Gut microbiome composition and function – including transposase gene abundance - varies with age, but not senescence, in a wild vertebrate

Wild gut microbiome changes with age

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Abstract

Studies on wild animals, mostly undertaken using 16S metabarcoding, have yielded ambigous evidence regarding changes in the gut microbiome (GM) with age and senescence. Furthermore, variation in GM function has rarely been studied in such wild populations, despite GM metabolic characteristics potentially being associated with host senescent declines. Here, we used seven years of longitudinal sampling and shotgun metagenomic sequencing to investigate taxonomic and functional changes in the GM of Seychelles warblers (*Acrocephalus sechellensis*) with age and senescence. Our results suggest that taxonomic GM species richness declines with age and in the terminal year, with this terminal decline occurring consistently across all ages. Taxonomic and functional GM composition also shifted with host age. However, all the changes we identified occurred linearly with adult age, with little evidence of accelerated change in late life or during their terminal year. Therefore, the results suggest changes that changes in the GM with age are not linked to senescence. Interestingly, we found an increase in the abundance of a group of transposase genes with age, which may accumulate passively or due to increased transposition induced as a result of stressors that arise with age. These findings reveal taxonomic and functional GM changes with age in a wild vertebrate and provide a blueprint for future wild functional GM studies linked to age and senescence.

Keywords: gut microbiome, age, senescence, metagenomics, transposase, *Acrocephalus sechellensis*

Introduction

Senescence - a decline in physiological function in later life- occurs in most organisms [1,2]. However, its onset and rate often differ greatly among individuals within populations [1,3]. One factor that may contribute to individual differences in senescence is variation in host-associated microbial communities. The intestinal tract of animals contains a diverse collection of microbes and their genomes (the gut microbiome; GM), which play an important role in host adaptation and fitness [4,5]. The GM influences the regulation of essential processes, such as digestion, reproduction, and immune function [6,7]. However, shifts in GM composition can be detrimental to the host; certain microbes may be pathogenic, while overall dysbiosis may impair host function [8,9].

Studies in humans and laboratory animals have shown that GM composition generally changes rapidly in early life [10,11] before stabilising during adulthood [12]. This is often followed by greater GM instability in advanced age including a loss of diversity and changes to composition [13–15]. These late-life compositional shifts are generally characterised by a loss of commensal or probiotic bacteria and an increase in pathogenic microbes [16]. GM functional changes with age have also been identified. For example, healthy ageing has been associated with microbes that enable increased biodegradation and metabolism of xenobiotics [16,17], whereas unhealthy ageing has been linked to increased production of detrimental microbial metabolites [16].

Studies have demonstrated links between the GM and senescence in humans and laboratory animals, however, their GM composition varies markedly from their counterparts living in natural environments because of the artificial environments they are exposed to [18,19]. It remains unclear if these effects can be generalised to wild animals [18–20].

Recent studies on wild organisms have not reached a consensus on what characterises the ageing microbiome. Some have documented altered GM composition [21–23], increased GM diversity [22,24], and reduced GM stability [25] with increasing age. Other studies have indicated that GM characteristics remain relatively stable throughout adulthood [25–27]. However, these studies have been based on 16S rRNA gene metabarcoding, which is limited in resolution [28–30]. Shotgun metagenomic sequencing enables higher taxonomic resolution (species or strain level), as well as informing on the functional potential of microbial communities based on gene content [31–33]. In humans and captive primates, metagenomics has revealed an increase in pathogenic microbial genes, and a decrease in beneficial genes, with age [17,34,35]. To our knowledge, no previous studies have investigated GM functional changes with age and senescence using shotgun metagenomics in a wild population.

Also, most GM studies on wild animals have relied on a cross-sectional sampling of differently aged individuals [36–38] and, therefore, may be confounded by the selective

appearance/disappearance of individuals with particular GM characteristics. A lack of longitudinal samples also makes it difficult to infer changes in GM stability with age [39]. Understanding what drives this GM variation is important, as it may lead to a deeper comprehension of the evolution of senescence and life-history trade-offs [3], and enhance our ability to prolong healthy lifespans. As senescence occurs at different rates across individuals, a longitudinal approach is crucial for accurately evaluating age-associated effects [40]. Given this rate variation, and because declines are expected to be greatest at the end of life, GM changes may be more closely associated with proximity to death than chronological age. Including such information in analyses requires accurate estimates of the point of death that are not confounded by dispersal.

The long-term study of the Seychelles warbler population on Cousin Island provides a powerful natural system in which to study GM variation and host senescence [3]. Its isolated nature allows for the longitudinal sampling of uniquely marked, known-age individuals across their entire lifespan and the collection of accurate survival and reproductive success data [41,42]. Previous studies using 16S metabarcoding have demonstrated that Seychelles warbler GM composition is linked to subsequent survival [43] but identified no overall patterns of GM senescence [26].

Here, we use shotgun metagenomics to assess fine-scale changes in the GM with age and senescence in the Seychelles warbler. First, we determine how GM taxonomic diversity and composition change with host age, particularly in a bird's terminal year when GM dysregulation is expected to be at its greatest. Then we test the hypothesis that GM functional characteristics (assessed via microbiome gene content) will change with age, senescence, and in the terminal year.

Materials and Methods

Study system and sample collection

Seychelles warblers are insectivorous passerines endemic to the Seychelles archipelago. The population on Cousin Island (29 ha; 04° 20′ S, 55° 40′ E) has been extensively monitored since 1985 in the winter (January – March) and summer (June – October) breeding seasons [3,44,45]. Each season nearly all new birds (offspring) are caught, in the nest or as dependent fledglings in the natal territory [45]. As many adult birds as possible are re-caught each season using mist nets. Bird age is determined using either lay/fledgling date [45] for the majority of individuals, if birds are first caught without a fledging date being recorded, eye colour is used to estimate age instead (see [45]).

The population on Cousin Island consists of ca. 320 individuals grouped into ca. 115 territories, defended year-round by a dominant breeding pair [46,47]. Territory quality is calculated each season using arthropod counts, vegetation density, and territory size information [45,48].

Nearly every bird in the population (> 96% since 1997 [49]) has been caught and marked with a unique combination of a British Trust for Ornithology (BTO) metal ring and three plastic colour rings, which enables them to be monitored throughout their lives [3,50]. Individuals almost never disperse between islands and the annual resighting probability is around 98% ± 1% [41,42,51]. If an individual is not seen for two consecutive seasons it is assumed to have died (an error rate of 0.04%) [41,42]. Death dates for individuals were set as the final day of the season in which the bird was last seen. Benign climatic conditions and a lack of predators result in relatively long-lived individuals (median lifespan 5.5 years, max lifespan 19 years) [46,52]. Extensive previous research shows that reproductive and actuarial senescence occurs in this population (Hammers et al., 2015, 2019, 2021).

Faecal samples were collected from caught birds and stored as described previously (see [26] and supplementary material). Contamination (hand) controls were collected from fieldworkers each season. The time-of-day that samples were collected and the number of days for which samples were stored at 4°C, were recorded. A ca 25 µl blood sample was also taken via brachial venepuncture and stored in 1 mL of absolute ethanol at 4°C.

DNA extraction and sequencing

Blood samples were processed with a salt extraction method [42] or Qiagen DNeasy Blood and Tissue Kit and the resulting DNA was used for molecular sexing [52,54]. DNA from faecal samples was extracted using the Qiagen DNeasy PowerSoil Kit with a modified protocol (see [55]). Individuals for which multiple longitudinal samples were available were prioritised for metagenomic sequencing to capture within-individual

changes. In total, 155 faecal samples from 92 individuals across 7 years were sequenced, as well as three positive controls (two extractions from a ZymoBIOMICS Microbial Community Standard (D6300), and one extraction from a ZymoBIOMICS Fecal Reference with TruMatrix™ Technology (D6323)), and six hand controls. Library preparation was performed in two lanes per run using the LITE protocol [56] and sequencing undertaken in two runs of 2 x 150 bp NovaSeq X platform. The D6300 extraction control was sequenced on both runs to compare extraction and batch effects.

Bioinformatics

Shotgun metagenomic sequence analysis was carried out using the MATAFILER pipeline (see [5] and supplementary materials). Briefly, MATAFILER removes host reads, assembles reads, predicts and annotates genes, builds metagenome-assembled genomes (MAGs) and metagenomic species (MGSs), and taxonomically assigned MGSs. Due to the high individuality of the Seychelles warbler GM and the high sequencing coverage required to assign MGS, Metaphlan4 was also used to taxonomically classify reads (see supplementary materials for details).

Gut microbiome analyses

A total of 162 samples were successfully processed bioinformatically (153 faecal samples, 4 controls). Positive controls were successfully recovered, and hand controls did not contribute to substantial contamination in samples (Figure S1).

The 153 faecal samples (Figure S2) included 71 from 40 females and 82 from 51 males. In total, 41 individuals had one sample, 41 had two, eight individuals had three, and one individual had four samples. Age at sampling ranged from 0.6-17.0 years (mean 5.7 $\pm$ 0.3 SE). Of these, 48 were from 22 individuals in their terminal year (the year in which they died); with ages in terminal year ranging from 1.4–17.0 years. From all these samples, 1025 unique metaphlan4 species-genome-bins assignments were used for the subsequent taxonomic analysis (mean 29.3 $\pm$ 2.0 SE per sample).

All statistical analysis was performed using R version 4.33 [57,58]. Variance Inflation Factor (VIF) scores (*car* version 3.1.2) were used to test for collinearity between variables in all models; all had a score <3 indicating no issues with collinearity [59].

Taxonomic GM changes with age

Taxonomic GM alpha diversity

A rarefaction curve of Metaphlan4 species was constructed with *iNEXT* version 3.0.1 to determine the read depth required to recover 95% of theoretically present species (Figure

S3) [60]. Taxonomic classifications were rarefied to a depth of 5,500 reads before alpha diversity analysis; two samples were removed due to insufficient read depth. Species richness and Shannon diversity metrics were calculated per sample using R packages *phyloseq* version 1.46.0 and *microbiome* 1.24.0 [61,62]. Wilcoxon rank sum tests were used to examine whether different sequencing plates affected species diversity (Shannon index, $p = 0.353$) and species richness (Observed index, $p = 0.124$), both were not significantly different.

A linear mixed effect model with a Gaussian distribution (lmer), and a generalised linear mixed effect model with a negative binomial distribution (glmer.nb), were used to model changes in species diversity (Shannon index) and richness (observed taxa), respectively, using *lme4* version 1.1-35.5 [63]. Fixed effect variables included in models were: host age (years); terminal year (yes/no); sex (male/female); breeding season (winter/summer); sample year (as a factor: 2017-2023); territory quality; storage at 4°C (days); time of day collected (minutes since sunrise at 6:00 am). Bird ID was included as a random effect. A quadratic age term, and an interaction between terminal year and host age, were tested to assess whether GM changes became more extreme in the terminal year but were dropped if not significant to allow interpretation of the main effects. Age was measured in years, but all samples taken when birds were >12 years of age were designated as 12 years because these samples were rare (n = 9, max age = 17 years). Model diagnostics were run using *DHARMa* version 0.4.6, with no significant issues in each chosen model [64]. Herein, all models were tested with the same variables unless stated otherwise.

A within-subject centering approach was used to separate between-individual (cross-sectional) GM differences with age (which could be driven by the selective appearance/disappearance of individuals with particular GM characteristics), from within-individual (longitudinal) change (which could indicate senescence) [65]. This involves calculating the mean age of each individual across all it's sampling events (mean age) and the within-individual deviation from that mean age at each separate sampling event (delta age). These terms replace host age in the model. The fixed effect of terminal year was also replaced by a "terminal year bird" term (yes/no) which indicates whether individuals have at least one sample collected in the terminal year or not. An interaction between the terminal year bird and delta age, as well as quadratic delta age, were tested to assess whether within-individual GM changes were more extreme in birds with a sample taken in the terminal year of life and/or in older individuals, respectively (which would be indicative of senescence). These were dropped if not significant to allow interpretation of the main effects.

Taxonomic GM composition

A permutational multivariate analysis of variances (PERMANOVA) was carried out on a Euclidean distance matrix calculated using centered log ratio (CLR)-transformed reads,

using the adonis2() function in *vegan* version 2.6.6 [66]. A blocking effect of Bird ID was used to account for repeated measures. The same predictors were included as for the main model in the Alpha diversity analysis above. Differences in composition were visualised with a principal component analysis (PCA) in *phyloseq* version 1.46.0 [62].

Taxonomic GM differential abundance analysis (DAA)

Two different DAA methods were used to identify differentially abundant GM species with host age (as recommended by [67,68]; *ANCOMBC2* version 2.4.0 and *GLLVM* version 1.4.3 [69,70]. A total of 22 common species, defined as species found in 20% of the population at more than 0.01% abundance, were retained. Species that were significantly differentially abundant in the same direction using both DAA methods were considered robustly significant. Variables included in each model were the same as in models above.

Functional GM changes with age

Functional GM alpha diversity

Initially, 4727 different eggNOG orthologues (mean = 3616.6 $\pm$ 64.4 SE per sample) were identified in our gene catalogues. A rarefaction curve of eggNOG orthologues was constructed using *iNEXT* to determine sample completeness [60]. Samples were then rarefied to 100,000 reads based on >95% completeness. One sample was removed due to insufficient reads. Following rarefication, 4685 eggNOG orthologues were retained (mean = 3054.3 $\pm$ 47.1 SE per sample). Due to the (negative) skewness of the observed richness and Shannon diversity of eggNOG annotations, a scaled exponential transformation and an exponential transformation were used for analyses, respectively, to improve residual fit. Both these alpha diversity indices were then analysed with linear mixed models containing the same predictors as for taxonomic alpha diversity above.

Functional GM composition

To test for changes in functional microbiome beta diversity, a PERMANOVA of Euclidean distances calculated from CLR-transformed read abundances per orthologue was used, using the same model structure as for taxonomic compositional analysis (described above). Differences in composition were visualized with a PCA plot as above.

Functional GM differential abundance analysis (DAA)

Differential abundance analysis was performed on eggNOG annotations using their assigned categories from the database of clusters of orthologous genes (COG) (Supplementary Table S5) [71] using *ANCOMBC2* and *GLLVM* as described above [69,70]. Post-hoc DAA were performed on individual eggNOG members found within

differentially abundant COG categories to establish the drivers of any significant differences (see Supplementary material for details).

Results

Taxonomic GM changes with age

Taxonomic GM alpha diversity

GM species richness declines with host age, and individuals in their terminal year had significantly lower species richness than those in a non-terminal year (Table S1 & Figure S4). However, Shannon diversity was not significantly associated with host age, and did not differ between samples taken in a terminal or non-terminal year (Table S2). A quadratic age term, and an interaction between host age and terminal year were not significantly associated with species richness or Shannon diversity ($p > 0.05$) and were dropped from the final model.

The within-individual centering approach revealed that the decline in GM species richness with host age occurred longitudinally within individuals (Table 1, Figure 1) with no evidence of between-individual selective disappearance effects (Table 1). Shannon diversity did not change significantly with mean or delta age (Table S3). There was no evidence of a quadratic relationship between within-individual delta age and species richness or Shannon diversity, hence the quadratic age term was dropped from the final model. This suggests that within-individual changes were not more extreme in older individuals and that declines in species richness happen equally in all mature individuals. We also tested for an interaction between within-individual age and whether an individual's final sample was in their terminal year, but this was not significant ($p > 0.05$) and was dropped. This result indicates that within-individual changes in species richness with age had a similar slope whether the bird was sampled in its terminal year or not.

Taxonomic GM composition

A PERMANOVA analysis found that cross-sectional host age was a marginally significant predictor of GM taxonomic composition (Table 2), but terminal year was not (Table 2). Sample year, season, and catch time were significant and explain the largest proportion of GM compositional variance (Table 2) followed by days sample stored at at 4°C and sex. An interaction between age and terminal year was not significant ($p > 0.05$). A PCA showed limited sample clustering according to age, which is consistent with the small amount of variance explained in the PERMANOVA (Figure S5).

Taxonomic GM differential abundance analysis (DAA)

Five of the 22 common GM species found in the Seychelles warbler population (i.e. in >20% individuals) differed significantly in relative abundance with age in the GLLVM analysis (*Escherichia coli*, *Lactococcus lactis*, *Brucella pseudogrignonensis*, *Lactococcus garvieae*, *Microbacterium enclense*), but none were differentially abundant with age in the ANCOMBC2 analysis (Figure S6A & S6B). Similarly, six species were differentially

abundant in the terminal year in the GLLVM analysis (*Lactococcus garvieae*, *Pantoea anthophila*, *Escherichia coli*, *Rothia* sp AR01, *Microbacterium enclense*, *Brucella pseudogrignonensis*), but none were differentially abundant with terminal year in the ANCOMBC2 analysis (Figure S6C & S6D). Thus, there is no clear consensus of significant variation in the abundance of specific GM species with age or in the terminal year.

Functional GM changes with age

Functional GM alpha diversity

Alpha diversity of eggNOG gene orthologues declined significantly with host age for both observed richness and Shannon diversity metrics (Table S4, Figure S7). Alpha diversity of eggNOG orthologues did not differ between terminal year and non-terminal year samples (Table S4). Additionally, the interaction between host age (or quadratic age) and terminal year was not significant ($p > 0.05$).

The decrease in functional alpha diversity with host age is best explained by within-individual longitudinal changes with age for both tested indices (Table 3, Figure 2). Cross-sectional, between-individual age was a marginally significant predictor of Shannon diversity but not observed richness. Alpha diversity did not differ between individuals that had at least one sample taken in their terminal year and those that did not. The interaction of terminal year bird and within-individual age as well as quadratic within-individual age were also not significant ($p > 0.05$) predictors of either index. Sample year was a significant variable of both eggNOG observed richness and Shannon diversity.

Functional GM beta diversity

A PERMANOVA analysis identified factors that were significantly related to GM functional composition (Table 4). Host age, but not terminal year, was a marginally significant predictor of functional composition (Table 4). An interaction between age and terminal year was not significant ($p > 0.05$). The largest effect sizes were found in relation to season, sample year, sex, and days stored at 4°C (Table 4). Time of day was not significant related to GM functional composition (in contrast to GM taxonomic composition). A PCA plot showed limited clustering of GM samples according to age, consistent with the small amount of variance explained by this variable (Figure S8).

Functional GM differential abundance analysis (DAA)

Only one cluster of orthologous genes (COG) category was differentially abundant in relation to age. The COG category "X", which represents mobilome COGs such as prophages and transposons, significantly increased in abundance with age in both the ANCOMBC2 and the GLLVM analyses (Figure 3). Several COG categories were significantly differentially abundant with environmental variables including Cat A (RNA

processing and modification) with season and Cat C (Energy production and conversion) with sample year (Figure S10, Figure S11).

Within category X (mobilome), only COG2801 (transposase genes) was found to significantly increase in abundance with age in both GLLVM and ANCOMBC2 analyses (Figure S9). A within-subject centering approach within a linear mixed model showed an increase in COG2801 was associated with both within-individual (longitudinal) age and between-individual (cross-sectional) age (Table S7, Figure 4). However, the interaction between within-individual age and terminal year was not significant ($p > 0.05$).

COG2801 located within MGSs (509 COG2801 copies from 160 MGS) were most closely related to the group insertion sequences (IS) 3 family of transposases (30%), other IS family transposases (12%), partial or putative transposases (33%) or other/unknown function (25%; Table S8). An increased abundance of COG2801 in the GM may be due to either an increase in the abundance of COG2801-carrying microbes or increased replication of the transposase gene itself. However, contrary to the first hypothesis, we found no relationship between the total abundance of COG2801-carrying MGSs ($n = 160$) and host age (Table S9). To further test this, COG2801-MGSs were matched with metaphlan4 annotations at the genus level; the abundance of COG2801-metaphlan4 genera was not significantly associated with host age (Table S10). Hence, the increase in COG2801 abundance with host age could not be attributed to an increased abundance of COG2801-carrying bacteria. Additionally, within COG2801, ten gene catalogues were commonly shared across 50% of samples. Each of these ten COG2801 gene catalogues was not significantly ($p > 0.05$) differentially abundant with age individually when tested using both ANCOMBC2 or GLLVM analysis (Figure S12). Thus, the increase in abundance of COG2801 with age was not being driven by the abundance of a single prevalent, gene catalogue but rather the cumulative abundance of many.

Discussion

We used a longitudinal metagenomic dataset from individuals in a Seychelles warbler population to investigate how GM taxonomic and functional characteristics varied with host age. We identified a linear decrease in species richness, and small shifts in GM taxonomic composition, with host age. Additionally, species richness was lower in samples taken during an individual's terminal year, but taxonomic composition did not differ between terminal and non-terminal samples. We also identified a linear decrease in the GM's functional richness and diversity, and differences in functional GM composition, with host age. Finally, COG categories representing the mobilome increased in prevalence with bird age, driven by an increase in the abundance of COG2801, a group of transposases.

The small reduction in GM richness, but not Shannon diversity, with age suggests a loss of rare taxa that is not linked with a major restructuring of the evenness of the GM. This also concurs with the small changes in GM composition with age we identified; i.e showing a limited number of differentially abundant taxa with increasing host age. This result is consistent with a previous 16S metabarcoding analysis of senescence of the Seychelles warbler GM despite the increased taxonomic resolution afforded by a metagenomics approach [26] Overall, the results support the conclusion that, taxonomically, most of the GM stays the same with increasing age, apart from the loss of a few rare taxa.

Taxonomic changes in GM species diversity and composition with age have been repeatedly demonstrated in humans and captive animals [16]. However, in these species, late-life changes in the GM may be due to external factors such as antibiotic use, lifestyle, and dietary changes [18,20]. An increasing number of wild animal studies are finding little evidence of a late-life shift in GM taxonomic diversity without such external factors (see [26,72]). Our study supports this conclusion despite the longitudinal sampling and increased resolution yielded by shotgun metagenomics, which can potentially reveal more nuanced changes at lower taxonomic levels.

Few studies have directly investigated functional changes in the GM with age in wild animals [73]. Some studies have been undertaken using functional inferences from metabarcoding sequence homology. However, this can be misleading due to being limited to variation within the same genus thus providing potentially inaccurate functional profiles. [74,75]. In our study using a higher resolution metagenomic approach, we found evidence of small, linear, changes in GM functional diversity and composition with age in the Seychelles warbler. Functional observed richness and Shannon diversity declined with age, which suggests not only that rare functions are lost, but that the evenness of these GM functions also changes linearly with adult age. Age-related decreases in functional richness and shifts in functional composition have previously been identified in elderly humans [76,77]. Such changes have been linked to the onset of specific disease states,

such as inflammation and pathogenesis and changes to diet degradation and digestion, in humans and laboratory mice [78]. However, other studies have either found no change in functional alpha diversity, or even an increase in microbial functional richness and diversity with age [35,79]. Whether the loss of functional diversity, and minor changes in functional composition, with host age in Seychelles warbler is linked to declines in health and condition remains unclear and requires further study.

Despite the small changes in functional diversity and composition with age in the Seychelles warbler, we only identified one specific functional category whose abundance was significantly associated with host age. An increase in the abundance of COG2801 transposases occurred with age. However, this was not due to an increase in COG2801-carrying microbes. COG2801 are a group of transposases that are primarily found in bacteria (89.5%) and have been shown to be the most widely transferred genes among prokaryotes [80]. Most COG2801 genes found within MGSs were group insertion sequences 3 (IS3), which use a copy-out-paste-in mechanism to replicate [81]. This could lead to an increased number of transposon copies in the same individual bacterial genome over time, or to horizontally transfer to other bacterial genomes. [82,83]. Thus, the increased abundance of COG2801 with age in Seychelles warbler GM's may be the result of self-replication, independent of microbial host cell DNA replication. An increase in transposition has been observed when bacteria are stressed and COG2801 is one of the most horizontally transferable eggNOG genes [84,85]. Therefore, as vertebrate hosts get older, the GM may be exposed to a greater number or intensity of stressors, such as mucus barrier thinning or inflammation, which may induce activation of COG2801 [86]. However, there was not an accelerated increase (i.e. a quadratic relationship) of COG2801 abundance with host age, which would be expected if the cumulative effects of host senescence were driving these changes. Therefore, stressors to the host that occur linearly in adulthood, such as cell death in the gastrointestinal autonomic nervous system [87,88], may better explain the increased abundance of COG2801 with host age

We also focused on assessing terminal year effects in the Seychelles warbler GM. Only species richness was found to be significantly lower in the final year of a bird's life. Moreover, the effect of terminal year was uniform across age, i.e. it was not more extreme in older individuals. Previous research has identified age-dependent terminal-declines in fitness components (reproductive success and survival probability) in the Seychelles warbler [89]. However, the lack of age-dependent terminal changes in GM characteristics identified in our study suggests that the GM does not undergo senescence in association with these other traits. As such, the declines in microbial species richness in terminal year samples (and linearly with age) may rather reflect the stabilisation of the GM with age rather than a senescence effect. These results concur with the previous 16S metabarcoding analysis of the Seychelles warbler GM which found little evidence of GM senescence [26].

Across analyses, environmental factors explained most of the variance in the Seychelles warbler GM. This concurs with previous research on this species [26,43,55] as well as studies of other taxa [21,90,91]. Temporal variation -specifically year and season- explained the most variance in both taxonomic and functional GM composition. This may be explained by many factors including climate variability, differences in insect prey availability, or host population density [92–94]. Most Seychelles warbler individuals breed in the summer rather than the winter season, and GM shifts may therefore reflect reproductive activity and related hormonal changes [24]. Time of day was also associated with GM composition. Differences in insect activity might drive this pattern due to light availability and/or temperature [95,96]. However, such patterns could also be due to host intrinsic circadian rhythms [97]. These factors lead to a substantial amount of noise in GM studies that can confound studies on ageing, reproduction, and disease outcomes in wild populations. Therefore, accounting for these factors is important when investigating the GM in natural systems.

Our findings highlight the need for more studies investigating the functional characteristics of wild microbiomes as taxonomic relationships might not capture functional GM changes that occur (e.g. the increased prevalence of COG2801). However, researchers should not totally discount the utility of 16S metabarcoding for investigating general GM questions, as it may, in many cases, provide sufficient taxonomic resolution to answer specific questions [28]. Indeed, we identified similar taxonomic patterns using shotgun metagenomics to those revealed by a previous metabarcoding study on the Seychelles warbler [26]. The cost-effectiveness of 16S rRNA allows greater sample sizes, and thus power, to resolve certain questions. A combination approach that harmonises both 16S metabarcoding and shotgun metagenomics has been proposed to maximise sample size, although such analyses are limited to genus-level comparisons [98]. On the other hand, shotgun metagenomics not only allows higher taxonomic resolution and functional analysis of the GM, but also an assessment of the interaction between taxa and their functions. As described with transposable elements, our functional analysis uncovered changes in GM function that were not detectable using 16S metabarcoding analysis.

In conclusion, while we found that the Seychelles warbler GM changes in terms of diversity, composition and even function with age, this happens gradually over the adult lifespan and there is little evidence of late-life GM senescence. Whilst species richness is lower in the terminal year, this occurs at all ages and is not more extreme in the oldest individuals. Interestingly, we found that the abundance of a group of transposase gene increases considerably with age in the GM, probably because of more frequent transposition within the GM community over time. Future work is required to determine exactly why these transposable element changes occur and what impact they may have. Additionally, work should investigate the generality of these conclusions by assessing whether functional changes occur in the GM of other wild vertebrates.

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Tables and figure legends

Table 1. A generalised linear mixed effect model with a negative binomial distribution (glmer.nb) investigating gut microbiome species richness in relation to within- (delta) and between- (mean) individual variation in age amongst Seychelles warblers (n = 151 samples, 91 individuals). Conditional R^2 = 47.1%. Reference categories for categorical variables are shown in brackets.

Predictor	Estimate	SE	*z*	*P*
(Intercept)	**2.69**	**0.32**	**8.41**	**< 0.001**
Delta Age	**-0.13**	**0.06**	**-2.10**	**0.036**
Mean Age	-0.03	0.02	-1.50	0.134
Terminal Year Bird (yes)	-0.19	0.14	-1.37	0.172
Season (winter)	0.00	0.16	-0.01	0.995
Sex (female)	-0.02	0.14	-0.11	0.916
Days at 4°C	-0.18	0.14	-1.31	0.190
Time of day	0.23	0.12	1.84	0.066
Territory quality	-0.07	0.13	-0.56	0.577
Sample Year (2017)				
2018	0.47	0.29	1.64	0.101
2019	0.45	0.33	1.38	0.169
2020	**0.80**	**0.35**	**2.25**	**0.025**
2021	**0.76**	**0.34**	**2.21**	**0.027**
2022	**0.74**	**0.35**	**2.12**	**0.034**
2023	**0.89**	**0.40**	**2.20**	**0.028**
Random				
Individual ID	151 observations	91 individuals	Variance	0.2075

Note: Significant ($p < 0.05$) predictors are shown in bold.

Table 2. A PERMANOVA analysis of gut microbiome taxonomic composition in relation to age and terminal year in the Seychelles warbler. The PERMANOVA was performed using a Euclidean distance matrix of CLR-transformed taxon abundances. N = 153 samples from 91 individuals. Bird ID was included as a blocking factor.

Predictor	*df*	R^2	F	*P*
Age	**1**	**0.009**	**1.368**	**0.043**
Terminal Year	1	0.007	1.051	0.569
Season	**1**	**0.013**	**2.021**	**0.001**
Sample Year	**6**	**0.056**	**1.479**	**< 0.001**
Sex	**1**	**0.007**	**1.096**	**0.064**
Days at 4°C	**1**	**0.008**	**1.193**	**0.034**
Time of day	**1**	**0.010**	**1.583**	**< 0.001**
Territory Quality	1	0.005	0.813	0.982

Note: Significant ($p < 0.05$) predictors are shown in bold.

Table 3. A linear mixed effect model investigating variation in gut microbiome functional diversity (observed richness and Shannon diversity) in relation to within- (delta) and between- (mean) individual age in Seychelles warblers (n = 152 samples, 90 individuals). Functional diversity is based on eggNOG annotations. Observed richness and Shannon diversity were transformed using a scaled exponential and exponential function, respectively. Conditional R^2 = 35.6% and 13.7% respectively. Reference categories for categorical variables are shown in brackets

Observed Richness					
Predictor	Estimate	SE	*df*	*t*	*P*
(Intercept)	**0.99**	**0.17**	**124.77**	**5.68**	**< 0.001**
Delta Age	**-0.12**	**0.04**	**137.00**	**-3.31**	**0.001**
Mean Age	-0.03	0.01	89.42	-1.97	0.052
Terminal Year Bird (yes)	0.01	0.08	83.34	0.17	0.870
Season (winter)	-0.06	0.10	136.94	-0.64	0.525
Sex (female)	-0.06	0.08	81.33	-0.79	0.430
Days at 4°C	**-0.19**	**0.09**	**127.35**	**-2.23**	**0.028**
Time of day	-0.07	0.08	137.00	-0.88	0.381
Territory quality	-0.07	0.08	129.62	-0.88	0.381
Sample Year (2017)					
2018	0.13	0.15	135.76	0.82	0.416
2019	0.08	0.18	135.88	0.46	0.647
2020	0.36	0.20	136.54	1.82	0.071
2021	**0.39**	**0.19**	**136.94**	**2.04**	**0.044**
2022	**0.56**	**0.19**	**128.48**	**2.90**	**0.004**
2023	**0.57**	**0.23**	**122.81**	**2.50**	**0.014**
Random					
Individual ID	152 observations	90 individuals	Variance		0.050
Shannon Diversity					
Predictor	Estimate	SE	*df*	t	*P*
(Intercept)	**757.59**	**182.06**	**119.47**	**4.16**	**< 0.001**
Delta Age	**-117.01**	**41.06**	**135.71**	**-2.85**	**0.005**
Mean Age	**-27.30**	**13.54**	**83.56**	**-2.02**	**0.047**
Terminal Year Bird (yes)	17.93	79.75	76.74	0.23	0.823
Season (winter)	173.07	104.67	127.74	1.65	0.101
Sex (female)	-4.98	80.46	69.67	-0.06	0.951
Days at 4°C	-48.55	95.70	133.26	-0.51	0.613
Time of day	-21.18	81.57	132.14	-0.26	0.796
Territory quality	-0.74	85.97	136.99	-0.01	0.993
Sample Year (2017)					
2018	88.02	168.08	136.67	0.52	0.601
2019	32.22	200.48	136.71	0.16	0.873
2020	169.50	210.62	131.73	0.81	0.422
2021	**464.12**	**206.85**	**136.39**	**2.24**	**0.026**
2022	**484.95**	**202.78**	**124.82**	**2.39**	**0.018**
2023	453.37	238.55	116.14	1.90	0.060
Random					

Individual ID	152 observations	90 individuals	Variance	5046

Note: Significant ($p < 0.05$) predictors are shown in bold.

Table 4. A PERMANOVA analysis of gut microbiome functional composition in relation to age (and other factors) in the Seychelles warbler. The PERMANOVA was performed using a Euclidean distance matrix calculated using CLR-transformed (eggNOG) abundances. N = 153 samples. 91 individuals. Bird ID was included as a blocking factor.

Predictor	df	R^2	F	*P*
Age	**1**	**0.007**	**1.096**	**0.044**
Terminal Year	1	0.006	0.890	0.292
Season	**1**	**0.011**	**1.823**	**0.042**
Sample Year	**6**	**0.052**	**1.374**	**0.020**
Sex	**1**	**0.008**	**1.250**	**0.001**
Days at 4°C	**1**	**0.010**	**1.569**	**0.007**
Time of day	1	0.008	1.200	0.139
Territory quality	1	0.007	1.094	0.413

Note: Significant ($p < 0.05$) predictors are shown in bold.

Figures

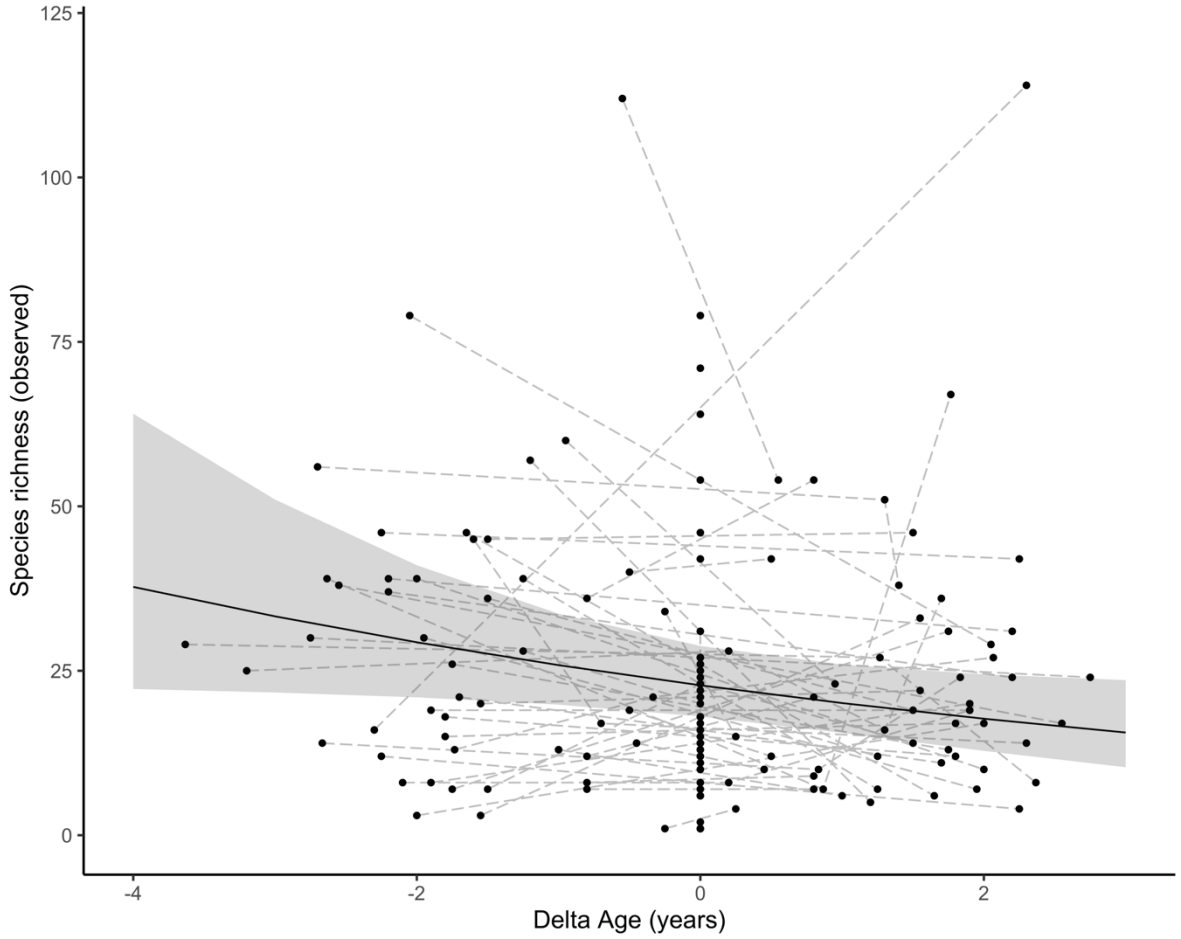

Figure 1. Gut microbiome species richness in relation to within-individual, longitudinal differences in age (delta age in years) in Seychelles warblers. The solid line represents model predictions with 95% confidence intervals calculated from the generalised linear mixed effect model (Table 1). Each point represents an individual gut microbiome sample, and the dashed grey lines connect samples from the same individual (n = 151 samples, 91 individuals).

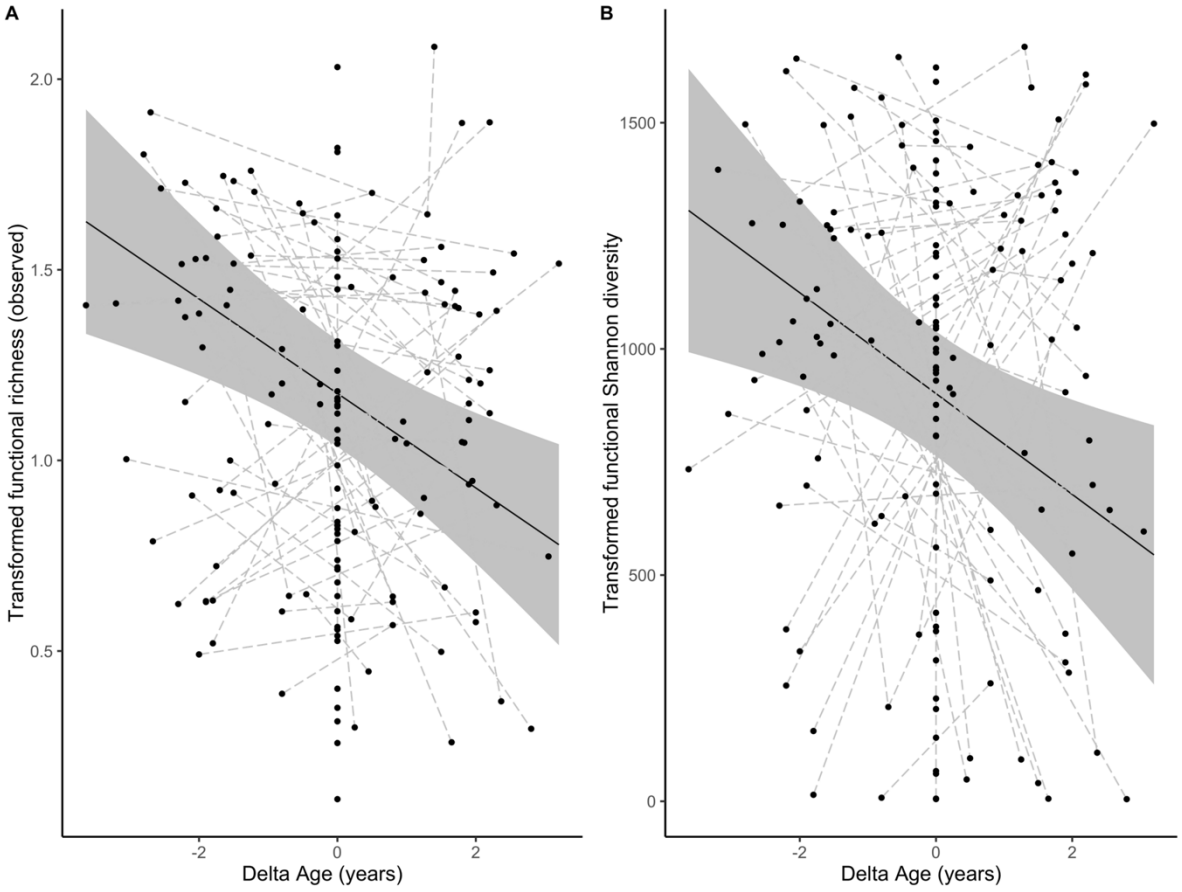

Figure 2. Gut microbiome functional diversity measured as (A) observed richness and (B) Shannon diversity in relation to within-individual host age (years). Functional diversity calculations are based on eggNOG orthologue groups. Solid lines represent model predictions (± 95% confidence interval) from linear mixed effects models. Each point represents a unique gut microbiome sample, and the dashed grey lines connect samples collected from the same individual (n = 152 samples, 90 individuals).

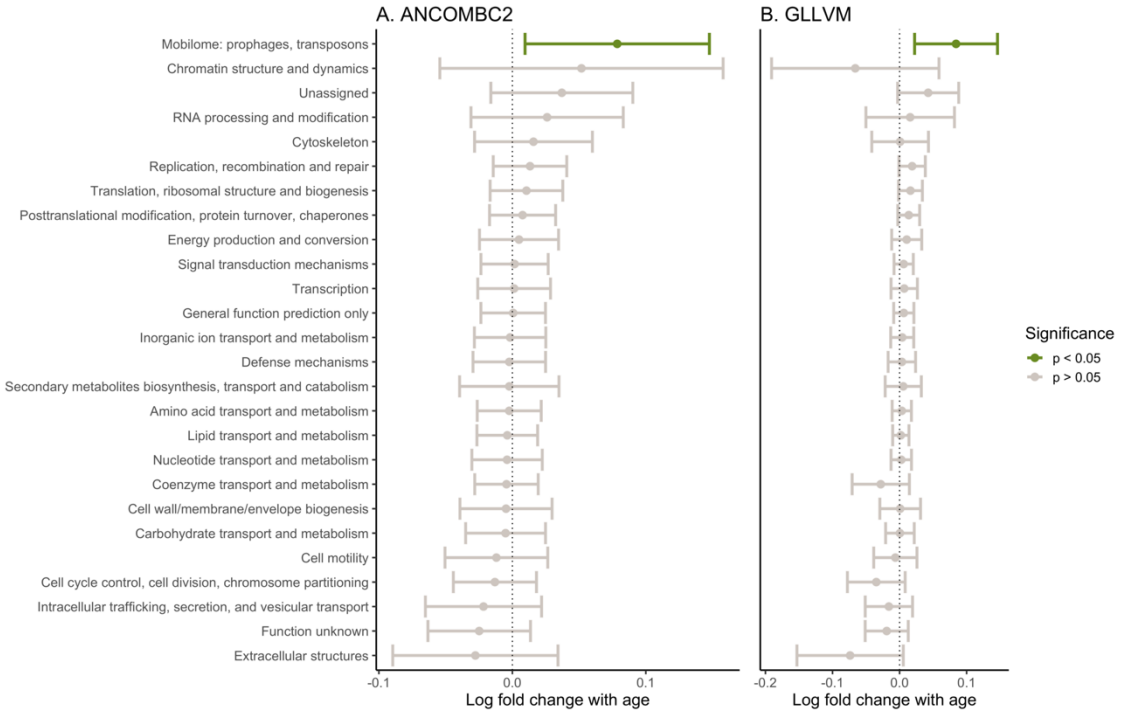

Figure 3. Differential abundance analysis of functional gut microbiome cluster of orthologous genes (COG) categories in Seychelles warblers using (A) ANCOMBC2 and (B) GLLVM. Each COG category is represented on the y-axis. Points and error bars are coloured according to significance (green: $p < 0.05$; grey: $p > 0.05$).

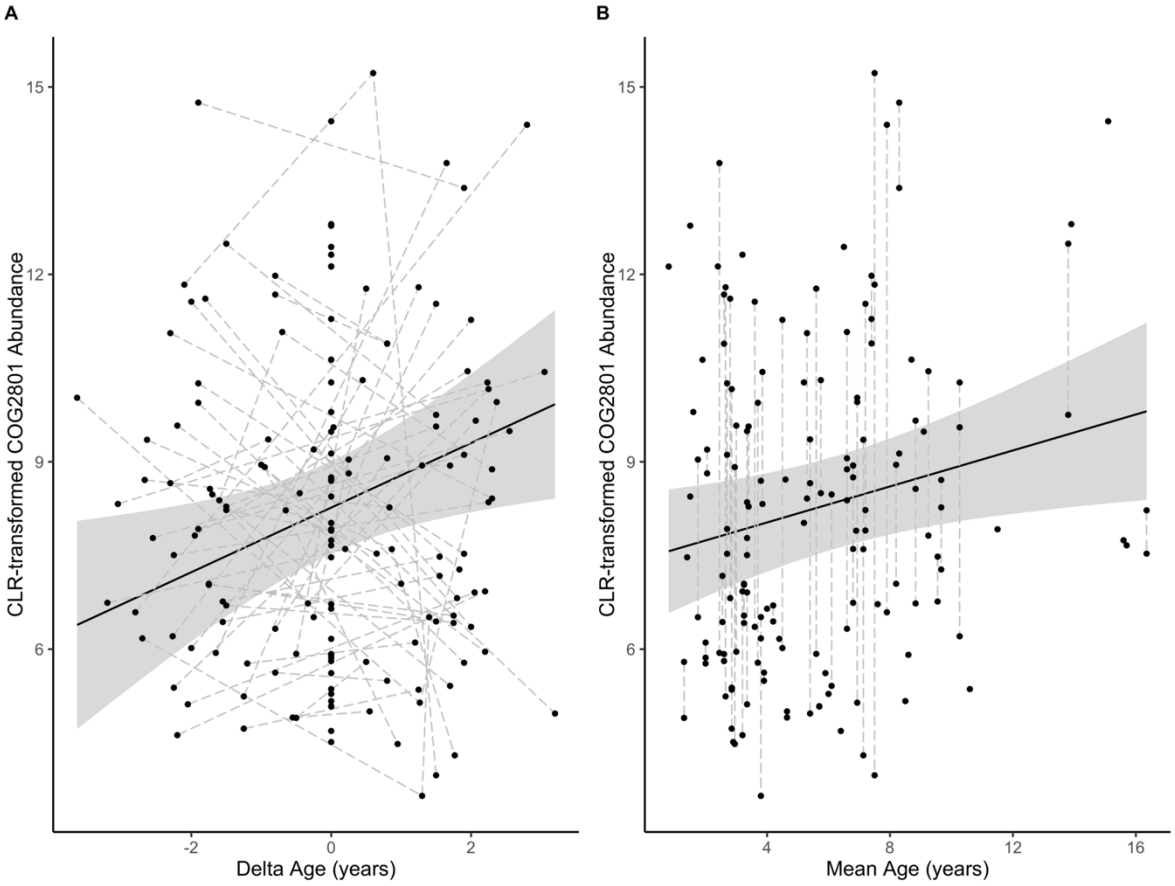

Figure 4. CLR-transformed COG2801 abundance in relation to (A) within-individual (delta) host age and (B) between-individual (mean) host age in the gut microbiome of Seychelles warblers. The solid line represents model predictions (± 95% confidence intervals) from a linear mixed effect model (Table 5). Each point represents a gut microbiome sample with dashed grey lines connecting samples from the same individual (n = 153 samples, 91 individuals).

Acknowledgements

We thank Nature Seychelles for facilitating fieldwork on Cousin Island and the Seychelles Bureau of Standards and the Ministry of Agriculture, Climate Change & Environment for providing permission to conduct fieldwork and sample collection. This study would not have been possible without the contribution of exceptional fieldworkers, laboratory technicians and database managers associated with the Seychelles Warbler Project. The research presented in this paper was carried out on the High-Performance Computing Cluster supported by the Research and Specialist Computing Support service at the University of East Anglia.

Compliance with ethical standards

Ethics Statement

Fieldwork was carried out in accordance with local ethical regulations and agreements (UEA ethics approval ID ETH2223-0665). The Seychelles Department of Environment and the Seychelles Bureau of Standards approved the fieldwork (permit number A0157).

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability statement

All raw sequence data have been submitted to the European Nucleotide Archive (ENA) database under the study accession number PRJEB81709.

Code availability statement

The data files and script necessary to reproduce the statistical analysis and plots are provided at https://github.com/Chuen-Lee/SW_Senescence_GM

Supplementary Information:

Gut microbiome composition and function – including transposase gene abundance - varies with age, but not senescence, in a wild vertebrate

Wild gut microbiome changes with age

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Supplementary methods

Sample collection and storage

Between 2017 and 2023 all caught birds were placed in a disposable flat-bottom waxed paper bag containing a sterilised plastic weighing tray underneath a sterilised metal grate [43,55,100]. This allows the bird to stand on the grate and faecal samples to fall into the sterile tray, minimising contact with the bird's surface. After ca 15 minutes (or when defecation was observed) the bird was removed. Any sample was collected, using a single-use sterile flocked swab, and placed into a microcentrifuge tube containing 1 mL of absolute ethanol. Samples were stored at 4°C in the field before being transferred to -80°C for long-term storage.

Bioinformatics

Briefly, host reads were removed by mapping sequences to the Seychelles warbler genome (unpublished; complete BUSCO = 96.0% with a total length = 1,081,018,985 bp), using Kraken 2 (version 2.1.3). Remaining reads underwent quality filtering using sdm software version 2.14 beta [101,102]. After trimming, two samples and five hand controls were removed because they did not return enough reads for subsequent analysis (< 300,000 reads). An average of 20,481,040 (±1,109,059 SE) paired-end reads per sample were retained across the remaining samples.

The same trimmed reads were also used for *de novo* metagenome assembly, as implemented in MATAFILER: MEGAHIT version 1.2.9 [103] was used for metagenomic assemblies, on these genes were predicted using Prodigal version 2.6.3 [104] and clustered into a gene catalogue (95 % identity) of 19,527,109 gene clusters, and a gene abundance matrix created using rtk2 [105]. Functional annotations of clustered genes were done using eggNOGmapper version 2.1.12 and the evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) database version 4 [82,106]. Subsequently, genome binning was done with SemiBin which created 4,176 bins (mean completeness = 34.95%, mean contamination = 1.41%) [107]. The bins were then filtered based on >80% completeness and <5% contamination using CheckM2 [108]; this retained 824 metagenome-assembled genomes (MAGs). MAGs were dereplicated across samples to generate 323 non-redundant metagenomic species (MGS) level bins, using clusterMAGs (https://github.com/hildebra/clusterMAGs). For MGSs, taxonomic assignment was performed using a marker-based approach with GTDB database version 214 [109]. Due to the high individuality of the warbler GM and the high sequencing coverage required to assign MGS, only one MGS was present in more than 50% of sequenced samples and relatively fewer MGSs were identified per sample (average 17 $\pm$ 1.3 SE per sample) which is likely to be an underestimate of the true diversity of the GM.

Therefore, Metaphlan4 version 4.1.0 (which is assembly-free and therefore requires lower coverage) was used to taxonomically classify reads using the default parameters [110]. Metaphlan4 assignments identified an average of 29.3 $\pm$ 2.0 species genome bins per

sample and were used for the subsequent taxonomic analysis and MGS was only used for tracking functional annotations back to their taxonomy.

Post-hoc functional differential abundance analysis

Posthoc investigations were performed on individual eggNOG members found within the COG categories that were significantly differentially abundant with age. Firstly, a linear model was performed for each significant eggNOG member to test whether age-related changes were driven by between- or within-individual processes. Second, we tested if changes in the abundance of significant eggNOG members could be driven by changes in the abundance of the taxa from which these genes originate. To test this, the total abundance of MGSs carrying the eggNOG gene orthologs of interest was used as the response variable and age was included as a predictor in a lmer model. Furthermore, genera of eggNOG-carrying MGSs were matched with metaphlan4 genera to test whether the total abundance of known eggNOG-carrying genera was significantly associated with host age. Lastly, a protein-protein Basic Local Alignment Search Tool (BLASTp) analysis of each eggNOG gene ortholog of interest embedded within each MGS was performed to determine the identity of genes [111,112]. To test if the differential abundance of eggNOG members was driven by changes in the abundance of a specific gene (versus the cumulative abundance of many genes), gene catalogues assigned to the eggNOG cluster of interest (filtered to those with > 20% prevalence and 0.1% detection) were tested for differential abundance.

Supplementary Figures and Tables

Components of positive controls were successfully recovered as high-quality MGSs in acceptable relative abundances (Figure S2). Only 2 out of the 18 MGS from controls were found in faecal samples, both were widespread species *Enterococcus faecalis* and *Klebsiella pneumoniae* [113,114]. *E. faecalis* was part of the positive control but not found in the hand controls. *K. pneumoniae* was found in hand controls as well as samples but due to the low abundance in hand controls, we decided to retain all species for taxonomic analysis.

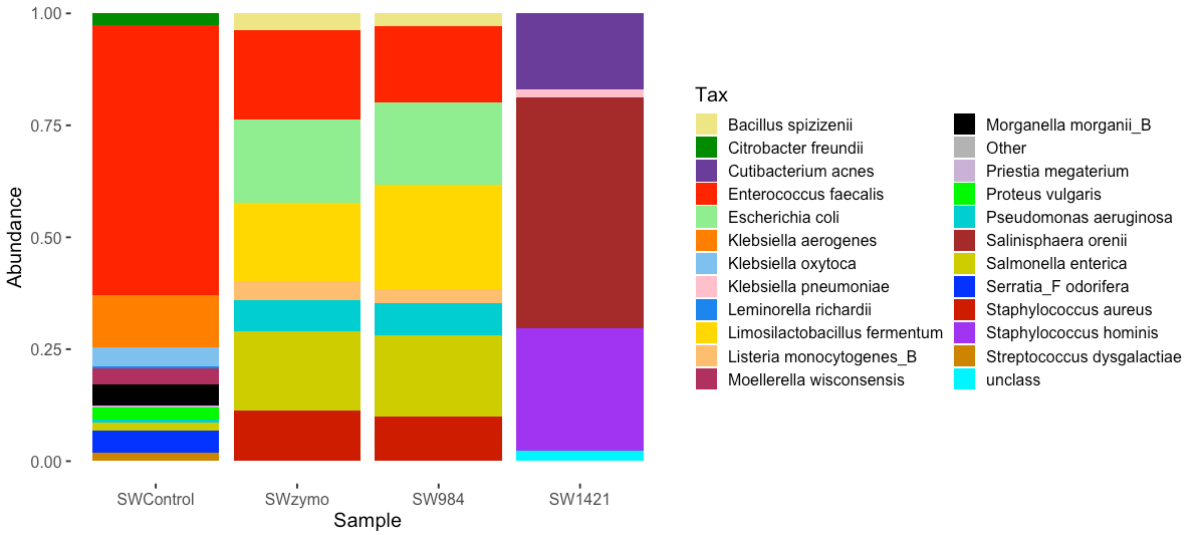


Figure S1. Controls and relative abundance of MGS at the species level. SWControl is positive control (ZymoBIOMICS Fecal Reference with TruMatrix™ Technology), SW984 and SWzymo are positive controls (ZymoBIOMICS Microbial Community Standard) sequenced separately, and SW1421 is a contamination (hand) control from 2023. We identified subspecies of *Bacillus subtilis* - *Bacillus spizizenii* and *Lactobacillus fermentum* – *Limosilactobacillus fermentum* . In SW1421 hand control, *Cutibacterium acnes* is linked to acne, *Klebsiella pneumoniae* is commonly found in the gut, *Salinisphaera orenii* are bacteria commonly isolated in high salinity environments, *Staphylococcus hominis* is commonly found to be harmless on human and animal skin.

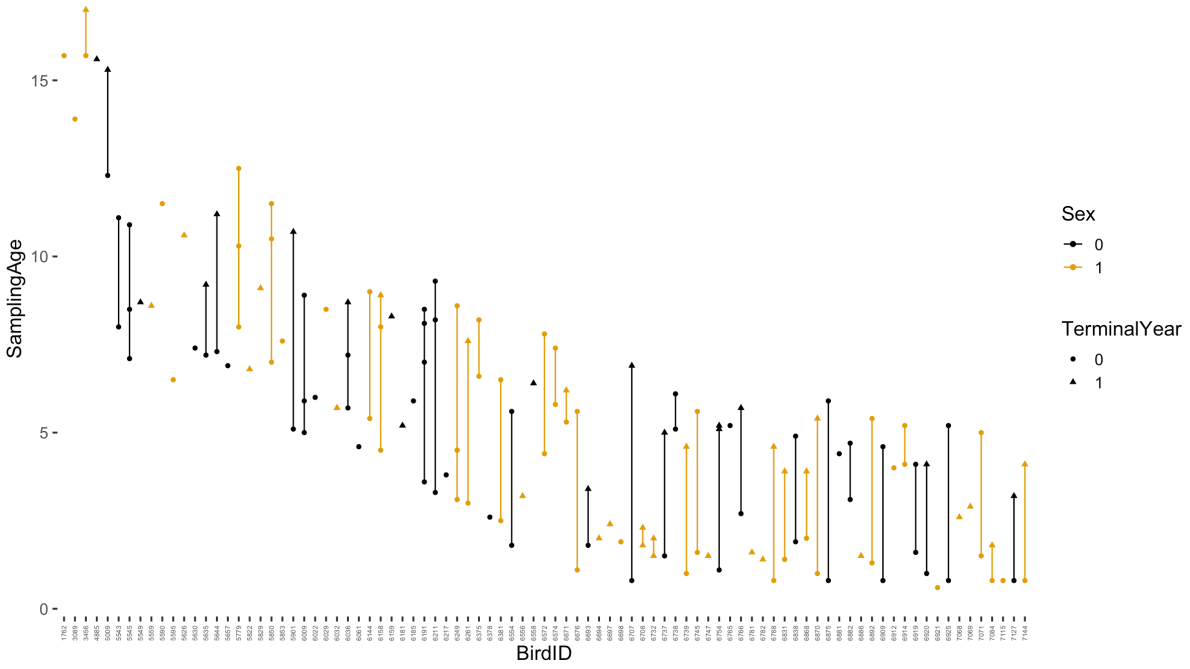


Figure S2. Seychelles warbler gut microbiome samples that were retained for analysis after sequencing and bioinformatics (n = 153 from 91 individuals). Points represent each sample, the y-axis represents individual's age at sampling, whilst the x-axis represents

individuals. Solid lines connect samples that were collected from the same individual. Colours represent the different sex (black = female, gold = male). Shape represents whether the sample was collected in the individual's terminal year (circle = no, triangle = yes).

Taxonomy

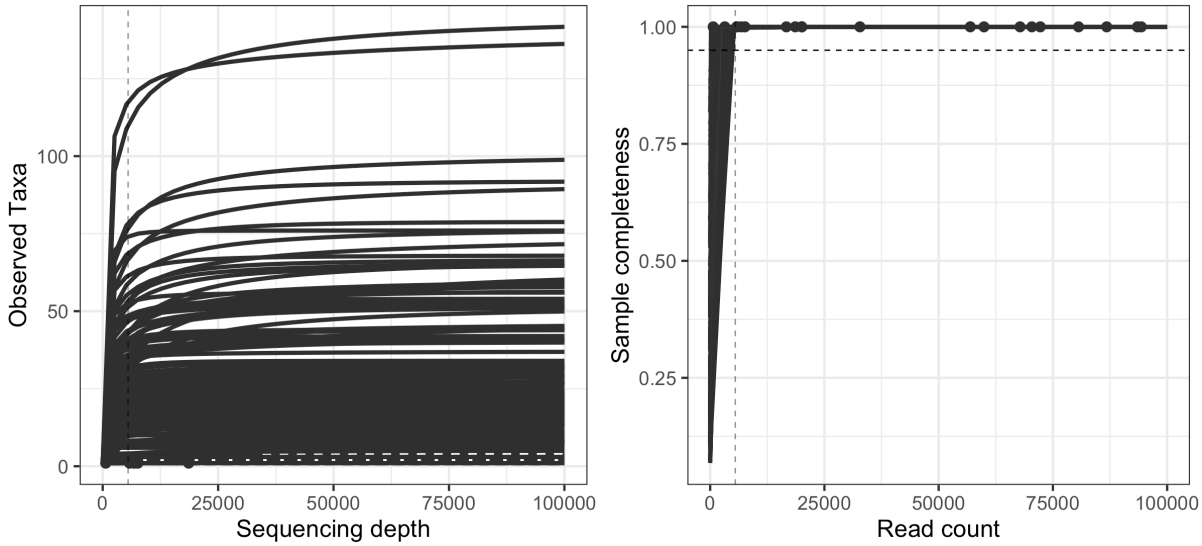


Figure S3. Sequencing depth against number of observed (metaphlan4) assembly-free taxonomic assignments (left) and read count against sample completeness (right) of each gut microbiome sample from Seychelles warblers (n = 153). 5500 reads at 95% completeness.

Table S1. A generalised linear mixed effect model with a negative binomial distribution investigating the relationship between age, terminal year, and species richness in the gut microbiome of Seychelles warblers (n = 151 samples, 91 individuals). Significant ($p < 0.05$) predictors are shown in bold. Conditional R^2 = 38.9%.

Predictor	Estimate	SE	*z*	*P*
(Intercept)	-125.20	71.62	-1.75	0.081
Age	**-0.04**	**0.02**	**-2.10**	**0.036**
Terminal Year (yes)	**-0.26**	**0.13**	**-2.06**	**0.039**
Season (winter)	0.01	0.13	0.09	0.932
Sex (female)	0.01	0.13	0.05	0.959
Time at 4°C	-0.18	0.14	-1.33	0.183
Time of day	0.22	0.12	1.82	0.069
Territory quality	-0.08	0.12	-0.67	0.506
Sample Year	0.06	0.04	1.79	0.073
Random				
Individual ID	151 observations	91 individuals	Variance	0.14

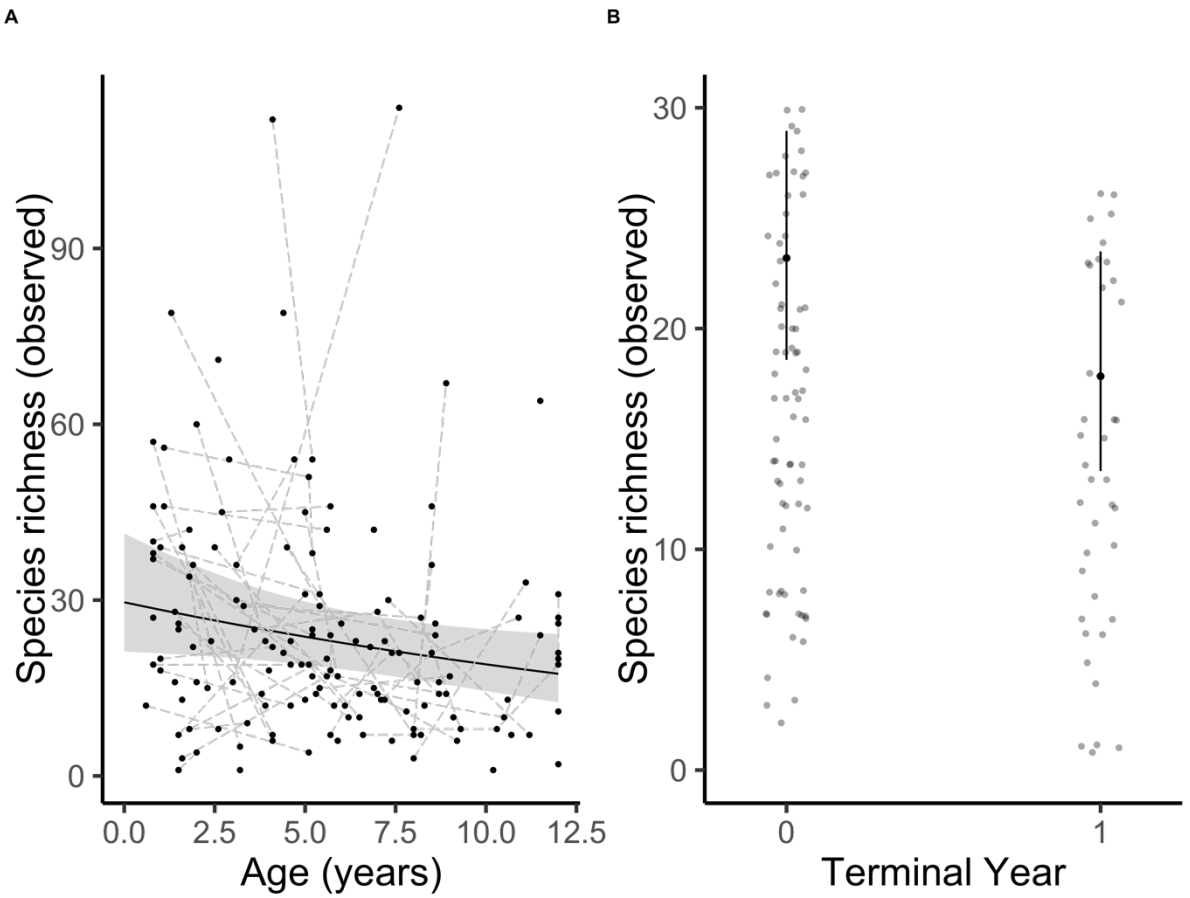


Figure S4. Species richness prediction from glmer.nb of the gut microbiome in the Seychelles warblers (n = 151 samples from 91 individuals). (A) Species richness against host age in years, solid black line and grey shaded area represent model predictions and confidence intervals respectively, points represent raw data. (B) Species richness against terminal year (0: No, 1: Yes), black dot and lines represent model predictions and error bars respectively, grey dots represent raw data points.

Table S2. A linear mixed effect model of Shannon diversity with chronological age and terminal year in the gut microbiome of Seychelles warblers (n = 151 samples, 91 individuals). Significant ($p < 0.05$) predictors are shown in bold. Conditional R^2 = 46.4%.

Predictor	Estimate	SE	*df*	*t*	*P*
(Intercept)	**-152.40**	**76.85**	**142.00**	**-1.98**	**0.049**
Age	-0.01	0.02	86.36	-0.46	0.644
Terminal Year (yes)	-0.16	0.14	133.79	-1.17	0.244
Season (winter)	-0.12	0.17	130.60	-0.69	0.491
Sex (female)	0.10	0.16	74.64	0.63	0.529
Time at 4°C	**-0.32**	**0.15**	**113.36**	**-2.15**	**0.034**
Time of day	-0.01	0.13	133.62	-0.10	0.920
Territory quality	-0.14	0.14	124.33	-1.02	0.311
Sample Year	0.08	0.04	142.00	2.00	0.047
Random					

Individual ID	151 observations	91 individuals	Variance	0.27

Table S3. A linear mixed effect model of Shannon diversity within- and between- individual age analysis, accounting for subsequent close-to-death samples in the gut microbiome of Seychelles warblers (n = 151 samples, 91 individuals). Significant ($p < 0.05$) predictors are shown in bold. Conditional R^2 = 49.7%.

Predictor	Estimate	SE	*df*	*z*	*P*
(Intercept)	**0.95**	**0.35**	**129.65**	**2.75**	**0.007**
Delta Age	-0.07	0.07	135.41	-1.12	0.265
Mean Age	-0.18	0.16	77.16	-1.14	0.257
Terminal Year Bird (yes)	-0.01	0.03	81.30	-0.24	0.809
Sample Year	0.09	0.06	105.90	1.60	0.11
Season (winter)	-0.12	0.17	128.97	-0.72	0.470
Sex (female)	0.10	0.16	75.58	0.62	0.535
Time at 4°C	**-0.33**	**0.15**	**112.75**	**-2.24**	**0.027**
Time of day	-0.02	0.13	131.47	-0.12	0.908
Territory quality	-0.15	0.14	122.92	-1.08	0.281
Random					
Individual ID	151 observations	91 individuals	Variance		0.3003

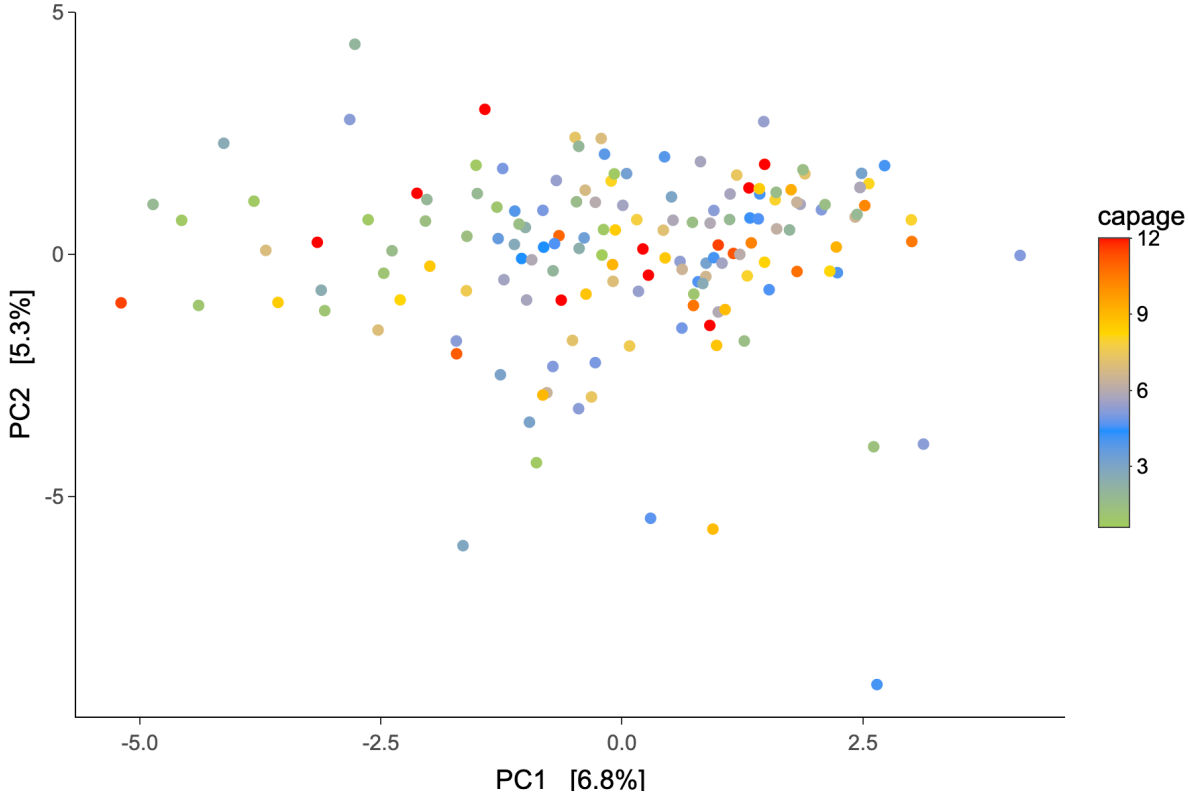


Figure S5. PCA plot of CLR-transformed reads in Euclidean distance, coloured by age.

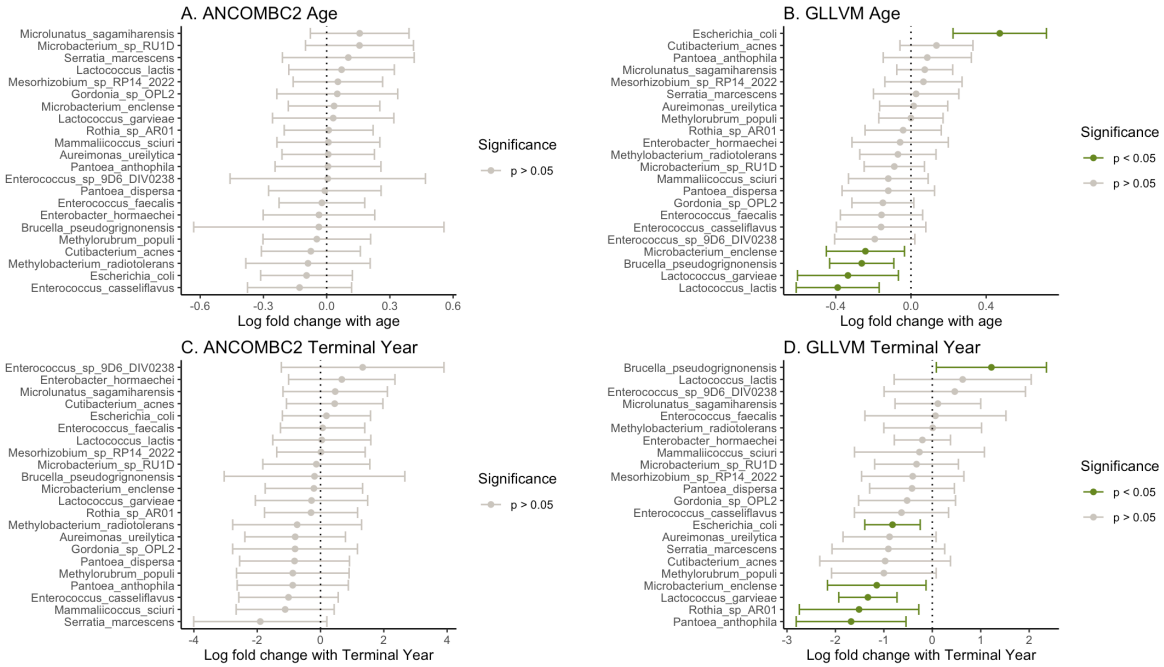


Figure S6. Taxonomic differential abundance analysis for common species (> 20% prevalence in the population). (A) ANCOMBC2 with age, (B) GLLVM with age, (C) ANCOMBC2 with terminal year, (D) GLLVM with terminal year. Significant ($p < 0.05$). Green = significant ($p < 0.05$) log fold change, grey = insignificant log fold change.

Table S4. A linear mixed effect model testing for age-related changes in functional scaled exponentially transformed observed richness and exponentially transformed Shannon diversity of eggNOG annotations in the gut microbiome of Seychelles warblers (n = 152 samples, 90 individuals). Conditional R^2 = 33.7% and 9.2% respectively.

Observed Richness					
Predictor	Estimate	SE	*df*	*t*	*P*
(Intercept)	-109.417	42.293	142.715	-2.587	0.011
Age (years)	**-0.036**	**0.013**	**92.620**	**-2.877**	**0.005**
Terminal Year (yes)	-0.124	0.077	142.784	-1.605	0.111
Season (winter)	-0.080	0.078	141.089	-1.024	0.307
Sex (female)	-0.080	0.080	78.890	-1.008	0.317
Days at 4°C	**-0.198**	**0.082**	**130.818**	**-2.422**	**0.017**
Time of day	-0.027	0.071	142.930	-0.373	0.710
Territory quality	-0.074	0.072	134.361	-1.030	0.305
Sample Year	**0.055**	**0.021**	**142.686**	**2.618**	**0.010**
Random					
Individual ID	152 observations	90 individuals	Variance		0.047
Shannon Diversity					
Predictor	Estimate	SE	*df*	*t*	*P*
(Intercept)	-92473.06	46119.45	143.00	-2.01	0.047

Age (years)	**-31.31**	**12.59**	**143.00**	**-2.49**	**0.014**
Terminal Year (yes)	-20.41	83.74	143.00	-0.24	0.808
Season (winter)	105.32	85.76	143.00	1.23	0.221
Sex (female)	-21.32	78.14	143.00	-0.27	0.785
Time at 4°C	-36.85	92.11	143.00	-0.40	0.690
Time of day	27.32	76.97	143.00	0.36	0.723
Territory quality	-1.21	79.70	143.00	-0.02	0.988
Sample Year	46.31	22.85	143.00	2.03	0.045
Random					
Individual ID	152 observations	90 individuals	Variance		108.9

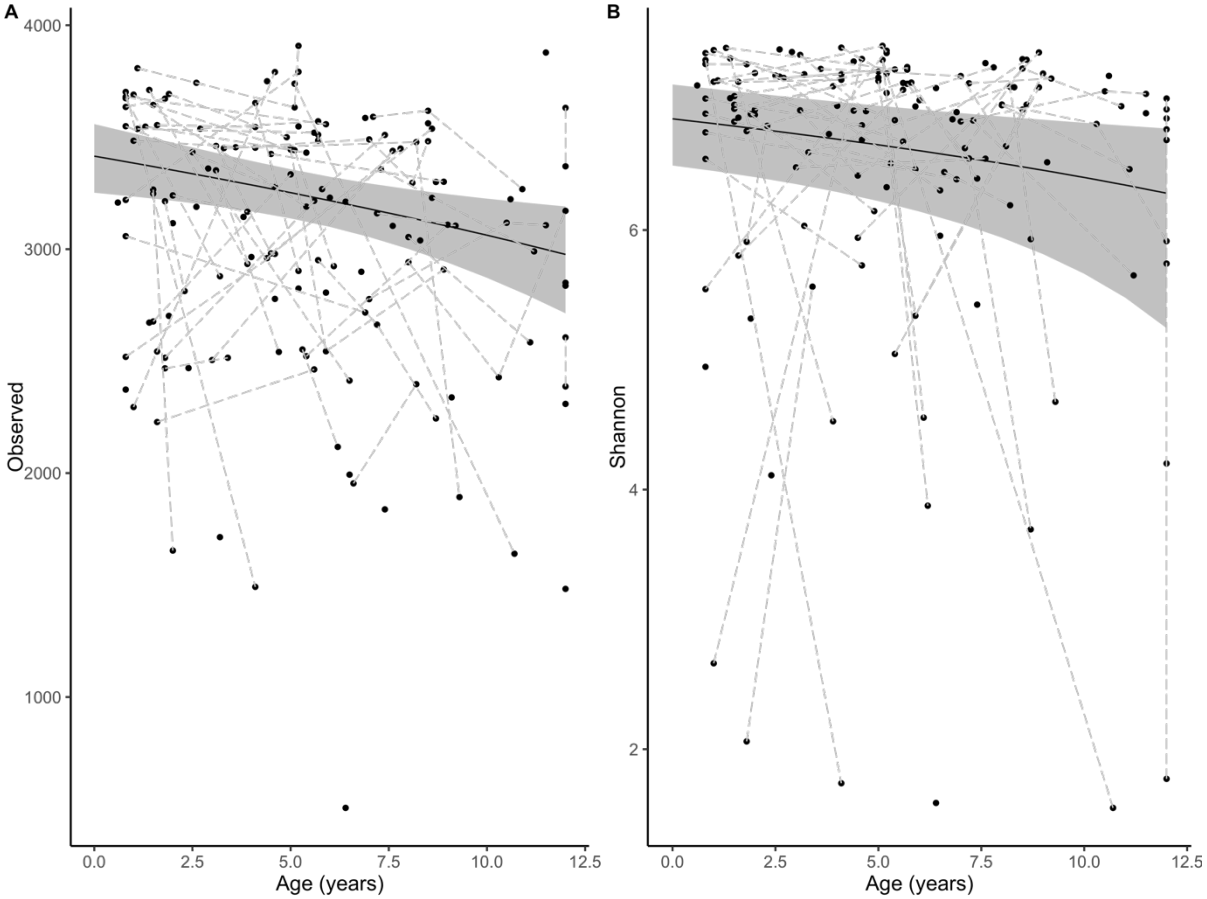


Figure S7. Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) (A) observed richness and (B) Shannon diversity against host age (years) model prediction from linear mixed effect model in the gut microbiome of Seychelles warblers. The solid line represents model predictions and ribbon-shadding represent confidence intervals from model predictions. Each point represents a sample, and the dashed grey lines connect samples collected from the same individual (n = 152 samples from 90 individuals).

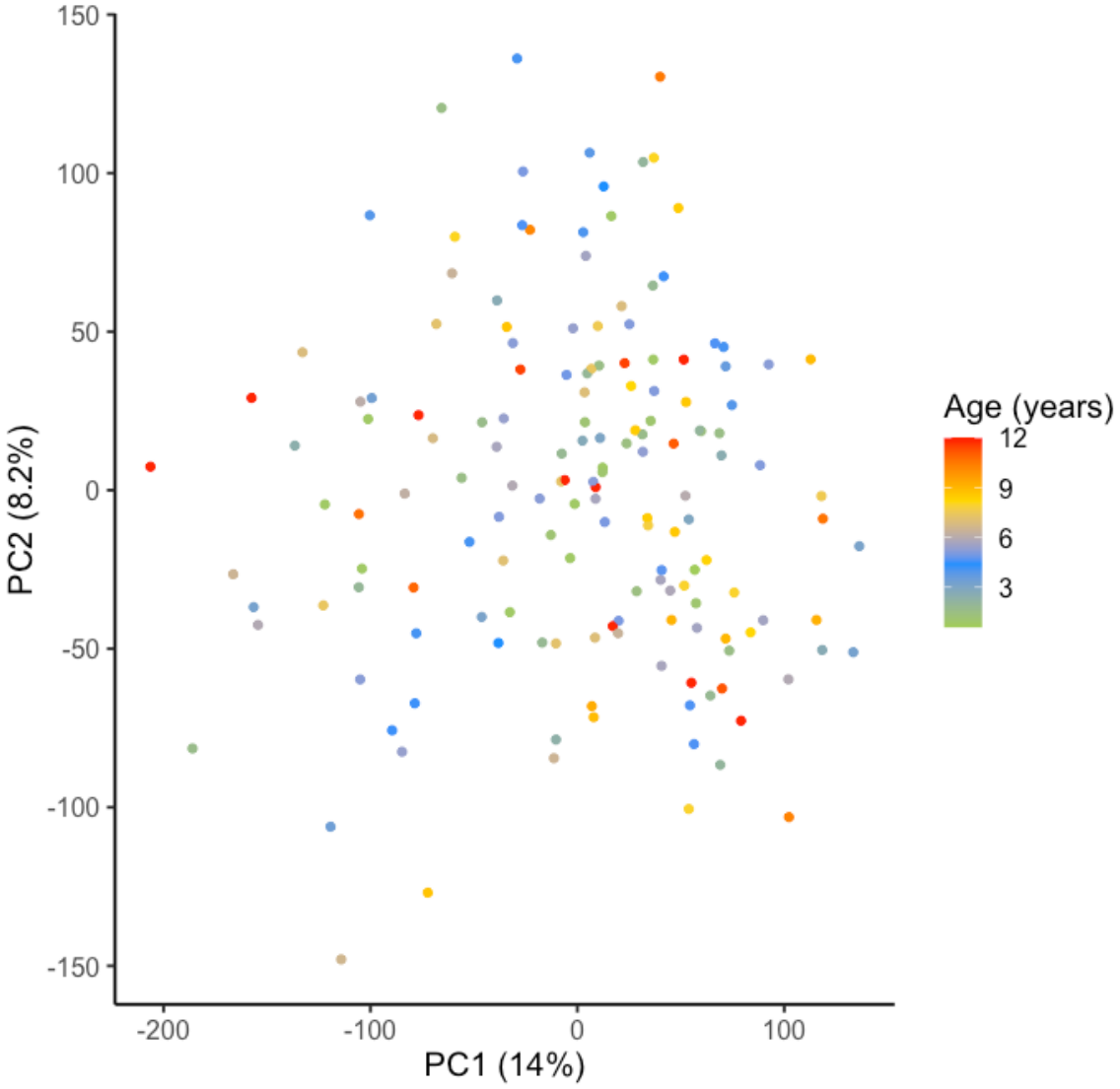


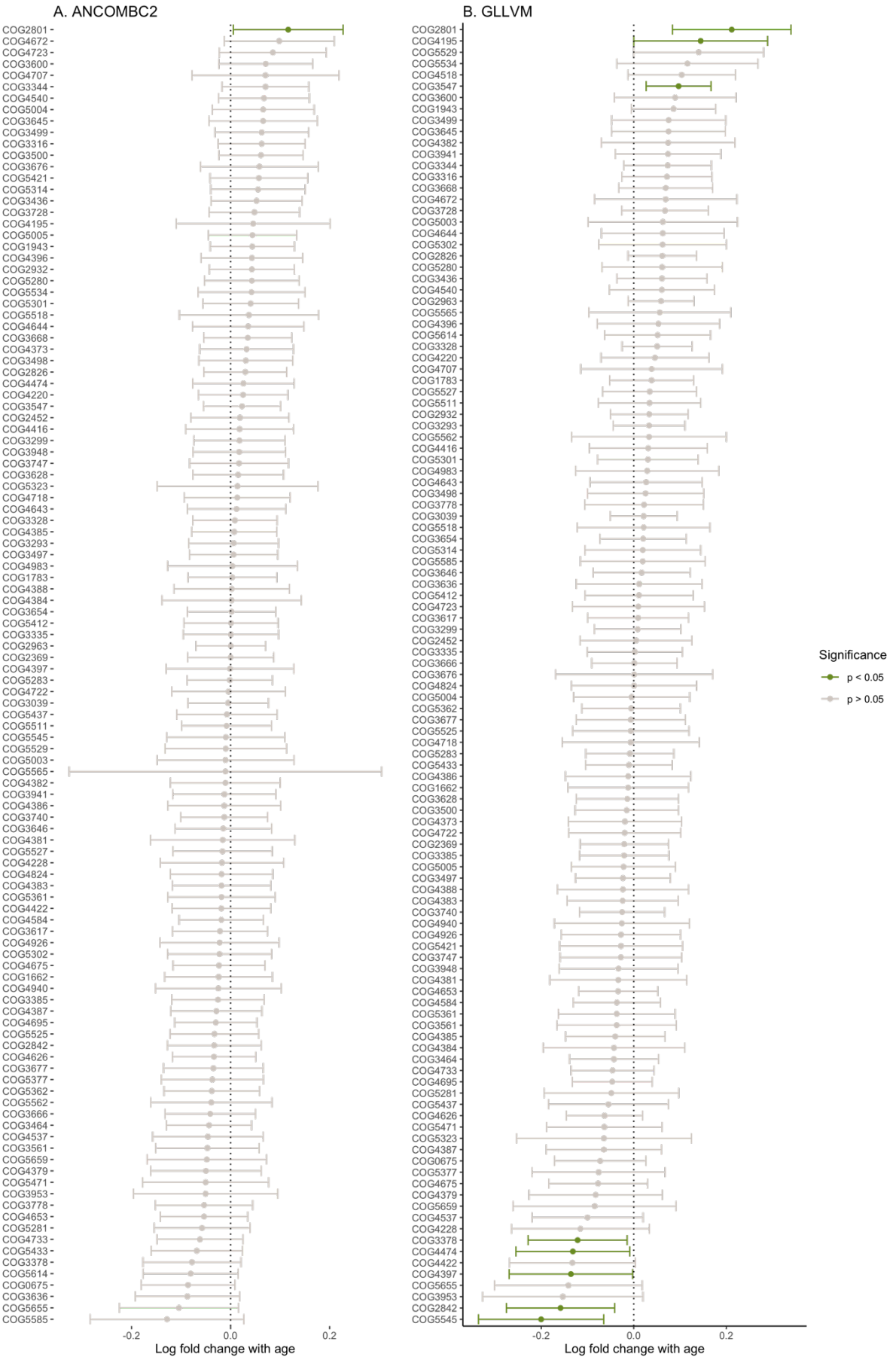
Figure S8. Functional PCA plot of CLR-count, euclidean distances of eggNOG annotations

Table S5. COG functional categories [71]

Abbreviation	COG Functional Categories
A	RNA processing and modification
K	Transcription
L	Replication, recombination and repair
B	Chromatin structure and dynamics
D	Cell cycle control, cell division, chromosome partitioning
V	Defense mechanisms
Y	Nuclear structure
T	Signal transduction mechanisms
M	Cell wall/membrane/envelope biogenesis
N	Cell motility
Z	Cytoskeleton
W	Extracellular structures

U	Intracellular trafficking, secretion, and vesicular transport
O	Posttranslational modification, protein turnover, chaperones
X	Mobilome: prophages, transposons
C	Energy production and conversion
G	Carbohydrate transport and metabolism
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
H	Coenzyme transport and metabolism
I	Lipid transport and metabolism
P	Inorganic ion transport and metabolism
R	General function prediction only
Q	Secondary metabolites biosynthesis, transport and catabolism
S	Function unknown
`	Unassigned

1027


A. ANCOMBC2
B. GLLVM
Log fold change with age
Log fold change with age
-0.2
0.0
0.2
Significance
p < 0.05
p > 0.05

1028

Figure S9 Differential abundance of COG X eggNOG members (A) ANCOMBC2 and (B) GLLVM.

Table S7. A linear mixed effect model of COG2801 abundance in the gut microbiome of Seychelles warblers in relation to within- (delta) and between- individual (mean) age. n = 153 samples, 91 individuals. Significant ($p < 0.05$) predictors in bold. Conditional R^2 = 14.7%. Reference categories for categorical variables are shown in brackets

Predictor	Estimate	SE	df	t	*P*
(Intercept)	9.700	0.971	115.370	9.989	**< 0.001**
Delta Age	**0.549**	**0.218**	**141.991**	**2.516**	**0.013**
Mean Age	**0.157**	**0.062**	**85.606**	**2.534**	**0.013**
Terminal Year Bird (yes)	0.028	0.420	69.803	0.067	0.947
Season (winter)	-0.502	0.553	132.368	-0.908	0.365
Sex (female)	0.219	0.422	63.434	0.520	0.605
Days at 4°C	-0.196	0.495	136.509	-0.396	0.693
Time of day	-0.313	0.428	136.421	-0.730	0.466
Territory quality	-0.315	0.452	141.901	-0.697	0.487
Sample Year (2017)					
2018	-1.662	0.902	140.921	-1.844	0.067
2019	-1.457	1.068	141.645	-1.363	0.175
2020	-2.200	1.129	134.384	-1.949	0.053
2021	**-2.911**	**1.119**	**140.585**	**-2.601**	**0.010**
2022	**-3.341**	**1.098**	**118.243**	**-3.042**	**0.003**
2023	**-3.215**	**1.289**	**111.442**	**-2.495**	**0.014**
Random					
Individual ID	153 observations	91 individuals		Variance	0.1776

Table S8. BLASTp top hits for each COG2801 found in the genomes of all constructed metagenomics species (MGS) from the gut microbiome of Seychelles warblers (n = 153 from 91 individuals).

Top hit (contains keyword)	**Count**	**Percentage**
IS3 transposase	154	30%
otherIS transposase	64	13%
transposase	170	33%
integrase	30	6%
Mobile element protein	4	1%
Helix-turn-helix	19	4%
Hypothetical protein	45	9%
Unknown	23	5%

Table S9. Linear mixed model on the CLR-transformed abundance of metagenomic species in the gut microbiome of Seychelles warblers (n = 2589 from 89 individuals). To test if COG2801-carrying MGS significantly differed in abundance with host age. Significant ($p < 0.05$) predictors are shown in bold. Conditional R^2 = 46.9%.

Predictor	Estimate	SE	*df*	t	*P*
(Intercept)	5.44	0.41	233.52	13.34	< 0.001
Age	0.03	0.04	69.32	0.79	0.432
Terminal Year (yes)	0.24	0.19	339.71	1.26	0.210
Season (winter)	-0.09	0.22	394.36	-0.43	0.671
Sex (female)	0.01	0.26	69.10	0.02	0.982
Time at 4°C	**-0.44**	**0.18**	**434.74**	**-2.40**	**0.017**
Time of day	**-0.35**	**0.18**	**395.12**	**-2.01**	**0.045**
Territory quality	**-0.47**	**0.17**	**379.46**	**-2.85**	**0.005**
Sample Year (2017)					
2018	**-0.77**	**0.41**	**402.12**	**-1.90**	**0.059**
2019	**-1.69**	**0.46**	**416.15**	**-3.71**	**0.000**
2020	**-1.19**	**0.48**	**360.43**	**-2.50**	**0.013**
2021	-0.70	0.46	334.48	-1.53	0.127
2022	-0.56	0.45	266.74	-1.25	0.213
2023	-0.65	0.49	239.09	-1.33	0.186
Random					
Individual ID	874 observations	85 individuals		Variance	1.042

Table S10. Linear mixed model on the CLR-transformed abundance of metaphlan4 genera in the gut microbiome of Seychelles warblers (n = 4477 from 91 individuals). To test if known COG2801-carrying genera significantly differed in abundance with host age. Significant ($p < 0.05$) predictors are shown in bold. Conditional R^2 = 16.8%.

Predictor	Estimate	SE	df	t	*P*
(Intercept)	9.08	0.45	316.13	20.37	< 0.001
Age	0.04	0.04	77.18	0.91	0.363
Terminal Year (yes)	0.30	0.22	272.48	1.37	0.173
Season (winter)	-0.30	0.27	271.10	-1.09	0.276
Sex (female)	0.15	0.27	70.01	0.54	0.589
Time at 4°C	**-0.52**	**0.22**	**373.62**	**-2.34**	**0.020**
Time of day	**-0.60**	**0.21**	**224.79**	**-2.82**	**0.005**
Territory quality	0.03	0.21	486.10	0.13	0.898
Sample Year (2017)					
2018	-0.15	0.47	519.08	-0.33	0.745
2019	-0.85	0.54	423.70	-1.57	0.116
2020	-0.80	0.55	380.92	-1.46	0.145
2021	**-1.13**	**0.52**	**377.58**	**-2.20**	**0.029**
2022	-0.56	0.49	363.36	-1.14	0.254

2023	-0.06	0.55	281.62	-0.11	0.916
Random					
Individual ID	1794 observations	89 individuals		Variance	0.995

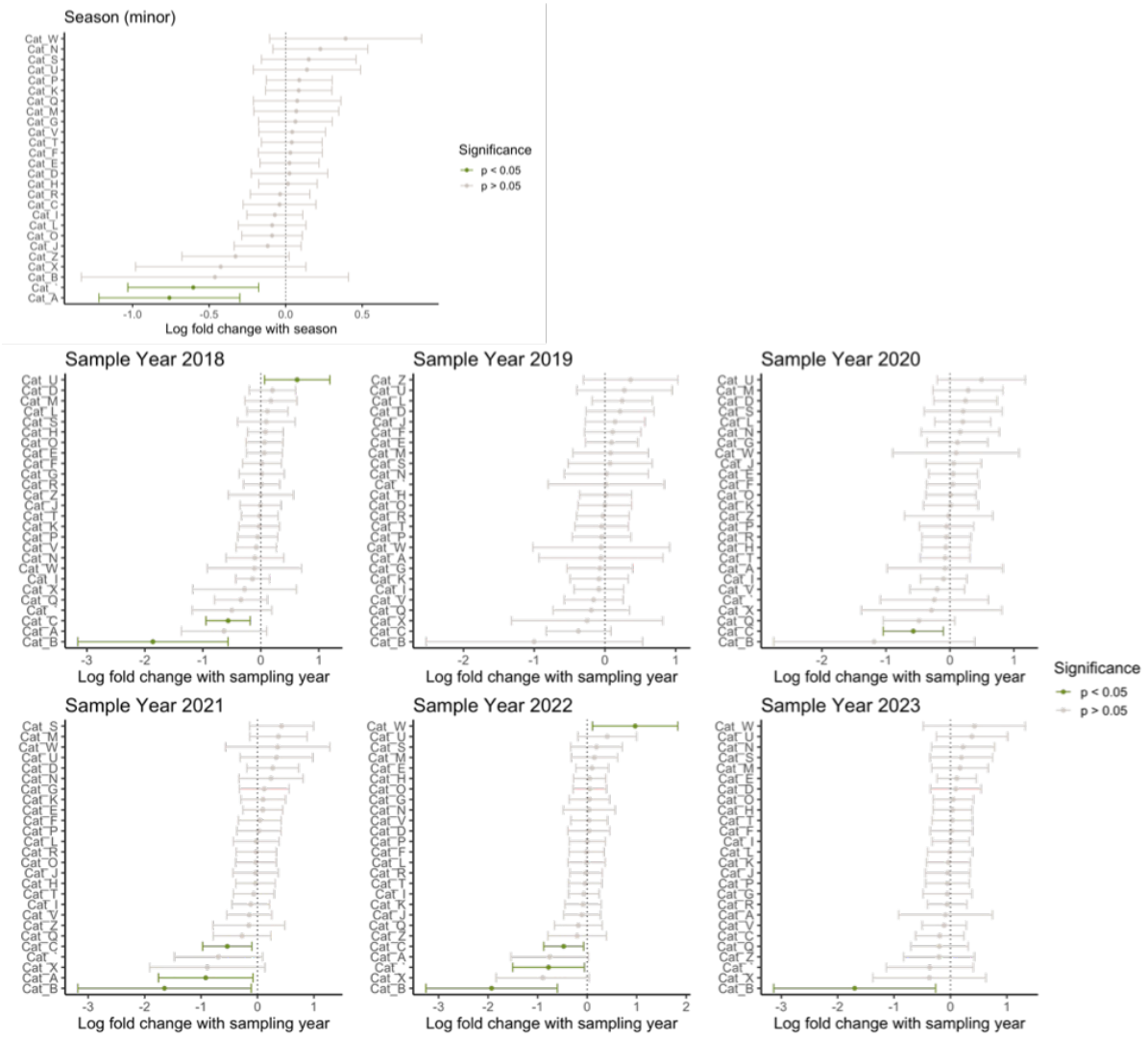


Figure S10. Differential abundance analysis of functional gut microbiome cluster of orthologous genes (COG) categories in Seychelles warblers using ANCOMBC2 with season and sample year. Each COG category is represented by a letter on the y-axis. Details of all COG categories are given in Table S5 [71]. "Cat_`" represents eggNOG annotations that were not assigned a COG category. Points and error bars are coloured according to significance (green: $p < 0.05$; grey: $p > 0.05$).

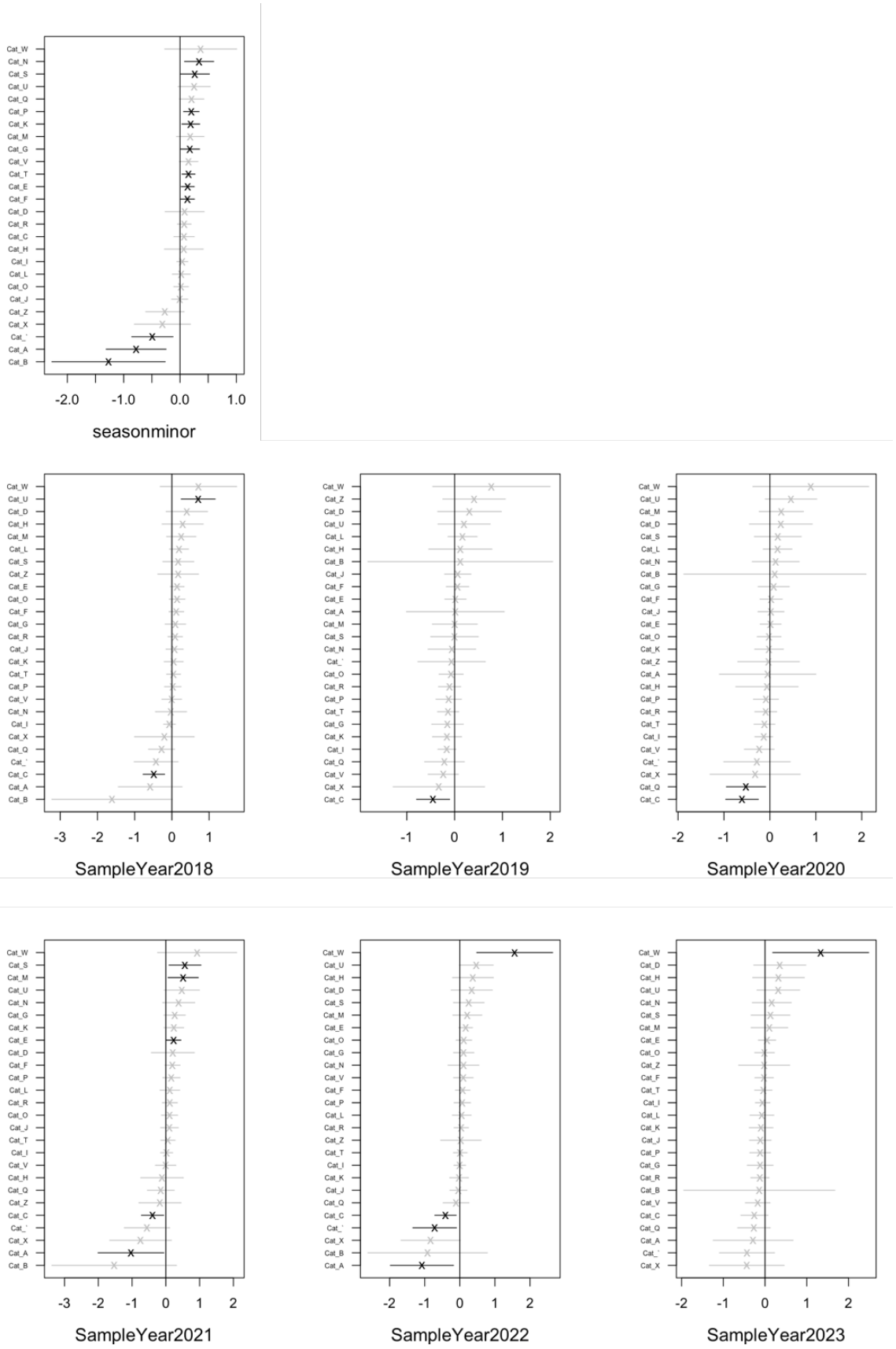


Figure S11. Differential abundance analysis of functional gut microbiome cluster of orthologous genes (COG) categories in Seychelles warblers using GLLVM with season and sample year. Each COG category is represented by a letter on the y-axis. Details of all

COG categories are given in Table S5 [71]. “Cat_`” represents eggNOG annotations that were not assigned a COG category. Points and error bars are coloured according to significance (black: $p < 0.05$; grey: $p > 0.05$).

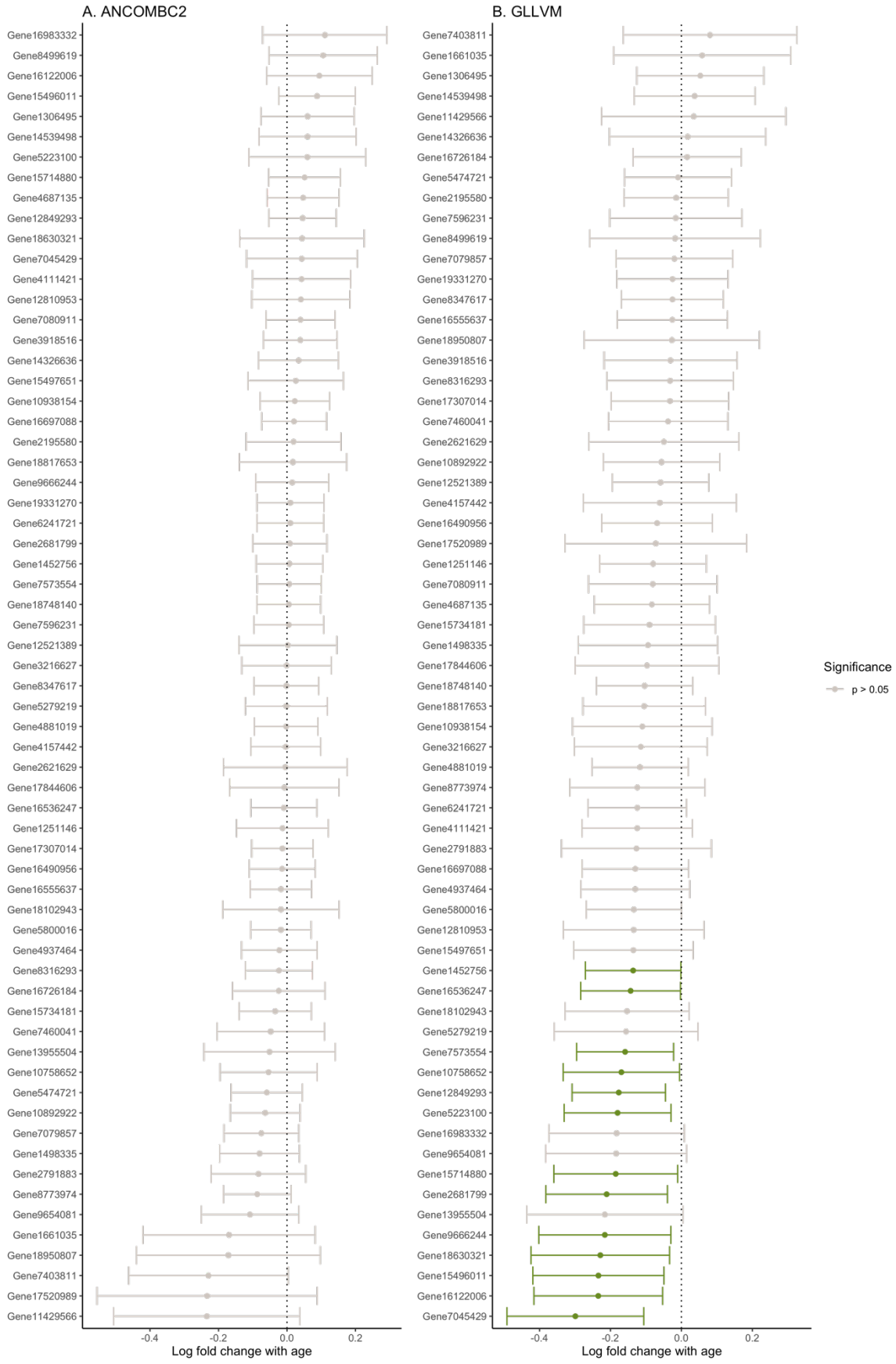


Figure S12. Differential abundance analysis of functional gut microbiome COG2801 gene catalogue that were commonly (20% prevalence) found in Seychelles warblers using (A)

ANCOMBC2 and (B) GLLVM. Each gene catalogue (95% average nucleotide identity) are represented on the y-axis by their gene catalogue number. Points and error bars are coloured according to significance (black: $p < 0.05$; grey: $p > 0.05$).