maximum of 4.8 years between longitudinal samples for putatively senescent individuals (mean 1.4 years ± 0.2 SE, Additional file 1: Figure S2). These sample sizes are either comparable to, or greater than, the few studies that have identified statistically significant effects of age on GM structure in other wild systems (e.g. Bennett et al. (2017), N = 14 post-prime individuals sampled cross-sectionally; Trosvik et al. (2018), N = 70 post-prime individuals sampled cross-sectionally; Sadoughi et al. (2022), N = 11 senescent individuals sampled longitudinally over 1.5 years). Furthermore, our study also included 97 individuals (out of a total of 273 adults) that had a sample taken in their terminal year of life allowing us to additionally test for changes in the GM close to death. Thus, this was a robust dataset with which to investigate the relationships between host age, senescence, and the GM. As such, the absence of statistical significance is unlikely to be due to a lack of power to detect effects that are large enough to be biologically meaningful to the host.

A wealth of studies on humans have identified a decline in bacterial diversity and shifts in GM composition in older age groups [e.g. 13, 15, 20]. Only a few studies have been undertaken in the wild however, cross-sectional studies on lemurs (*Lemur catta*) [28] and geladas (*Theropithecus gelada*) [29] have also demonstrated shifts in GM composition, although not GM alpha diversity, between reproductively mature and post-prime adult individuals. We found no statistical evidence of an association between age and GM alpha diversity, and only identified extremely limited shifts in GM composition in adult Seychelles warblers. This was the case even after controlling for the possibility of differential rates of damage accumulation by investigating GM differences in the terminal year of life [34]. This is consistent with the findings of several other studies on wild non-human primate populations in which GM diversity and composition remained largely invariable with respect to chronological age during adulthood [31, 32]. However, these analyses were either cross-sectional with small sample sizes

for old individuals (N = 12 samples from old individuals in [31]) or, where longitudinal sampling did take place, did not control for the time interval between sampling points when comparing GM similarity [32]. Cross-sectional analyses can suffer from issues such as selective disappearance which mask processes occurring within individuals, and GM turnover can confound comparisons made between samples taken at different time points. A recent study on wild meerkats also demonstrated that GM diurnal cycling remained consistent with chronological age, suggesting that functionality is largely maintained even in old individuals [33]. However, although repeat samples were taken from individuals, this study did not investigate within individual dynamics *per se*. Furthermore, none of the aforementioned studies controlled for differential damage accumulation by including a measure of biological condition such as time to death. Thus, we provide a more robust test of the association between age, senescence, and the GM and, by doing so, corroborate the results of these previous studies.

The discrepancy between studies on wild animals and those on humans might be explained, in part, by lifestyle and behavioural factors that change with age in human populations but that don't exist in wild systems. For example, in humans, medication intake and the probability of living in residential care increase with age, whilst physical activity and dietary quality decrease, all of which can directly impact the GM [70, 71]. It is possible that certain factors that impact the GM also vary with age in some wild mammalian species. For example, dental wear and tooth loss increase with age in some non-human primates [72, 73] which can, in turn, influence an individual's food choices and their ability to extract nutrients from dietary components [74]. However, Seychelles warblers ingest their food whole, feed almost exclusively on insects throughout their lives, and show no decline in foraging efficiency with age [75]. Thus, such effects are unlikely to be universal across wild species. This could potentially explain some of the observed variation in GM-ageing patterns identified amongst different wild taxa.

Our findings are also in contrast to studies on captive animals which have demonstrated shifts in overall GM composition with increasing age [e.g. 14, 16, 76]. However, captive animals are often housed in highly controlled conditions and frequently harbour unrealistically low levels of GM diversity [22, 77]. In wild systems, environmental variation can strongly impact the GM [33, 78, 79] and may override host intrinsic effects observed in the laboratory. Indeed, time of day, seasonal differences, and changes in mean territory quality were strong determinants of GM composition in Seychelles warblers, consistent with previous studies on this system [6, 45, 80]. GM similarity also declined with an increasing number of days between samples suggesting a high level of turn-over within the GM. Thus, although Seychelles warblers demonstrate both reproductive and survival senescence [43, 44], constant environmental uptake of microbes may override any largescale effects of senescence on the GM.

In addition to the lack of change in overall GM composition, only six (out of 54) individual core genera were associated with age in adult Seychelles warblers. One genus that decreased in abundance with increasing host age was in the family *Ruminococcaceae.* This is one of the most abundant families in the human GM [81]. Members of the *Ruminococcaceae* are obligate anaerobes, interact directly with the mucosal layer of the gut epithelium and produce important short chain fatty acids such as butyrate [82]. The abundance of this family has also been shown to decrease with age in several long-term human

studies [11, 83, 84] and reduced abundances have been associated with increased gut inflammation and chronic intestinal disorders such as Crohn's disease [85, 86]. Thus, it is possible that certain members of the GM are linked to senescent declines in the Seychelles warbler. However, this *Ruminococcaceae* genus made up a relatively small fraction of the overall GM (c.a. 1% average relative abundance in adults). Thus, further tests of its functionality, for example through metagenomic sequencing, would be needed to understand its role within the Seychelles warbler GM and whether a decline in its abundance in older age groups is biologically significant to the host.

Aside from *Ruminococcaceae*, the core genus *Gordonia* also decreased in abundance with increasing host age, while four other genera (*Kineococcus*, *Pseudonocardia*, *Quadrisphaera*, and a genus in the family *Micromonosporaceae*) increased with age. These genera are all in the phylum *Actinobacteria* which is widely distributed in the environment. They are also aerobic, and produce a range of natural products including antimicrobials [87–91]. It's possible that environmental bacteria may increase in abundance within the GM as host immune function declines with age and/or as other key bacterial genera, such as the *Ruminococcaceae*, are lost. However, due to their widespread distribution, it is also plausible that these bacteria are constantly acquired from the host's environment (e.g. via their diet) and may gradually accumulate and persist within the GM by outcompeting other microbes. Each actinobacterial genus that was associated with age was present at < 1% mean relative abundance in adults, suggesting that they may only play a very minor role within the gut ecosystem. Furthermore, despite these few patterns, the vast majority of core genera showed no association with age suggesting senescence was not associated with a large-scale restructuring of the GM.

Many more taxa were associated with variation in environmental factors, including time of sampling, season, and mean territory quality across sampling periods. This is consistent with other studies showing that environmental dynamics can play a significant role in structuring the GM of wild animals [33, 78, 79]. For example, a study on wild meerkats showed that diurnal shifts in GM composition outweighed an association between the GM and host chronological age [33]. Circadian GM dynamics have been identified in human and wild animal studies, and may be driven by differences in foraging regimes and dietary intake throughout the day [33, 92]. In the Seychelles warbler, the genera *Lactococcus* and *Enterococcus* showed the largest increases in abundance between samples collected in the morning and afternoon. Both genera are lactic acid producing bacteria and play an important role in dietary carbohydrate fermentation [93]; as such, these genera may gradually increase in response to an influx of nutrients as individuals start to forage [92]. However, both genera are also found in insect microbiomes [94–96] and so it is possible that these taxa accumulate passively as feeding increases throughout the day and are mainly transient colonisers of the GM that don't carry out a fermentative function.

Aside from time of day, differences in mean territory quality between sampling periods and season were also associated with GM differences. As territory quality (a measure of insect abundance) is likely to be linked to climatic factors, such as rainfall and temperature, which may also influence microbial abundances, it is possible that these GM differences were driven by differential exposure to microbes in the external environment. Seasonal GM differences could also be driven by similar processes. However,

variation in the GM could also be linked to physiological change associated with host stress under low quality conditions [97]. Furthermore, since Seychelles warbler predominantly reproduce in the major breeding season (June-September) some of the seasonal differences in adult GM composition could be linked to physiological changes associated with host reproduction [98, 99]. Further work, including dense sampling of the same individual pre- and post-reproductive attempts would be needed to understand if this is the case.

We found no evidence that changes in the GM were more extreme in an individual's terminal year of life when damage accumulation is expected to be at its greatest due to senescence, and only two genera were more abundant in terminal year samples (*Friedmanniella* and *Microbacterium*). These are both environmental microbes that are frequently isolated from insects [100, 101] and neither of them were associated with host age. The Seychelles warbler benefits from very low levels of extrinsic mortality during adulthood [40]. Indeed, an absence of natural predators, lack of human disturbance, and a relatively constant and benign climate, enables many individuals to reach an old age, providing the opportunity to detect and study senescence in this species [2]. Thus, although it is possible that a very small proportion of mortality in our study was stochastic, it is very unlikely that this would be at a level high enough to override any significant association between senescence and the GM. Therefore, the fact that we found little change in GM alpha diversity, composition, or stability in the terminal year, and that this relationship did not depend on age, suggests that the GM is largely unaffected by host senescence in the Seychelles warbler. Our previous work has shown that differential survival is associated with differences in both bacterial and fungal GM composition in the Seychelles warbler, however, in both cases, survival was assessed over much shorter periods post sampling (in most cases less than 3 months post sampling, but in some cases less than 5 months) [45, 80]. In this study we chose to look at changes over a longer period (the terminal year) to try to identify factors linked to senescence rather than the more immediate changes in the GM just prior to death which could be a consequence of a rapid decline in health. We expected senescent changes in the GM to accumulate gradually over longer periods, but we found no evidence that this was the case.

Aside from overall GM alpha diversity and composition, we found no evidence of increased GM personalisation, or reduced within-individual GM stability, with increasing age or in the year leading up to death in adult Seychelles warblers. This also contrasts with long-term studies on humans [15, 46, 102], and a recent longitudinal study on wild macaques (*Macaca assamensis*) [30], which identified increasing GM heterogeneity amongst individuals in older age groups. These studies were unable to determine the cause of these differences; whilst greater heterogeneity could be driven by reduced GM stability, it may also be driven by other factors such as reduced social contact amongst elderly individuals [11, 30]. Social interaction strength predicts GM similarity in wild mice, *Apodemus sylvaticus* [69], and reduced social network size has also been associated with lower GM diversity [69, 103]. However, social isolation may not be a hallmark of ageing in all species. Seychelles warblers are cooperative breeders that often live in groups consisting of a breeding pair and subordinate individuals, some of which may help with reproductive attempts [104–106]. The presence of subordinates does not decline as dominant breeding individuals age and, indeed, the recruitment of helpers increases in elderly females [41, 107]. Thus, there

is currently no evidence to suggest that warblers become less social with age. Consequently, there may be ample opportunity for microbes to be shared amongst individuals in this population regardless of age.

In conclusion, our study finds little evidence of senescent changes in the GM of the Seychelles warbler. Although the abundance of a very small number of individual genera were associated age, overall GM alpha diversity and composition remained stable throughout adulthood in this species. Instead, environmental factors were the major driver of GM differences amongst adult warblers. Whilst this contrasts with studies on humans and captive animals, our findings add to the growing body of literature reporting mixed effects of age in wild populations. Further work is needed to better understand whether variation in lifestyle and behavioural factors drive the observed variation in senescent changes in the GM of different taxa.

Declarations

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Authors' contributions

The study was conceived by SFW and DSR. SFW, CSD, CZL, and DSR performed the fieldwork. SFW, CSD, and MEM conducted the microbiome laboratory work. SFW performed the bioinformatics and statistical analyses and drafted the manuscript with input from DSR. DSR, HLD, JK and TB managed the Seychelles Warbler Project. All authors read and approved the final manuscript.

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Availability of data and materials

All 16S rRNA gene amplicon sequences have been submitted to the European Nucleotide Archive (ENA) database under the study accession numbers PRJEB45408 (samples taken in 2017 and 2018) and

PRJEB47095 (samples taken in 2019 and 2020) and PRJEB67634 (samples taken in 2021 and 2022). The scripts and metadata to reproduce all analyses and figures can be accessed via the GitHub repository, https://github.com/Seych elle-Warbler-Project.

Ethics approval and consent to participate

Fieldwork was carried out in accordance with local ethical regulations and agreements. The Seychelles Department of Environment and the Seychelles Bureau of Standards approved the fieldwork.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

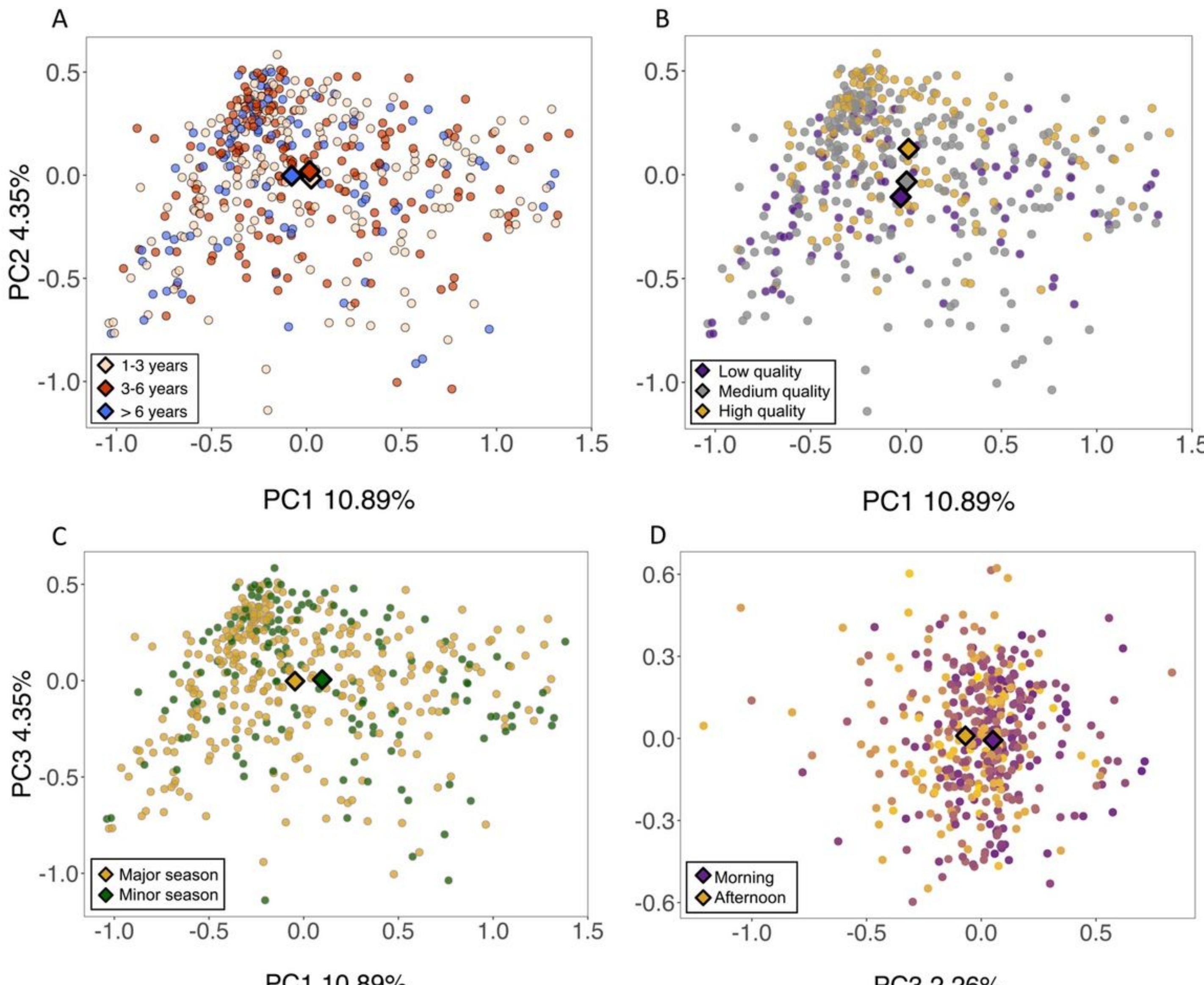

Figure 1

Shifts in gut microbiome composition according to A) host age B) mean territory quality C) season and D) time of day in adult Seychelles warblers. PCA ordination was carried out using Aitchison distances calculated on Centred Log Ratio (CLR)-transformed amplicon sequencing variant (ASV) abundances.

Each point represents a unique gut microbiome sample (N=462 samples from 273 individuals). Large diamonds represent the group centroids. For clarity, samples were grouped into discrete categories for plotting: A) Age: 1-3 years, 3-6 years, or >6 years; B) Territory quality: low (lower quartile <17136), medium (interquartile range), or high (upper quartile >36602); C) Season: major or minor; D) Time of day: samples collected in the morning (<6 hours after sunrise at 06:00 am) or afternoon (>6 hours after sunrise). Principal components 1, 2, 3 & 4 explained 10.9%, 4.4%, 2.3% and 1.9% of the variation in gut microbiome structure, respectively.

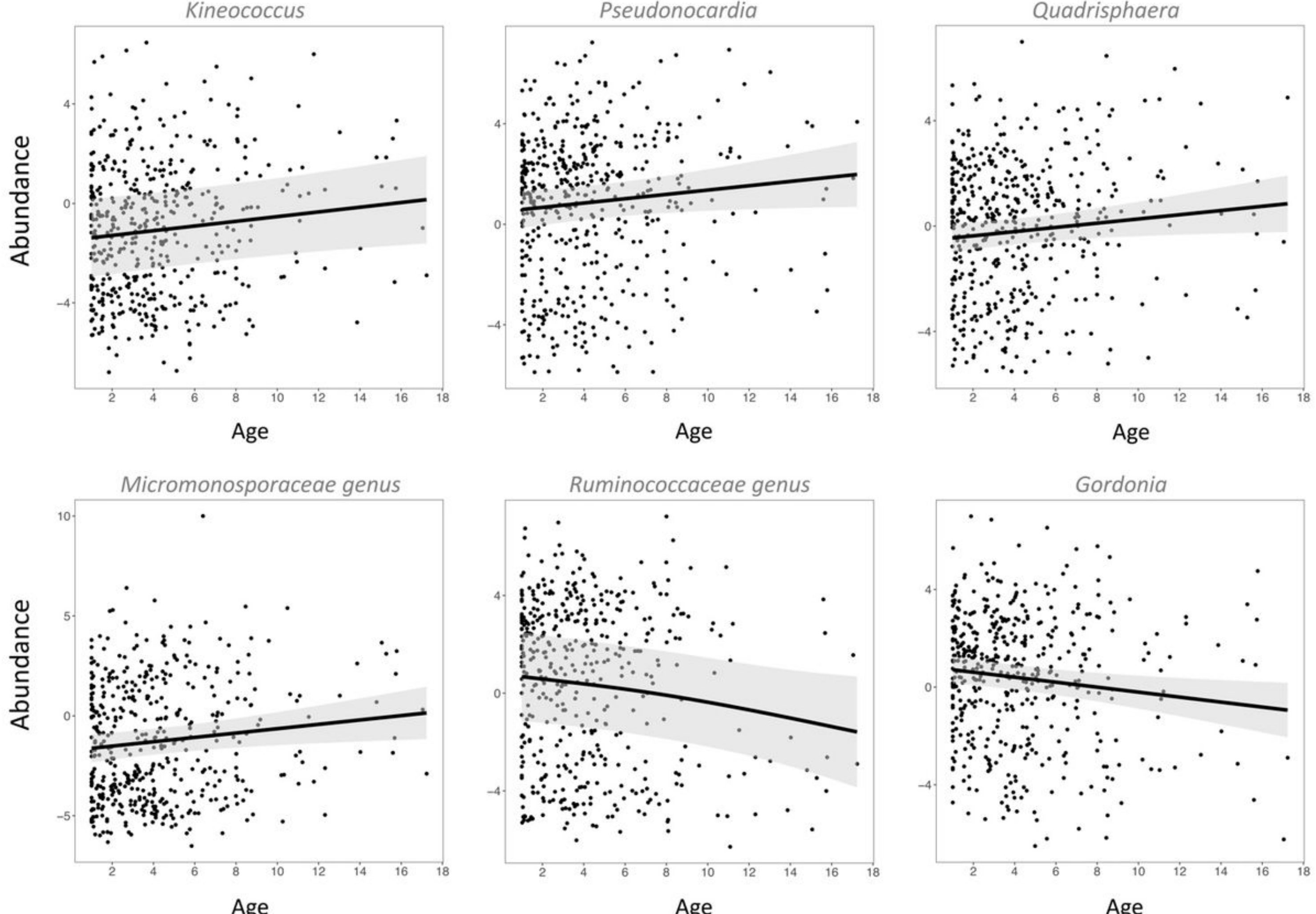

Figure 2

Changes in the abundance of six core bacterial genera with host age. Abundances are Centred Log Ratio (CLR) transformed to control for the compositionality of the dataset. Fitted lines are model predictions with 95% confidence intervals calculated from GAMM models (P< 0.05 in GAMMs and a GLLVM model). N=462 samples from 273 adult individuals. The results of GAMMs are presented in full in Additional file 1: Table S5.

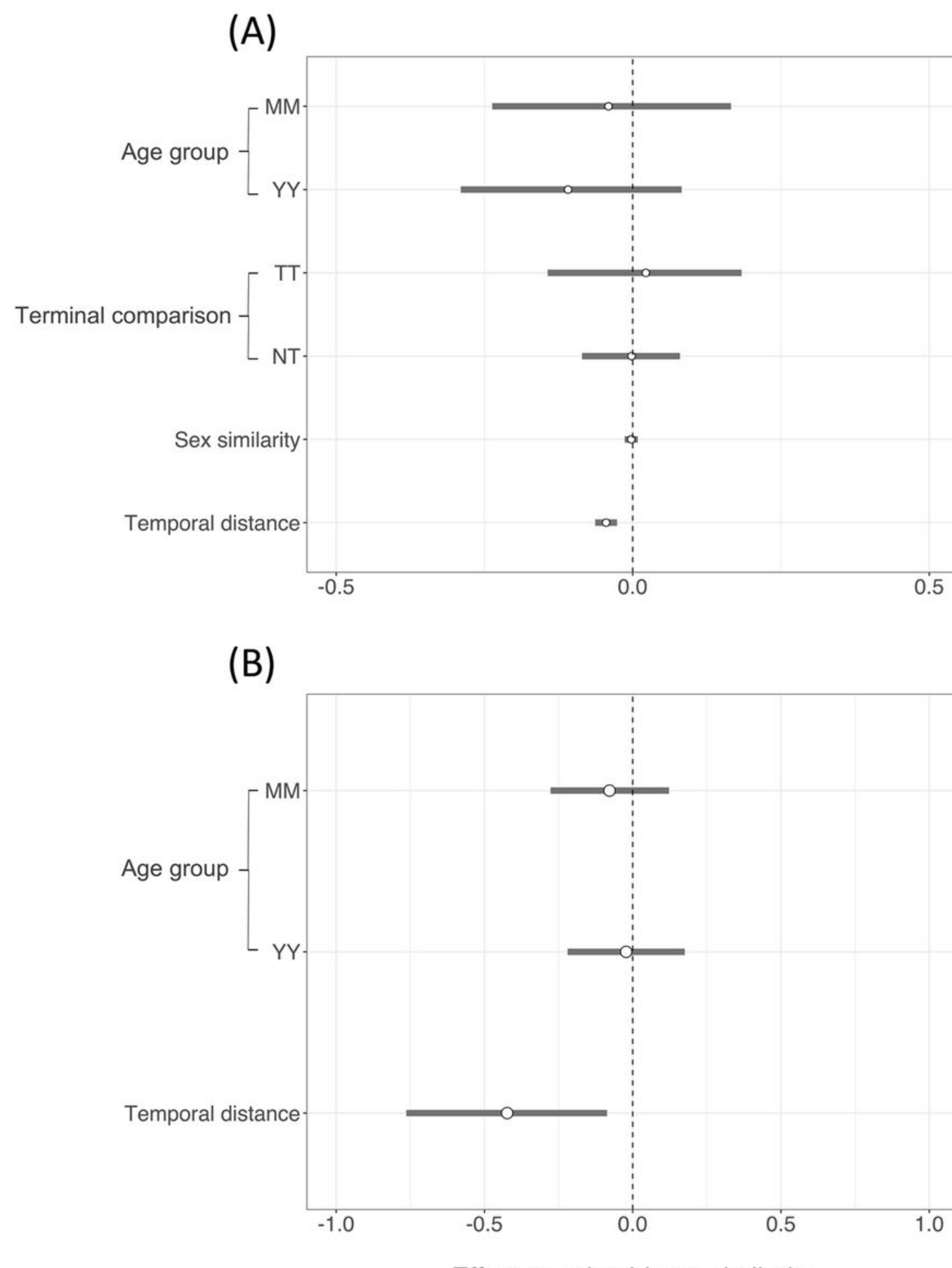

Figure 3

Compositional similarity of adult Seychelles warbler gut microbiome samples taken from A) different individuals or B) from the same individual. Effect sizes (points) are plotted with their 95% credible intervals and were calculated using Bayesian dyadic regression models. Pairwise GM Aitchison similarities among samples were uses as the response in these models. Comparisons were made between samples taken in the same age group: YY = young adult comparisons (individuals 1-3 yrs); MM

= middle aged comparisons (3-6 years). Old adult comparisons (OO, >6 years) were used as the reference category in the model. In (A) the terminal comparison term indicates whether a pairwise comparison was made between a non-terminal sample and a sample taken in a bird's terminal year (NT), or between two terminal year samples (TT). NN (two non-terminal year samples) was used a reference. Sex similarity (same or different) was also included in (A). Temporal distance indicates the time interval (in days) between samples in each pairwise comparison. Significant predictors are those where the credible intervals do not overlap zero.

Supplementary Files

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- AdditionalFile1Final.docx