Associations between brood size, gut microbiome diversity and survival in great tit (*Parus major*) nestlings

Liukkonen[1*], M., Hukkanen[2,5] M., Cossin-Sevrin[2,], N., Stier[2,4], A., Vesterinen[2], E., Grond[6], K. & Ruuskanen[1,2], S.

[1] Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland
[2]Department of Biology, University of Turku, Turku, Finland
[4] Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA, F-69622, Villeurbanne, France
[5] Institute for Molecular Medicine Finland, University of Helsinki, Helsinki, Finland
[6] Department of Biological Sciences, University of Alaska Anchorage, Anchorage, AK 99508, USA

* Corresponding author: Martta Liukkonen
email: martta.liukkonen@gmail.com or martta.a.liukkonen@jyu.fi

Keywords: avian microbiome, brood size, gut microbiome, *Parus major*, 16S rRNA gene

ABSTRACT

Background

The gut microbiome forms at an early stage, yet data on the environmental factors influencing the development of wild avian microbiomes is limited. The early studies with wild gut microbiome have shown that the rearing environment may be of importance in gut microbiome formation, yet the results vary across taxa, and the effects of specific environmental factors have not been characterized. Here, wild great tit (*Parus major*) broods were manipulated to either reduce or enlarge the original brood soon after hatching. We investigated if brood size was associated with nestling bacterial gut microbiome, and whether gut microbiome diversity predicted survival. Fecal samples were collected at mid-nestling stage and sequenced with the 16S rRNA gene amplicon sequencing, and nestling growth and survival were measured.

Results

Gut microbiome diversity showed high variation between individuals, but this variation was not explained by brood size or body mass. Additionally, we did not find a significant effect of brood size on body mass or gut microbiome composition. Furthermore, we found no significant association between gut microbiome diversity and short-term (survival to fledging) or mid-term (apparent juvenile) survival.

Conclusions

Early-life environment can lead to variation in offspring condition and gut microbiome and therefore, understanding how and which changes in the rearing environment are associated with offspring development is of importance. However, we did not find an association between brood size, gut microbiome diversity and

survival, indicating that future studies should expand into other early-life environmental factors e.g., diet composition and quality, and parental influences.

INTRODUCTION

The digestive tract hosts a large community of different microorganisms (i.e., gut microbiome) and is known to be a fundamental part of organismal health and a powerful proximate mechanism affecting host performance [1-2]. The gut microbiome has been studied across a wide range of animal taxa e.g., humans [3-5], fish [6], and economically important species such as poultry [7], and data from wild populations is slowly increasing, as reviewed by Hird 2017 [8]. Generally, a more diverse gut microbiome is considered beneficial for individual health [9], but there are also community structure effects that define the functionality [10]. For example, laboratory-bred mice with a less diverse gut microbiome have a substantially lower chance of surviving an influenza infection compared to their wild counterparts unless receiving a gut microbiota transplant from their wild counterparts [11-12]. Moreover, gut microbiome had been linked to host fitness and survival in the Seychelles warbler (*Acrocephalus sechellensis*): individuals that harbored opportunistic pathogens in their gut microbiome showed higher mortality [13-14]. Therefore, understanding how gut microbiome affects fitness within and between individuals is necessary for not only understanding species survival but also evolution [15-17].

Gut microbiome is largely defined at a young age and remains somewhat stable in adulthood as found for example in germ-free mice [18-20]. Dysbiosis at a young age

could result in both short-term and long-term effects in the gut microbiome [21-22]. Of the environmental effects, diet [23], including e.g., macronutrient balance (carbohydrates, fats, amino acids; [3, 24] have been concluded to be major determinants of murine gut microbiome, and this effect has recently been seen in avian models as well [25-28]. Moreover, macronutrient balance has been linked to intestinal microbiome composition [3, 24] and the functioning of individual immune response [29-30]. However, as a large part of the prior research has focused strictly on humans or species living in controlled environments in which environmental effects on both the microbiome and host are sidelined [31-32], many species, including the majority of birds [8], are only now attracting more attention, as reviewed by Bodawatta et al. [33].

The mechanisms of bacterial colonization of the bird gut are somewhat unique as avian life-histories differ significantly from those of e.g., mammals [34]. With mammals, the offspring are exposed to bacterial colonization during vaginal birth [35] and lactation [e.g., 36-37], whereas bird hatchlings are first exposed to bacteria upon hatching [20, 38; but see Trevelline et al. [39] for *in ovo* bacterial colonization. Genetics [40-42] as well as the post-hatch environment [20, 43-46] have a significant effect on the formation of the avian gut microbiome. Once hatched, most altricial birds feed their young which exposes the hatchlings to various bacteria that originate from the parents i.e., via vertical transmission [47]; but see [20] Grond et al. 2017. It has also been shown that environmental factors are major contributors in the formation of gut microbiome [48-51], one these being the rearing environment (nest) [44].

As early-life environment is connected to the establishment of gut microbiome, variation in brood size may affect gut microbiome [52]. Brood size is often associated with parents' performance and ability to feed their young without risking parents' survival [53]. The trade-off between offspring quality and quantity has been studied widely [e.g., 54-55] food quantity per nestling can decrease in enlarged broods because even if parents are able to increase food acquisition, they cannot fully compensate for the amount an enlarged brood requires e.g., [56-57]. For example, in great tits (*Parus major*) it has been shown that nestlings from reduced broods may have a higher body mass [58] and tend to survive better to the following autumn and breeding season [59]. Importantly, great tit nestling body mass has been connected to gut microbiome diversity and composition: body mass positively correlates with gut microbiome richness [52]. This could imply that good physiological condition and high food availability would allow the host to have a diverse gut microbiome that promotes a healthy gut.

Altered early-life gut microbiome could have long-term consequences on individual performance [e.g., 60], yet such effects have rarely been studied in wild organisms. In wild birds, some bacterial taxa have been linked to better survival, for example, abundance in the order *Lactobacillales* (of the phylum *Firmicutes*) in adult birds is related to higher individual fitness [14, 61] and is known for the benefits for bird health in economically important species such as poultry where *Lactobacilli* are used as probiotics to boost immune functioning [62]. Besides *Lactobacillales* gut bacteria belonging to the genera *Clostridium* and *Streptococcus* are important in degrading non-starch polysaccharides and known for synthesizing essential molecules such as the

short-chain fatty acids [e.g., 63-64]. Short-chain fatty acids are important in host energy metabolism [65] and therefore crucial for performance. Changes in nestling's early-life gut microbiome could affect these key physiological processes, which could influence for example nestling body mass which is tightly linked to survival to fledging [58-59]. Moreover, changes in the rearing environment can affect individual physiology and these effects can carry over to later stages of an individual's life such as survival to fledging and lifetime reproductive success [66].

Here, we use an experimental approach to investigate whether brood size influenced wild great tit nestlings' bacterial gut microbiome diversity (on day 7 post-hatch), nestling body mass on day 7 and 14 post-hatch, and whether gut microbiome predicts short-term (i.e., survival to fledging) and mid-term (i.e., apparent juvenile) survival. The great tit is a well-studied species in the fields of ecology and evolution, and it is easy to monitor in the wild due to its habit of breeding in nest boxes. Great tit nestlings' gut microbiome undergoes profound shifts during early life [52], and it has been linked to nestling natal body mass and body size [52, 61], yet studies focusing on gut microbiome associations with survival are still scarce. Here, we manipulated wild great tit broods by reducing and enlarging the original brood size and analyzing the gut microbiome diversity and composition. In large broods nestlings need to compete for their food more [67-68], and the lower food availability could result in a lower gut microbiome diversity, which might impair nestling body mass and fitness prospects [13, 52]. We incorporated a partial cross-fostering in the study design that enabled us to disentangle the relative contributions of genetic background (and early maternal effects) and rearing environment (parents, nest and nestmates) on gut microbiome.

Furthermore, we used an unmanipulated control group in which no nestling was cross-fostered to control for moving the nestlings between nests (i.e., if early human handling such as marking and weighing at day 2 post-hatch influences gut microbiome later on). We hypothesized that 1) in reduced broods nestlings would have a higher body mass, 2) in reduced broods nestling gut microbiome would be more diverse than in enlarged broods, and 3) higher gut microbiome diversity would explain higher short-term (survival to fledging) and mid-term (apparent juvenile survival). Our results bring new knowledge about gut microbiome in wild passerine bird population and how the early-life environment may associate with nestling gut microbiome, body mass, and short-term and mid-term survival.

METHODS

Study area and species

The great tit is a small passerine bird, which breeds in secondary holes and artificial nest-boxes, making it an easy model to study in the wild. Great tits breed throughout Europe and inhabit parts of Northern Africa and Asia as well, and the breeding areas differ in environment and diet [69]. In Finland the great tit is a common species with an estimate of 1.5 to 2 million breeding pairs. They lay 6 to 12 eggs between April and May and the female incubates the eggs for 12 – 15 days. The nestlings fledge approximately 16 to 21 days after hatching. The study was conducted during the breeding season (May-July 2020) during which we manipulated brood sizes of great tits in the study population on Ruissalo island (60° 25' 59.99" N 22° 09' 60.00" E).

Brood size manipulation experiment

Nest boxes were first monitored weekly and later daily when clutches were close to the estimated hatching date. Brood size manipulation took place on day 2 after hatching. Changes in great tit brood size can lead to lowered weight in both the nestlings and adults [e.g., 70-75], and our decision on the number of manipulated nestlings followed the previous studies. We had four treatment groups (see Fig 1, incl. sample sizes) : in the 'enlarged group (E)', we increased the brood size by two individuals that were taken from a 'reduced brood'; In the 'reduced group (R)', we decreased the brood size by two individuals, that were added to the enlarged broods; in the 'control group (C)', we swapped nestlings between nests but did not change the

brood size; and lastly, in the 'unmanipulated control group (COU)', we only weighed and collected fecal samples on day 7 but did not move the nestlings between nests. We also moved nestlings between the reduced nests to ensure that all nests (except COU) had both original and fostered nestlings. Control nests were used to control for potential cross-fostering effects. Additionally, in the unmanipulated control group nestlings were not moved or weighed at all on day 2 to control any handling effects per se. This study design enabled us to test the potential impacts of handling nestlings and swapping the nest early after hatching. We aimed to move approximately half of the chicks in the manipulated nests, so that the number of original and the fostered nestlings would be the same in each nest after manipulation.

Before they were moved, nestlings were weighed using a digital scale (0.1 g) and identified by clipping selected toenails. We aimed to add/remove nestlings that were of similar weight and to avoid changing the sibling hierarchy in the brood. The moving procedure was performed as quickly as possible to limit stress and the nestlings were kept in a warmed box during transportation. For each pair of nests in the brood size manipulation experiment, we aimed to select nests that had a similar initial clutch size, and then randomly chose which clutches to enlarge and reduce and which to use as control and unmanipulated control. To avoid potential bias from hatching date, we allocated nests in any given day evenly to each treatment. We also checked that the treatments had an equal clutch size on average i.e., we did not want to only reduce the larger clutches and enlarge the smaller clutches. Average brood size (mean±s.d.) before manipulation was 7.650±1.309 in the enlarged group (E), 8.375±1.637 in the reduced group (R), 7.565±1.805 in the control group (C), and

7.810±2.112 in the unmanipulated control group (COU). Brood sizes between treatment groups did not differ significantly from each other prior to cross-fostering (ANOVA: F_3=1.021, p=0.388). Average brood size after manipulation was 9.650±1.309 in E, 6.125±2.028 in R, and 7.565±1.805 in C. As the COU group was not manipulated at all, the average brood size remained the same in the COU group. Brood size differences between treatment groups were statistically significant post cross-fostering (ANOVA: F_3=13.244, p<0.000) except for C and COU group (t_{84}=0.437, p=0.972) which were intended to be of similar size. More information on brood size differences between treatment groups pre and post cross-fostering can be found in supplements (SI 1). We further tested whether treatment groups had similar average hatching dates and found that there were some differences among the groups (ANOVA: F_3=3.964, p=0.011). COU group showed on average a 4 days later hatching date compared to groups E (t_{84}=2.961, p=0.020) and C (t_{84}=2.983, p=0.019) but there were no statistical differences between other groups. Hatching date was included as a covariate in the model to control for these differences.

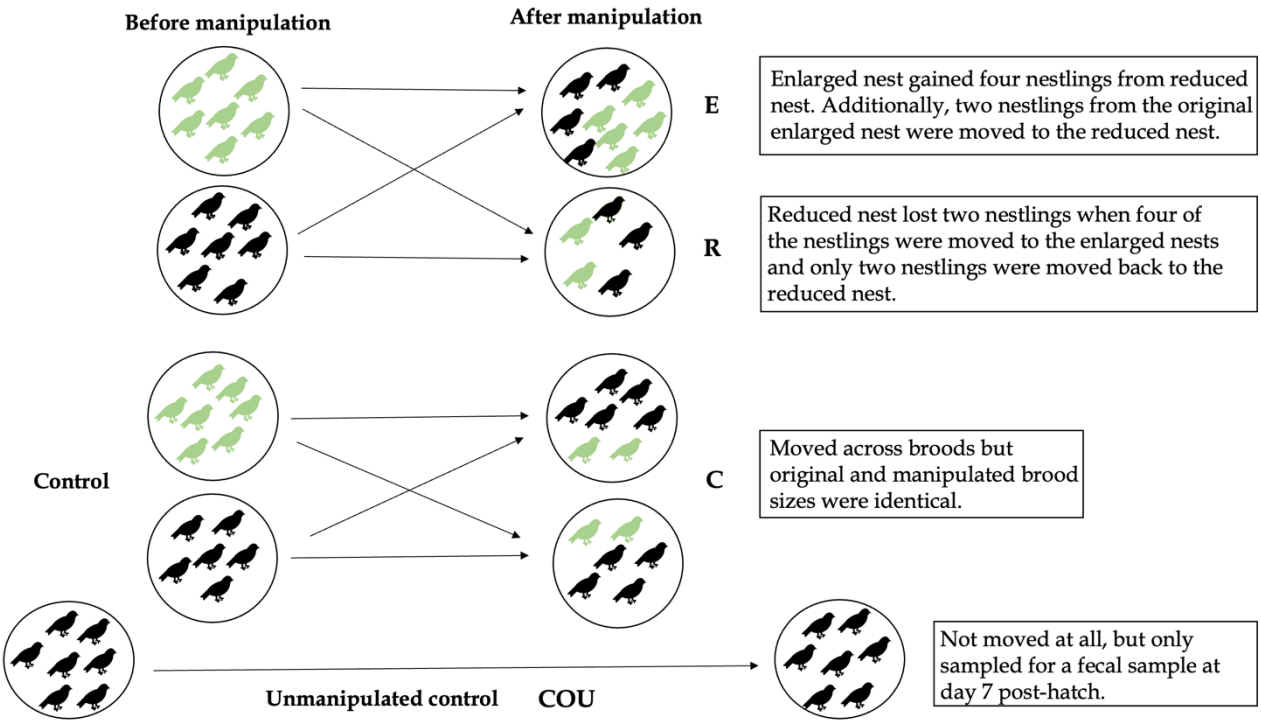

Figure 1. Brood size manipulation experiment schematic diagram. 2-day-old nestlings were moved between nestboxes to enlarge or to reduce original brood size (an example with brood size of seven is given). Some nests were kept as control nests (nestlings were moved but brood size remained the same) and some were kept as unmanipulated control nests (nestlings were not moved at all to test whether early-life handling affects gut microbiome). The original brood size varied between nests. In the complete brood size manipulation experiment (Cossin-Sevrin et al., *unpublished data*), the number of nestlings per nest was as follows: E=236/25, R=154/25, C=150/20, COU=113/17. The number of nestlings per nest that were included in the nestling body mass and gut microbiome analyses used in this paper were as follows: C=23/15 nests, COU=22/13, E=23/15, R=24/16 (see text).

Fecal sample collection

To study the effects that brood size may have on the nestling gut microbiome and its links to individual nestling body mass, survival to fledging and apparent juvenile survival, we used a subset of the before-mentioned dataset (Cossin-Sevrin et al., *unpublished data*). In this subset, we use individuals from which fecal samples were collected on day 7 after hatching and analyzed for microbiome diversity and composition (C=23 nestlings/15 nests, COU=22/13, E=23/15, R=24/16) We aimed to collect four samples (two from original and two from foster nestlings) per nest. Fecal

samples from the nestlings were collected gently by stimulating the cloaca with the collection tube. Samples were collected straight into a sterile 1.5 ml Eppendorf tube to avoid possible contamination of the sample. At time of sampling, each nestling was weighed (0.1g), their wing-length was measured with a metal ruler (1mm), and the nestlings were ringed for individual identification. The samples were stored in cool bags onsite and afterwards moved to a -80 °C freezer for storage until DNA extraction.

Apparent juvenile survival

On day 14 post-hatch, the sampled nestlings were weighed, and wing-length was measured to detect if the manipulation had any effects on nestling growth. Nests were subsequently monitored for fledging success, and we used fledging success as a proxy to measure short-term survival. Additionally, we monitored the Ruissalo population for apparent juvenile survival (i.e., mid-term survival) after the breeding season (i.e., approximately 3 months after fledging) to assess the association between gut microbiome and post-fledging survival. We captured juvenile great tits by mist netting during the autumn-winter 2020 at six different feeding stations that had a continuous supply of sunflower seeds and suet blocks. Feeding stations were located within the previously mentioned nest box population areas. For each site mist netting with playback was conducted on three separate days during October-November 2020 for three hours at a time, leading to a total of 69 hours of mist netting. A total of 88 individuals from the brood size manipulation experiment were caught, and the caught juvenile great tits were weighed, and wing length was measured. Our catching method provides an estimate of post-fledging survival yet, it could be slightly biased based on dispersal. In a previous study in our population, none of the birds ringed as

nestlings were recaptured outside the study area (Ruuskanen S., *unpublished data*), suggesting that dispersal is likely limited [76].

DNA extraction and sequencing

We chose two samples per nest for DNA extraction, yet in such a way that both fledged and not-fledged nestlings would be included in the dataset. The number of nestlings/experimental nests that were included in the gut microbiome analyses were as follows: C=23 nestlings/15 nests, COU=22/13, E=23/15, R=24/16. DNA was extracted from nestling fecal samples using the Qiagen QIAamp PowerFecal Pro DNA Kit (Qiagen; Germany) following the manufacturer's protocols. Additionally, we included negative (RNAse and DNAse free ddH_2O) controls to control for contamination during DNA extraction and additional controls to confirm successful amplification during PCR. A short fragment of hypervariable V4 region in the 16S rRNA gene was amplified using the purified DNA samples as template with the following primers: 515F_Parada (5′ - GTGYCAGCMGCCGCGGTAA -3′) and 806R_Apprill (5′ - GGACTACNVGGGTWTCTAAT - 3′) [77-78]. PCRs were performed in a total volume of 12 µL using MyTaq RedMix DNA polymerase (Meridian Bioscience; Cincinnati, OH, USA). The PCR cycling conditions were as follows: first, an initial denaturation at 95 °C for 3 minutes followed by 30 cycles of 95 °C for 45 sec., 55 °C for 60 sec., and 72 °C for 90 sec., and finished with a 10-minute extension at 72 °C. After the first round of PCR, a second round was conducted to apply barcodes for sample identification [79]. For this, PCR cycling conditions were as follows: first, an initial denaturation at 95 °C for 4 minutes followed by 18 cycles of 98 °C for 20 sec., 60 °C for 15 sec., and 72 °C for 30 sec., and finished with a 3-minute

extension at 72 °C. We performed replicate PCR reactions to control for errors during the amplification. Further on, the PCR products were measured for DNA concentration with Quant-IT PicoGreen dsDNA Assay Kit (ThermoFischer Scientific; Waltham, MA, USA) and for quality with TapeStation 4200 (Agilent; Santa Clara, CA, USA). The samples from each of the PCR replicates were pooled equimolarly creating two separate pools and purified using NucleoMag NGS Clean-up and Size Select beads (Macherey-Nagel; Düren, Germany). Finally, pooled samples were sequenced (2 x 300 bp) on the Illumina MiSeq platform (San Diego, CA, USA) at the Finnish Functional Genomic Center at the University of Turku (Turku, Finland).

Sequence processing and statistical analysis

Sequence processing All statistical analyses were performed with R (v. 4.11.0; R Development Core Team 2021) unless otherwise stated. The demultiplexed Illumina sequence data was first processed with Cutadapt version 2.7 [80] to remove locus-specific primers from both R1 and R2 reads. Then, the DADA2 pipeline (v. 1.24.0; [81]) was used to filter the reads based on quality, merge the paired-end (R1 and R2) reads, to define Amplicon Sequence Variants (ASV), and to construct a 'seqtab' (a matrix also known as otutable or readtable: ASVs in columns, samples in rows, number of reads in each cell) using default parameter settings. ASVs (9748 in total) were assigned to taxa against the SILVA v132 reference database [82]. In total, our seqtab consisted of 6,929,537 high-quality reads (average: 25,570; range: 0-108,112). Singleton reads were removed from the dataset by the DADA2 pipeline. To control for contamination, negative DNA extraction and PCR controls were used to identify contaminants (60 ASVs) using the decontam package (v. 1.12; [83]) and all were removed from the

dataset. Sequencing runs (replicate PCR's) were merged using the phyloseq package (v. 1.32.0) and non-bacterial sequences (mainly *Chlorophyta*) were removed from the data as they were not of interest in this study resulting in a total of 4,045,542 high-quality reads (average: 29,530; range: 0-189,000). Data was further analyzed with the phyloseq package (v. 1.32.0; [84]), and the microbiome package (v. 1.18.0; [85]) and visualized with the ggplot2 package (v. 3.3.6; [86]).

Our 92 samples contained a total of 3,161,696 reads (average: 34,366.26; range 108 – 189,300 reads), which belonged to 6,505 ASVs. The dataset was then rarefied for alpha diversity analyses at a depth of 5000, as this was where the rarefaction curves plateaued (SI 2). The rarefied dataset contained 4,791 ASVs in 88 samples. For beta diversity, the unrarefied dataset was used after confirming that the beta diversity statistics were quantitatively similar for the rarefied and unrarefied datasets. Bacterial relative abundances were summarized at the phylum and genus level and plotted based on relative abundance for all phyla and genera. A Newick format phylogenetic tree with the UPGMA algorithm to cluster treatment groups together was used to visualize sample relatedness and was constructed using the DECIPHER (v. 2.24.0; [87]), phangorn (v. 2.8.1; [88]), and visualized with ape (v. 5.6-2; [89]), and ggtree (v. 3.4.0; [90]) packages (SI 3).

Nestling body mass First, to analyze whether brood size affected nestling body mass in the control (C), enlarged (E), and reduced (R) treatment groups, we used the *lmer* function for linear mixed-effects models (LMMs) with the lme4 package (v. 1.1-29; [91]). We used body masson day 7 or 14 as the response variable and brood size

manipulation treatment, hatching date, body mass on day 2 post-hatch and original brood size as the covariates. Hatching date is used as a covariate because it is known to affect nestling body mass during the breeding season [92] and there were significant differences in hatching date between treatment groups (see above). We also included the interaction between original brood size and brood size manipulation treatment as the effect of manipulation may depend on the original brood size (e.g., stronger effect of enlargement in already large broods). Nest of origin and nest of rearing were used as random intercepts to control for the non-independence of nestlings sharing the same original or foster nests. Here, we did not include the unmanipulated control group (COU) in the analysis because we wanted to measure the effects of treatment (reduced, enlarged, or only moved but no change in brood size) on nestling body mass.

Second, to analyze whether final brood size affected nestling body mass, we used final brood size as a continuous variable to explain body mass on day 7 and day 14 post-hatch. Hatching date and body mass on day 2 post-hatch were used as covariates and nest of origin and nest of rearing as random intercepts to control for the non-independency of samples. We included the interaction between final brood size and hatching date because the effect of brood size may depend on the hatching date (e.g., hatching date reflects environmental conditions and large broods may perform poorly late in the season due to poorer food availability). Unmanipulated control (COU) group was excluded from this model to see which of the two random effects, nest of origin or nest of rearing, explained a larger portion of variation in the treatment groups. In the COU group, nest of origin and nest or rearing were the same, which

meant we could not include both random effects in models where all treatment groups were present due to the model failing to converge. Nest of rearing explained more of the variation in the first model and therefore, we used it in the full model with all treatment groups: C, COU, E and R. In this model, nestling body mass on day 7 and on day 14 post-hatch was used as a response variable and final brood size as the explanatory variable. Hatching date and body mass on day 2 post-hatch were set as covariates. Nest of rearing was used as a random effect. The significance of factors included in the models were tested using the F-test ratios in analysis of variance (ANOVA).

Alpha diversity For alpha diversity analyses, we used LMMs with the lme4 package (v. 1.1-29; [91]) to measure if brood size manipulation and final brood size (as a continuous variable) were associated with gut microbiome diversity. Shannon Diversity Index (number of bacterial ASVs and their abundance evenness within a sample) and Chao1 Richness (estimation of the number of different bacterial ASVs in a sample) were tested to check if alpha diversity results were consistent across different metrics. Each diversity index was used as the response variable at a time and either brood size manipulation treatment or final brood size as an explanatory variable. Original brood size, weight on day 7 post-hatch and hatching date were set as covariates in the model. We included interaction in both models (between brood size manipulation and original brood size, and final brood size and weight on day 7 post-hatch) as brood size manipulation may be affected by original brood size (e.g., stronger effect of enlargement in already large broods) and final brood size may affect the size of the nestlings (e.g., smaller final brood size may cause the nestlings to be

larger in body mass). We also tested whether alpha diversity predicted weight on day 7 post-hatch, as weight and gut microbiome diversity have been connected in previous studies. The analysis included the same covariates and random effects as above. In these sets of models, we first excluded the unmanipulated control (COU) group to see which of the two random effects, nest of origin or nest of rearing, explained a larger portion of variation in the treatment groups (See SI 6). In the COU group, nest of origin and nest or rearing were the same, which meant we could not include both random effects in models where all treatment groups were present due to the model failing to converge. Nest of rearing explained more of the variation in this model as well and therefore, we used it in the full model with all treatment groups: C, COU, E and R. The significance of factors included in the models were tested using the F-test ratios in analysis of variance (ANOVA).

Short-term survival To explore whether alpha diversity associated with survival to fledging (i.e., short-term survival) and with apparent juvenile survival in Autumn 2020 (i.e., mid-term survival), we used the *glm* function for generalized linear models (GLMs) with binomial model (v. 1.1-29; lme4 package, [91]), and then tested the significance of factors with type 2 ANOVA from the car package (v. 3.0-13; [93]). Type 2 ANOVA was used because the model did not contain interaction between covariates and there was no order between covariates (could not be ranked). Survival to fledging and recapture in Autumn 2020 were used as the binomial response variable (yes-no) in each model, and weight on day 7 post-hatch (same time as sampling the fecal gut microbiome), hatching date and final brood size were included as covariates in the model. We did not include brood size manipulation treatment in the survival models

as not enough birds from each treatment group were recorded for fledging and juvenile survival. Moreover, we excluded random effects from this model as the model failed to converge. Regarding fledging success 65 nestlings fledged successfully, while 8 nestlings were found dead in nest boxes. 15 nestlings had no fledging record, so these were excluded from the survival to fledging analysis. In apparent juvenile survival, 19 birds out of 92 (with data on microbiome diversity) were recaptured as juveniles. For all analyses, the R package car (v. 3.0-13; [93]) was used to test Variance Inflation Factors (VIFs) and the package DHARMa (v. 0.4.5; [94]) to test model diagnostics for LMMs and GLMs.

Beta diversity For visualizing beta diversity (gut microbiome composition), non-metric multidimensional scaling (NMDS) was used with three distance matrices: Bray-Curtis [95], weighted UniFrac, and unweighted UniFrac [96]. Permutational multivariate analysis of variance (PERMANOVA) using the Euclidean distance matrix and 9999 permutations was used with the *adonis2* function within the R package vegan (v. 2.6-2; [97]) to determine which variables (brood size manipulation treatment, hatch day, and weight on day 7 post-hatch) contributed to the variation in gut microbiome composition. Nest of rearing was set as a blocking factor in the perMANOVA to control for repeated sampling of foster siblings. The betadisper function was used to measure the homogeneity of group dispersion values.

RESULTS

The effects of brood size manipulation on nestling body mass

Brood size manipulation did not significantly affect nestling body mass on day 7 post-hatch (ANOVA: $F_{2, 25.832}$=0.441, p=0.648, Table 1). Moreover, there was no significant interaction between brood size manipulation and original brood size (ANOVA: $F_{2, 24.610}$=0.678, p=0.517, Table 1). On day 14 post-hatch, brood size manipulation did not significantly affect nestling body mass (ANOVA: $F_{2, 24.335}$=0.831, p=0.448, Table 1). However, body mass increased with increasing hatching date (ANOVA: $F_{1 24.070}$=13.367, p=0.001, Table 1). See supplements for results on other covariates (SI 5).

Table 1. A linear mixed effects model investigating the effects of brood size manipulation on nestling body mass on day 7 and day 14 post-hatch. This basic model includes control (C), enlarged (E), and reduced (R) groups. Interactions between manipulated brood size and original brood size were removed from final models as there was no significant interaction and are shown in the table below. Nest of origin and nest of rearing were included as random effects to control for the non-independency of samples.

Weight D7						
	estimate	**s.e.**	**df**	***t***	**p**	
(Intercept)	5.93456	2.582	25.522	2.298	0.030	*
Enlarged brood size	-0.387	0.595	26.154	-0.649	0.522	
Reduced brood size	0.146	0.518	24.937	0.282	0.780	
Original brood size	-0.075	0.159	26.391	-0.473	0.640	
Hatching date	0.057	0.039	24.444	1.436	0.164	
Weight D2	0.662	0.146	31.682	4.522	< 0.000	***
(Interactions)						
(Enlarged * original brood size	-0.105	0.421	25.254	-0.251	0.804)	
(Reduced * original brood size	0.322	0.370	23.902	0.871	0.393)	
Random effects						
	variance	**s.d.**				
Nest of origin	0.668	0.817				
Nest of rearing	0.396	0.629				
Residual	0.560	0.749				

Weight D14						
	estimate	**s.e.**	**df**	***t***	**p**	
(Intercept)	5.241	3.290	24.404	1.593	0.124	
Enlarged brood size	-0.527	0.778	24.655	-0.678	0.504	
Reduced brood size	0.359	0.684	23.788	0.526	0.604	
Original brood size	0.093	0.194	24.460	0.479	0.636	
Hatching date	0.184	0.050	24.070	3.656	0.001	**
Weight D2	0.089	0.167	30.925	0.532	0.599	
(Interactions)						
(Enlarged * original brood size	0.054	0.521	22.643	0.104	0.918)	
(Reduced * original brood size	0.316	0.464	22.215	0.681	0.503)	
Random effects						
	variance	**s.d.**				
Nest of origin	1.525	1.235				
Nest of rearing	0.510	0.714				
Residual	0.291	0.539				

Next, we did not find any significant associations between final brood size and nestling body mass (ANOVA for weight on day 7: $F_{1, 35.121}$=2.188, p=0.148; ANOVA for weight on day 14: $F_{1, 29.491}$=2.156, p=0.153, Table 2). See supplements for results on covariates (SI 5).

Table 2. A linear mixed effects model investigating the effects of final brood size on nestling body mass on day 7 and day 14 post-hatch. The analysis includes all treatment groups i.e., the full model: control (C), unmanipulated control (COU), enlarged (E), and reduced (R). Nest of origin was included as a random effect to control for the non-independency of samples.

Weight D7						
	estimate	**s.e.**	**df**	***t***	**p**	
(Intercept)	6.469	2.470	33.299	2.619	0.013	*
Final broodsize	-0.154	0.104	35.121	-1.479	0.148	
Hatching date	0.052	0.038	32.704	1.361	0.183	
Weight D2	0.717	0.147	39.933	4.868	<0.000	***
(Interactions)						
(Final brood size * Hatching date	-0.027	0.024	35.323	-1.104	0.277)	
Random effects						
	variance	**s.d.**				
Nest of origin	1.312	1.146				
Residual	0.561	0.749				

Weight D14						
	estimate	**s.e.**	**df**	***t***	**p**	
(Intercept)	7.157	3.000	28.886	2.386	0.024	*
Final broodsize	-0.166	0.113	29.491	-1.468	0.153	
Hatching date	0.181	0.047	28.208	3.874	0.001	***
Weight D2	0.120	0.151	36.957	0.797	0.431	
(Interactions)						
(Final brood size * Hatching date	-0.025	0.027	32.208	-0.945	0.352)	
Random effects						
	variance	**s.d.**				
Nest of origin	1.894	1.376				
Residual	0.284	0.533				

Alpha diversity

As 7-day-old nestlings, the majority of bacterial taxa belonged to the phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria* (Fig. 2). On genus level, the most abundant genera were *Candidatus_Arhtromitus, Ureaplasma, Mycobacterium, Afipia, Erwinia,* and *Turicibacter* (Fig. 3).

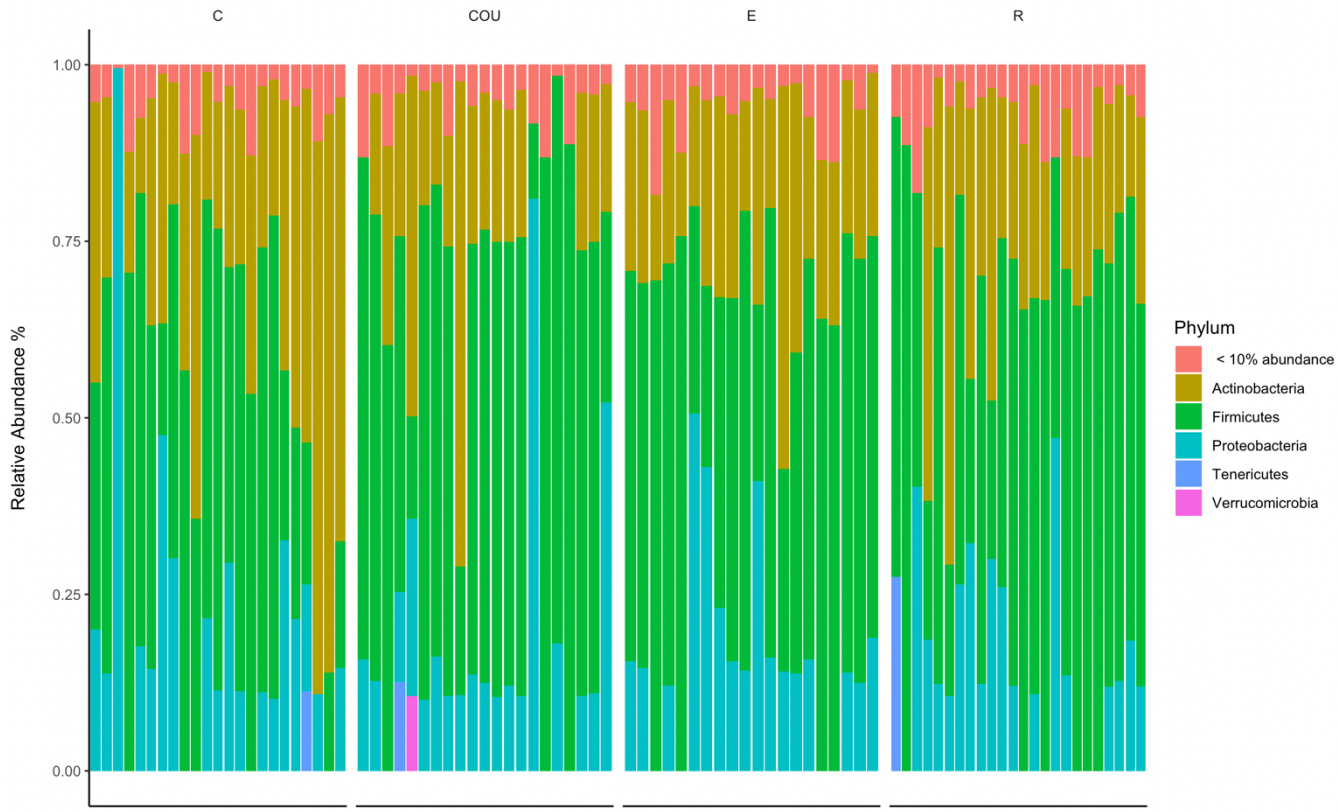

Figure 2. Bacterial relative abundances on Phylum level across the four treatment groups. Each bar represents an individual sample. Treatment groups are control (C), unmanipulated control (COU), enlarged (E), and reduced (R). N=88 samples divided into treatment groups as follows: C=23, COU=21, E=20, R=24. Phyla with less that 10 % in relative abundance is collapsed into the category "< 10 % abundance."

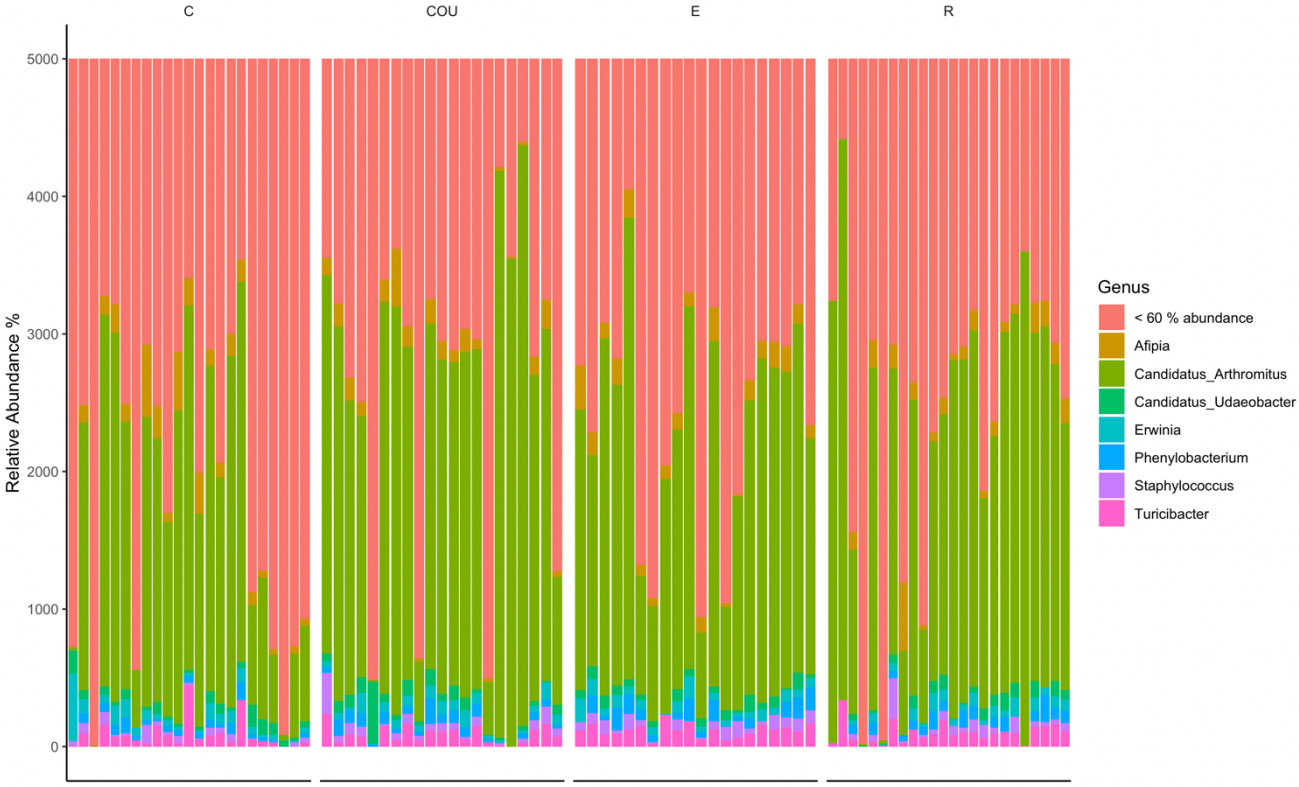

Figure 3. Bacterial relative abundances of top genera across the four treatment groups. Each bar represents an individual sample. Treatment groups are control (C), unmanipulated control (COU), enlarged (E), and reduced (R). N=88 samples divided into treatment groups as follows: C=23, COU=21, E=20, R=24. Genera that made up <60 % in relative abundance are collapsed into the category "< 60 % abundance" as this group contained 29 different genera and would have made the plot difficult to read.

Nest of rearing explained a larger proportion of variance in alpha diversity (σ^2=0.170, s.d.=0.413) than nest of origin (σ^2=0.067, s.d.=0.258). Brood size manipulation did not significantly influence alpha diversity (Shannon Diversity Index) (ANOVA: $F_{3, 47.485}$=1.020, p=0.392, Table 3, Fig. 4). Moreover, original brood size (ANOVA: $F_{1, 50.683}$=0.433, p=0.514, Table 3), weight on day 7 post-hatch (ANOVA: $F_{1, 80.546}$=0.003, p=0.954, Table 3), and hatching date (ANOVA: $F_{1, 50.306}$=1.087, p=0.302, Table 3) did not significantly associate with alpha diversity. There was no significant interaction between brood size manipulation and original brood size (ANOVA: $F_{3, 48.364}$=0.139,

p=0.936, Table 3). Results for Chao1 Richness, were quantitatively similar: brood size manipulation did not affect alpha diversity (ANOVA: $F_{3, 45.971}$=0.363 p=0.780, Table 3, Fig. 4).

Table 3. A linear mixed effects model investigating the associations between alpha diversity (Shannon Diversity Index and Chao1 Richness) and brood size manipulation. The model includes all four treatment groups i.e., the full model: control (C), unmanipulated control (COU), enlarged (E), and reduced (R). Interactions between brood size manipulation and original brood size were removed as there was no significant interaction and are shown in the table below. Nest of rearing was included as a random effect to control for the non-independency of samples.

Shannon Diversity Index					
	estimate	s.e.	df	*t*	p
(Intercept)	1.583	1.201	51.940	1.318	0.193
Control brood size	0.345	0.285	42.107	1.213	0.232
Enlarged brood size	0.493	0.285	48.406	1.727	0.091
Reduced brood size	0.292	0.274	46.882	1.065	0.292
Original brood size	-0.037	0.056	50.683	-0.658	0.514
Weight D7	-0.003	0.051	80.546	-0.058	0.954
Hatching date	0.018	0.018	50.306	1.043	0.302
(Interactions)					
(Control treatment * Original brood size	-0.070	0.142	44.646	-0.490	0.626)
(Enlarged treatment * Original brood size	-0.075	0.175	53.875	-0.430	0.669)
(Reduced treatment * Original brood size	0.005	0.151	44.285	0.032	0.974)
Random effects					
	Variance	s.d.			
Nest of rearing	0.229	0.478			
Residual	0.388	0.623			

Chao1 Richness					
	estimate	s.e.	df	*t*	p
(Intercept)	94.010	60.272	49.235	1.560	0.125
Control brood size	6.206	13.982	38.535	0.444	0.660
Enlarged brood size	-5.804	14.234	46.665	-0.408	0.685
Reduced brood size	-5.071	13.621	44.551	-0.372	0.711
Original brood size	-1.471	2.795	50.890	-0.526	0.601
Weight D7	-2.124	2.748	80.494	-0.773	0.442
Hatching date	0.891	0.877	49.233	1.015	0.315
(Interactions)					
(Control treatment * Original brood size	5.703	7.088	45.184	0.805	0.425)
(Enlarged treatment * Original brood size	6.666	8.920	57.402	0.747	0.458)
(Reduced treatment * Original brood size	4.981	7.563	44.041	0.659	0.514)
Random effects					
	Variance	s.d.			
Nest of rearing	269.9	16.43			
Residual	1424.6	37.74			

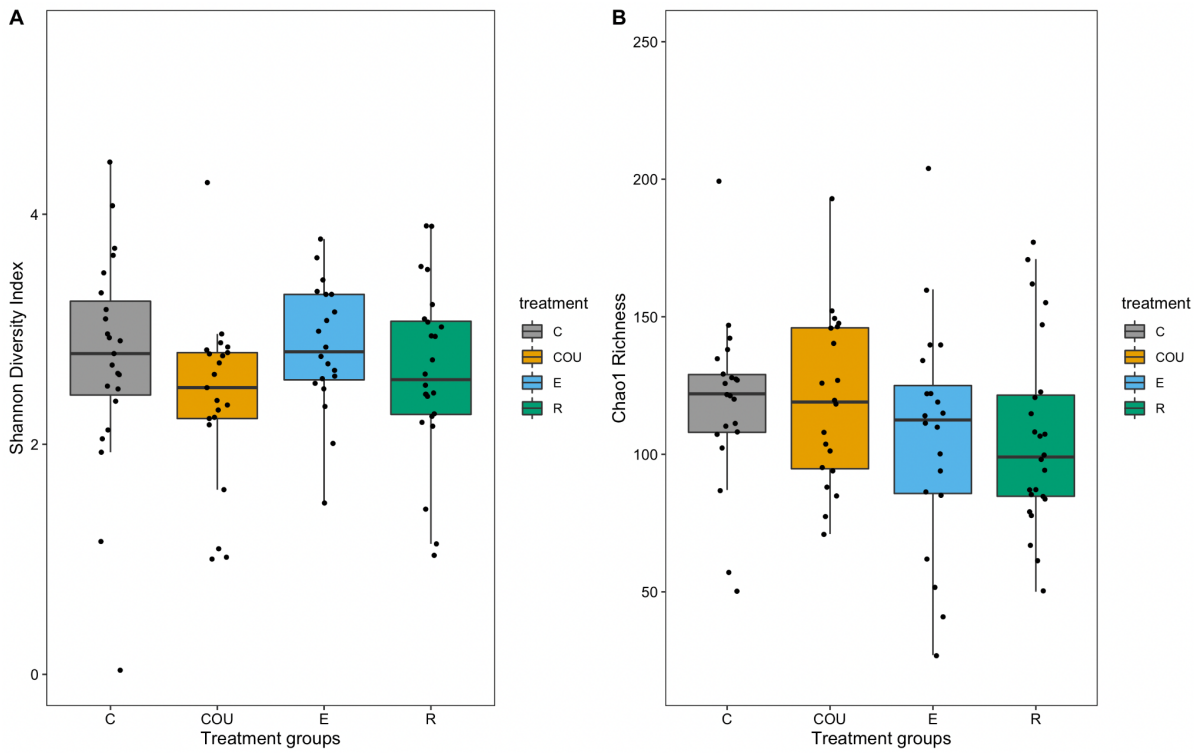

Figure 4. The gut microbiome alpha diversity of 7-day-old great tit nestlings across the four treatment groups visualized with two diversity metrics: A) Shannon Diversity Index and B) Chao1 Richness. The black dots represent each observation within a treatment group. The whiskers represent 95 % confidence intervals. Treatment groups are control (C), unmanipulated control (COU), enlarged (E), and reduced (R). N=88 samples divided into treatment groups as follows: C=23, COU=21, E=20, R=24.

Next, we tested whether the final brood size as a continuous variable was associated with alpha diversity (Shannon Diversity Index), but found no significant association (ANOVA: $F_{1, 60.451}$<0.000, p=0.999, Table 4) in this analysis either. Weight on day 7 post-hatch (ANOVA: $F_{1, 82.769}$=0.016, p=0.901, Table 4) and hatching date (ANOVA: $F_{1, 59.639}$=0.140, p=0.709, Table 4) did not correlate with alpha diversity in this model either. There was no significant interaction between final brood size and weight on day 7 post-hatch (ANOVA: $F_{1, 82.291}$=0.002, p=0.966, Table 4). Results for Chao1 Richness were quantitatively similar (ANOVA: $F_{1, 61.463}$=0.164, p=0.687, Table 4): final brood size did not affect alpha diversity, and neither did weight on day 7 post-hatch

(ANOVA: $F_{1\ 83.573}$=0.672, p=0.415, Table 4) nor hatching date (ANOVA: $F_{1\ 57.039}$=1.093, p=0.300, Table 4).

Table 4. A linear mixed effects model investigating the association between alpha diversity (Shannon Diversity Index and Chao1 Richness) and final brood size. The model includes all four treatment groups i.e., the full model: control (C), unmanipulated control (COU), enlarged (E), and reduced (R). Interactions between alpha diversity and final brood size were removed as there was no significant interaction and are shown in the table below. Nest of rearing was included as a random effect to control for the non-independency of samples.

Shannon Diversity Index						
	estimate	**s.e.**	**df**	***t***	**p**	
(Intercept)	2.354	1.053	65.130	2.237	0.029	*
Final brood size	<0.000	0.042	60.450	-0.001	0.999	
Weight D7	-0.001	0.005	82.770	-0.125	0.901	
Hatching date	0.001	0.002	59.640	0.374	0.709	
(Interactions)						
(Final brood size * weight D7	-0.001	0.022	82.291	-0.042	0.966)	
Random effects						
	Variance	**s.d.**				
Nest of rearing	0.248	0.498				
Residual	0.377	0.614				

Chao1 Richness					
	estimate	**s.e.**	**df**	***t***	**p**
(Intercept)	92.525	52.425	63.899	1.765	0.082
Final brood size	-0.845	2.089	61.463	-0.405	0.687
Weight D7	-2.226	2.715	83.573	-0.820	0.415
Hatching date	0.833	0.797	57.039	1.046	0.300
(Interactions)					
(Final brood size * weight D7	-0.560	1.142	81.132	-0.491	0.625)
Random effects					
	Variance	**s.d.**			
Nest of rearing	238.7	15.45			
Residual	1419.0	37.67			

Alpha diversity and short/mid-term survival

Next, we explored whether alpha diversity (Shannon Diversity Index and Chao1 Richness) contributed to predicting short/mid-term survival (survival to fledging and apparent juvenile survival). Survival to fledging was not predicted by alpha diversity (Chisq.=0.010, df=1, p=0.919, Table 5), final brood size (Chisq.=0.140, df=1, p=0.709, Table 5), weight on day 7 post-hatch (Chisq.=0.381, df=1, p=0.537, Table 5) or hatching date (Chisq.=0.452, df=1, p=0.501, Table 5).

Apparent juvenile survival was not significantly associated with alpha diversity (Chisq.=1.920, df=1, p=0.166, Table 5, Fig. 4). Moreover, there was no significant interaction between alpha diversity and final brood size (Chisq.= 1.160, df=1, p=0.282, Table 5). However, apparent juvenile survival was negatively associated with hatching date (Chisq.=4.923, df=1, p=0.027, Table 5). Additional analyses to check for the consistency of results were tested the following way: survival to fledging with nestlings from the unmanipulated control group (COU) removed and apparent juvenile survival without the nestlings with no recorded survival for fledging (see methods and SI 4). These results were quantitatively similar as in the whole dataset for both Shannon Diversity Index (survival to fledging: Chisq.= 2.274, df=1, p= 0.132; apparent juvenile survival: Chisq.=1.508, df=1, p=0.219, SI 4) and Chao1 Richness (survival to fledging: Chisq.=0.659, df=1, p=0.417; apparent juvenile survival: Chisq.=2.623, df=1, p=0.105, SI 4).

Table 5. A generalized linear model exploration into alpha diversity's (Shannon Diversity Index and Chao1 Richness) association with short-term (survival to fledging) and mid-term (apparent juvenile) survival. Random effects were excluded as the model failed to converge.

Survival to fledging (Shannon Diversity Index)	estimate	s.e.	z value	p	
(Intercept)	-1.434	4.165	-0.344	0.731	
Shannon	-0.046	0.457	-0.101	0.920	
Weight D7	0.141	0.229	0.616	0.538	
Hatching date	0.043	0.063	0.679	0.497	
Final brood size	-0.067	0.181	-0.370	0.712	

Survival to fledging (Chao1 Richness)	estimate	s.e.	z value	p	
(Intercept)	-1.244	4.215	-0.295	0.768	
Chao1	-0.003	0.009	-0.345	0.730	
Weight D7	0.132	0.229	0.576	0.564	
Hatching date	0.046	0.065	0.716	0.474	
Final brood size	-0.071	0.183	-0.389	0.697	

Recapture as juvenile (Shannon Diversity Index)	estimate	s.e.	z value	p	
(Intercept)	2.626	3.384	0.776	0.438	
Shannon	-0.503	0.369	-1.364	0.173	
Weight D7	0.273	0.185	1.475	0.140	
Hatching date	-0.103	0.049	-2.100	0.036	*
Final brood size	0.033	0.135	0.242	0.809	

Recapture as juvenile (Chao1 Richness)	estimate	s.e.	z value	p	
(Intercept)	2.572	3.396	0.757	0.449	
Chao1	-0.011	0.007	-1.516	0.129	
Weight D7	0.256	0.192	1.330	0.184	
Hatching date	-0.099	0.050	-1.979	0.048	*
Final brood size	0.021	0.133	0.159	0.874	

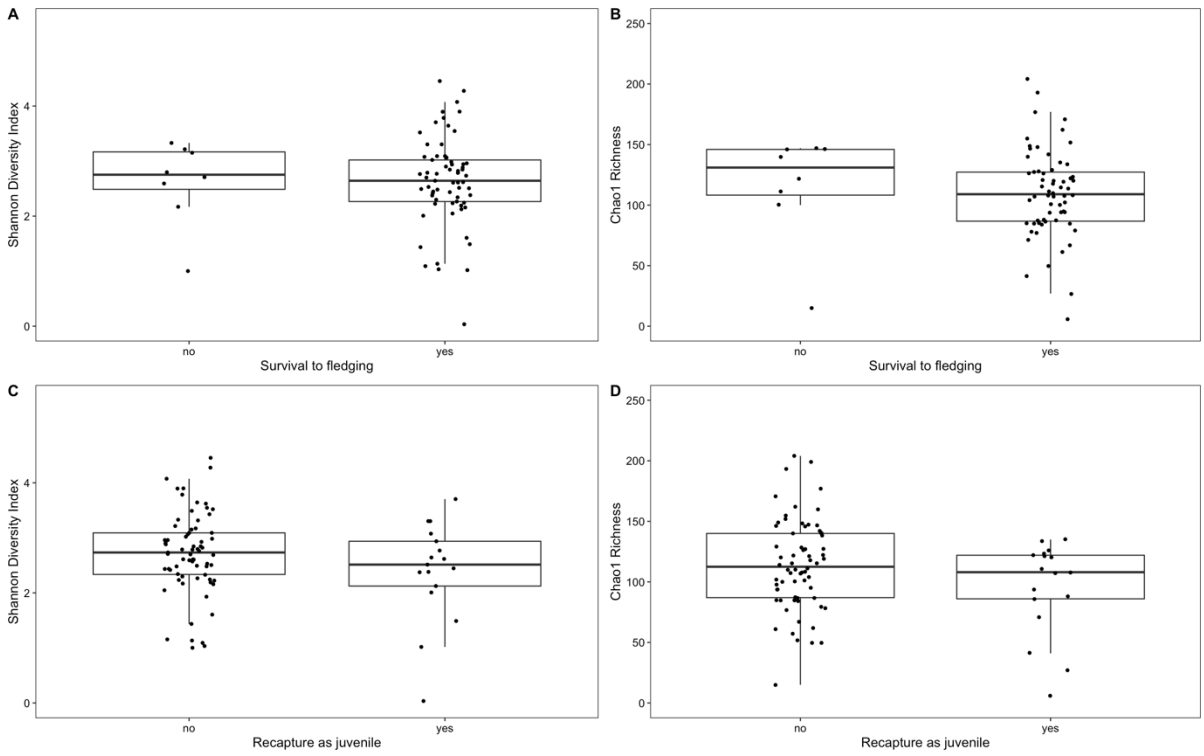

Figure 4. The gut microbiome alpha diversity (Shannon Diversity Index and Chao1 Richness) and short-term survival. In survival to fledging (A: Shannon Diversity Index; B: Chao1 Richness) 65 nestlings fledged successfully and 8 nestlings were dead. 15 nestlings had no fledging record, so these were excluded from the analysis. In recapture as juvenile (C: Shannon Diversity Index; D: Chao1 Richness) 19 out of 92 (with data on microbiome diversity) were captured. The black dots represent each observation within a treatment group. The whiskers represent 95 % confidence intervals.

Beta diversity

Non-metric multidimensional scaling (NMDS) using weighted and unweighted UniFrac and Bray-Curtis dissimilarity did not show clear clustering of samples based on brood size manipulation treatment (Fig. 5). The betadisper test for homogeneity of multivariate dispersions supported the visual assessment of the NMDS ($\text{Betadispersion}_{9999\ \text{permutations}}$: $F_{3,\ 0.069}$= 0.650, $p<0.001$). Pairwise PERMANOVA further indicated that the treatment (PERMANOVA: R^2=0.061, F=1.951, p=0.278), weight on day 7 post-hatch (PERMANOVA: R^2=0.015, F=1.387, p=0.091) or hatching date (PERMANOVA: R^2=0.0232, F=2.214, p=0.993) did not significantly contribute to the variation in gut microbiome composition between the treatment groups.

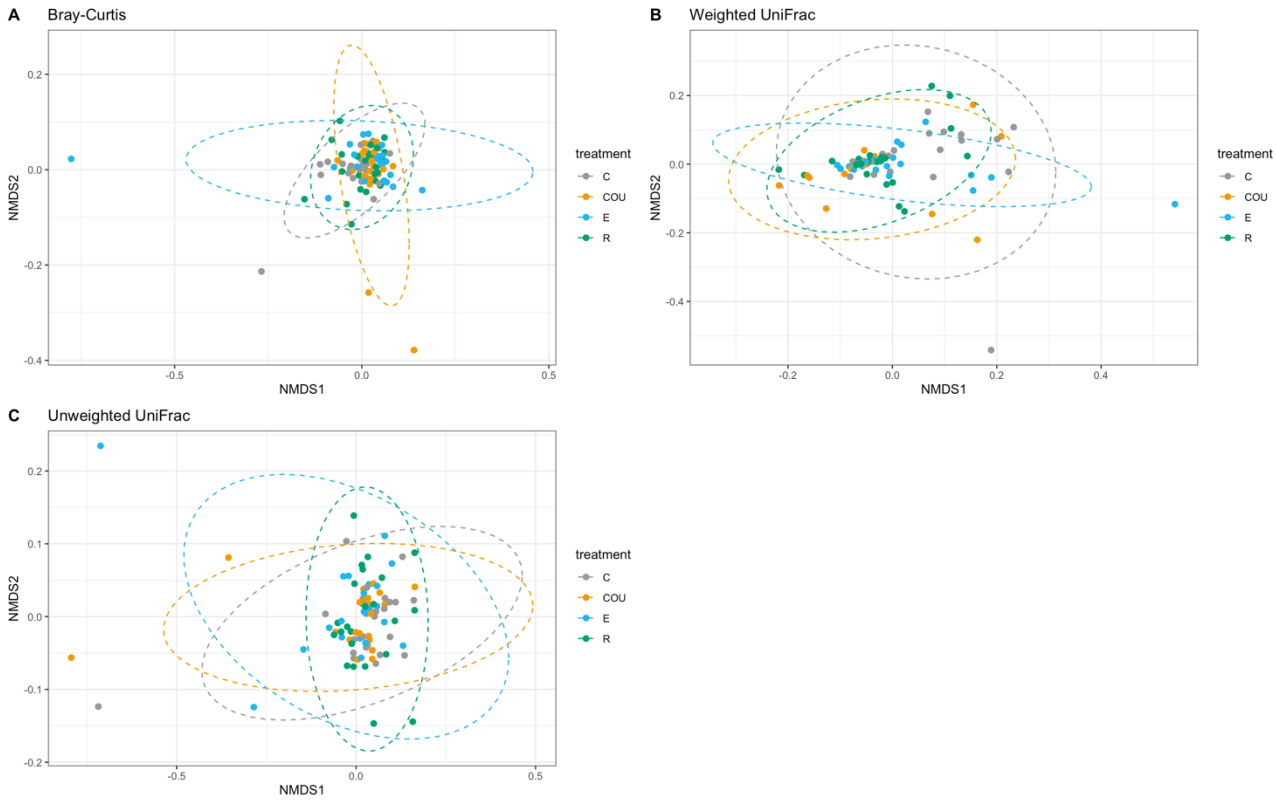

Figure 5. Ordination of the gut microbial communities. A) Weighted UniFrac, B) Unweighted UniFrac, and C) Bray-Curtis dissimilarity are displayed on NMDS ordinations. The color of the dots indicates which treatment, and the dashed ellipses represent 95 % confidence intervals.

DISCUSSION

In this study, we investigated the associations between great tit nestling gut microbiome, brood size, and nestling body mass by experimentally manipulating wild great tit broods to either reduce or enlarge the original brood size. The results show that even though there was individual variation in the nestling gut microbiome at the time of fecal sampling (Fig. 2), brood size did not significantly contribute to gut microbiome diversity, and gut microbiome diversity did not significantly explain short-term (survival to nestling) or mid-term (apparent juvenile) survival. Body mass was also not significantly affected by brood size manipulation. The unmanipulated control group (COU) that functioned as a control for any moving and handling effects, did not differ from the other groups, which suggests that human contact or handling nestlings 2 days post-hatch did not influence nestling gut microbiome or body mass. The partial cross-fostering enabled us to disentangle the relative contributions of rearing environment (i.e., parents, nest and nestmates) from genetic, prenatal maternal, and early post-natal effects. Nest of rearing did contribute more to the variation in nestling gut microbiome diversity than the nest of origin, which follows previous studies. However, nest of origin was a stronger contributor than nest of rearing on nestling body mass on day 7 and day 14 post-hatch.

Brood size manipulation and nestling body mass

First, we explored whether brood size was associated with nestling body mass, as such changes may explain the underlying patterns in gut microbiome [52]. Against our hypothesis, we found no significant association between nestling body mass and

brood size: neither the reduced nor the enlarged broods resulted in significant body mass differences in the nestlings on day 7 and day 14 post-hatch. While the result is supported by some studies in which associations between nestling body mass and brood size have been tested [e.g., 61, 98], the majority of the literature shows that brood size negatively correlates with nestling body mass: in larger broods nestlings are generally of lower mass [e.g., 52-53, 57, 67, 99-104]. We did observe that nest of origin contributed more to the variation in nestling body mass on both day 7 and day 14 post-hatch than the nest of rearing, which could be explained by optimal environmental conditions e.g., an abundance of food and good weather conditions, leading to little variation among rearing conditions [105].

There are a few possible explanations why brood size manipulation did not affect nestling body mass. Firstly, it could be that the enlarged brood size negatively influences some other physiological trait while body mass was retained at the expense of these other traits e.g., immune system functioning [106-107]. Secondly, if environmental conditions were good, parents may have been able to provide enough food even for the enlarged nests and thus, variance in brood size may not result in differences in nestling body mass between reduced and enlarged nests, or the number of nestlings transferred between enlarged and reduced nests should have been larger to observe changes in body mass (even though the decision to transfer +2/-2 was based on extensive previous literature) [103]. Moreover, our tests showed that hatching date had a significant effect on nestling body mass: nestlings that hatched later in the season were of lower weight. This could be a result of changes in the food items that great tits use. As the season progresses, different insect taxa become more

prevalent than others and the abundance of e.g., caterpillars can vary resulting in changes in nutrient rich food [103, 108]. Thirdly, it could be that the change in brood size was influencing the parents' condition instead of the nestlings [109-110]. In enlarged broods, parents are required to forage more which can lead to higher energy expenditure and increased stress levels in parents [72-73, 109].

Brood size manipulation and gut microbiome

We found large inter-individual differences in gut microbiome diversity, yet this variation was not explained by brood size or nestling body mass. It is possible that brood size did not result in differences in food intake (i.e., parents were likely able to provide an equivalent amount of food), given that body mass was not significantly affected by the brood size manipulation, and therefore brood size manipulation did not affect gut microbiome diversity through differences in nutrient uptake. Alternatively, in this study, fecal sampling took place 5 days after the initial brood size manipulation (day 2 post-hatch). It could be that sampling on a later date or at multiple timepoints [61, 111] would have led to different results, as (1) the time interval may not have been long enough to detect effects of the brood size manipulation and (2) it has been shown in previous studies that the nestling gut microbiome undergoes profound shifts at the nestling stage: overall gut microbiome diversity decreases but relative abundance in some taxa increases [52]. Therefore, we suggest that fecal samples could be collected on multiple days post-hatch to understand the potential day to day changes in the nestling gut microbiome.

Our results suggest that the variance in gut microbiome is a result of other factors than those linked to brood size. Firstly, one of these factors could be diet (i.e., food quality) which has gained attention in gut microbiome studies during the past years [e.g., 25, 27, 112-115]. The overall diversity in gut microbiome could be explained by adaptive phenotypic plasticity because it is sensitive to changes in the environment e.g., changes in diet [117]. The food provided by the parents can vary between broods in different environments [118], and this variation in diet can lead to differences in gut microbiome diversity [e.g., 115-116]. For example, abundance in certain dietary items such as insects or larvae can result in lower gut microbiome diversity than other dietary items [113-116]. As great tits have been reported to adapt their diet along the breeding season due to changes in insect taxa frequency [103, 108] this could affect the nestlings' gut microbiome diversity. However, using wild bird populations in gut microbiome studies limits the ability to control the consumed dietary items as parents may use variable food resources. Metabarcoding may help as it enables the identification of food items from e.g., fecal samples [119].

Secondly, breeding habitat may lead to differences in gut microbiome diversity [120]: adult birds living in deciduous forests have shown to harbor different gut microbiome diversity than their counterparts living in open forested hay meadows. Here, we used a cross-fostering design to study if the rearing environment contributed to the variation in gut microbiome diversity: Our study showed that the nest of rearing explained more of the gut microbiome variation than the nest of origin, which follows some previous results [43-44, 52]. For example, a study with great and blue tit (*Cyanistes caerulaeus*) nestlings showed that the nest of rearing contributed more to the

gut microbiome than the nest of origin [43], and another study with the brown-headed cowbird (*Molothrus ater*) concluded that the sampling locality had a significant contribution to the gut microbiome [44]. Teyssier et al. [52] conducted cross-fostering at day 8 post-hatch in great tits and found that the nest of rearing influenced the gut microbiome more than the nest of origin. Additionally, parents can pass down their bill and feather microbiome through vertical transmission, which could influence nestling gut microbiome [e.g., 20].

Results from beta diversity analysis were like that of alpha diversity: brood size manipulation did not contribute to the variation in gut microbiome composition. Overall, variation in gut microbiome composition could be a result of different genetic and environmental contributors. Firstly, great tit nestling gut microbiome composition could be explained by underlying genetic effects that we did not measure in this study. Phylosymbiosis (i.e., the matching of gut microbiome composition to host genetic structure) could be explained by underlying genetics that may translate into physiological differences that affect the gut microbiome e.g., founder effects or genetic drift [121]. Davies et al. [14] found that MHC genes correlate with gut microbiome composition: the expression of specific alleles in the MHC genes was connected to the abundance of specific bacterial taxa such as *Lactobacillales* and *Bacteroidales* that influenced host health. In a study by Benskin et al. [41] captive zebra finches (*Taeniopygia guttata*) showed significant variation in gut microbiome composition between individuals even though their diet and housing conditions were standardized. One explanation for this was suspected to be connected to individual homeostatic mechanisms that could link to naturally occurring differences in

individual gut microbiome [41]. Secondly, gut microbiome composition could have been affected by the same environmental effects that may have linked to the variation in gut microbiome diversity: diet and feeding behaviour [e.g., 115-116].

Gut microbiome and short-term and mid-term survival

Our results showed that gut microbiome diversity and brood size were not significantly associated with short-term (survival to fledging) or mid-term (apparent juvenile) survival. If anything, gut microbiome diversity tended to be lower in the individuals that showed better survival, which contradicts our hypothesis that higher gut microbiome diversity would enable better survival, as found in Davidson et al. [61]. However, while a more diverse gut microbiome is considered a possible indicator of a healthy gut microbiome, the effects of the gut microbiome on the host health may often be more complex and related to specific taxa [9-10]. For example, Worsley et al. [13] did not find a correlation between body condition and gut microbiome diversity, yet they found that specific taxa in the gut microbiome linked with individual body condition and survival. Not only environment, but also genetic background of the individual may contribute to gut microbiome and survival: In a study by Davies et al. [14], *Ase-ua4* allele of the MHC genes was linked to lower gut microbiome diversity and it was suspected that the variation in the MHC genes could affect the sensitivity to pathogens that could lead to variation in gut microbiome diversity and eventually, host survival. To gain a better understanding of gut microbiome diversity and the contribution of different taxa to host survival, functional analyses of the gut microbiome could be included in gut microbiome studies. Different bacterial taxa can have similar functions in the gut microbiome [122] and therefore, the absence of some

taxa may be covered by other functionally similar taxa, resulting in a gut microbiome that is functionally more stable [123]. Similarity in functions may also contribute to host's local adaptation e.g., to the changes in the host's early-life environment [122]: changes in brood size or dietary items could result in variation in the gut microbiome diversity, yet there may be no effects on host body condition.

The lack of association between brood size, nestling size and survival is in contrast to previous studies, but it should be noted that our sample size in the survival analyses was small, and it is hard to determine if the result was affected by the sample size. Firstly, nestling survival is often found to correlate with brood size and more specifically, with fledging mass and in particular, the ability to forage for food [61, 124]. Intra-brood competition may explain survival to fledging, as competition between nestlings can limit food availability and thus, leading to lower nestling body condition [68, 125]. A study with blackbirds (*Turdus merula*) showed that nestling body mass explained juvenile survival [126], and similar results have been shown with great tits and collared flycatchers (*Ficedula albicollis*; [31]). Contrastingly, Ringsby et al. [127] observed that in house sparrows (*Passer domesticus*) juvenile survival was independent of nestling massand brood size. Moreover, natal body mass is often positively correlated with survival to fledging and juvenile survival as heavier nestlings are more likely to be recruited [92, 128-129], yet we failed to demonstrate this in our study. Hatching date is also often positively correlated with fledging success [130] yet we did not find this association in our study, but instead found a significant association between hatching date and apparent juvenile survival.

CONCLUSIONS

Offspring condition can be affected by the early-life environment and early-life gut microbiome, thus highlighting the importance of understanding how changes in the rearing environment affect individual body mass and survival. Even though our results showed variation in nestling gut microbiome diversity, we did not find a significant link between brood size and nestling gut microbiome. Moreover, we did not find a significant association between nestling gut microbiome diversity and short-term or mid-term survival. This suggests that other environmental factors (e.g., diet quality) or genetic effects may contribute more to variation in nestling gut microbiome. Further research is needed to uncover the environmental factors that contribute to nestling gut microbiome in wild bird populations, and how gut microbiome may be linked to nestling survival. Gut microbiome can adapt faster to environmental changes than the host, which makes it important to understand the causes of inter-individual variation in microbiome, and how variation in microbiome possibly mediate adaptation to environmental changes.

DECLARATIONS

Ethics statement

All animal work was conducted under relevant national and international guidelines and legislation. The animal work was licensed by the environmental and ethical committee of Varsinais-Suomi (environmental permit license number

VARELY/890/2020; animal ethics permit number ESAVI/5454/2020). The birds were ringed from their nest-sites by permission from the Finnish Ringing Centre to SR (Finnish Museum of Natural History).

Consent for publication

Not applicable

Availability of data and material

All 16S rRNA gene amplicon sequences and metadata have been submitted to NCBI SRA (SUB11963078). Individual bird metadata and R scripts used to run the analyses are available in the Github Repository, https://github.com/marannli/bsm_analyses.

Competing interests

The authors declare that they have no competing interests.

Funding

ML was funded by the Emil Aaltonen Foundation. AS was funded by the Turku Collegium for Science and Medicine, who contributed to fund the field study. AS acknowledges funding from the European Commission Marie Sklodowska-Curie Postdoctoral Fellowship (#894963) at the time of writing. N.C.-S. was supported by EDUFI Fellowship (Opetushallitus).

Author contribution

The idea for this study was by AS, SR and NCS. Sample collection was done by NCS, MH, AS and SR. Laboratory analyses were done by ML with assistance from SR, KG and EV. Sequence processing and statistical analyses were done by ML with assistance from KG, EV (bioinformatics) and SR (statistics). Manuscript was written by ML and all authors commented and approved the manuscript.

Acknowledgements

We would like to thank Petri Papponen, Sari Viinikainen and Elina Virtanen at the University of Jyväskylä for their help with laboratory optimizations, as well as Jorma Nurmi, Robin Cristofari, Natacha Garcin, Toni Laaksonen at the University of Turku and volunteer bird ringers for their help in the field.

REFERENCES

[1] Kinross JM, Darzi AW & Nicholson JK. Gut microbiome-host interactions in health and disease. Genome medicine. 2011; 3:3:1-12.

[2] van Dongen WF, White J, Brandl HB, Moodley Y, Merkling T, Leclaire S, Wagner RH. (2013). Age-related differences in the cloacal microbiota of a wild bird species. BMC ecology. 2013; 13:1:1-12.

[3] David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Turnbaugh PJ et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014; 505:7484:559-563.

[4] Sánchez B, Delgado S, Blanco-Míguez A, Lourenço A, Gueimonde M & Margolles A. Probiotics, gut microbiota, and their influence on host health and disease. Molecular nutrition & food research. 2017; 61:1:1600240.

[5] Heintz-Buschart A & Wilmes P. Human gut microbiome: function matters. Trends in microbiology. 2018; 26:7:563-574.

[6] Egerton S, Culloty S, Whooley J, Stanton C & Ross RP. The gut microbiota of marine fish. Frontiers in microbiology. 2018; 9:873.

[7] Pan D & Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. Gut microbes. 2014; 5:1:108-119.

[8] Hird SM. Evolutionary biology needs wild microbiomes. Frontiers in Microbiology 2017;8.

[9] Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012; Sep;489:7415:220-30.

[10] Zaneveld JR, McMinds R & Vega Thurber R. Stress and stability: applying the Anna Karenina principle to animal microbiomes. Nature microbiology. 2017; 2:9: 1-8.

[11] Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, Rehermann B. Wild mouse gut microbiota promotes host fitness and improves disease resistance. Cell. 2017a; 171:5:1015-1028.

[12] Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, Rehermann B. Wild mouse gut microbiome protects laboratory mice against lethal influenza virus infection and colorectal cancer. The Journal of Immunology. 2017b; 198.

[13] Worsley SF, Davies CS, Mannarelli ME, Hutchings MI, Komdeur J, Burke T & Richardson DS. Gut microbiome composition, not alpha diversity, is associated with survival in a natural vertebrate population. Animal microbiome. 2021; 3:1:1-18.

[14] Davies CS, Worsley SF, Maher KH, Komdeur J, Burke T, Dugdale HL & Richardson DS. Immunogenetic variation shapes the gut microbiome in a natural vertebrate population. Microbiome. 2022; 10:1:1-22.

[15] Zilber-Rosenberg I & Rosenberg E. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. Fems Microbiology Reviews. 2008; 32:723-735.

[16] Round JL & Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nature Reviews Immunology. 2010; 9:313-323.

[17] Kopac SM & Klassen JL. Can they make it on their own? Hosts, microbiomes, and the holobiont niche. Frontiers in Microbiology. 2016; 7:6.

[18] Hansen CHF, Nielsen DS, Kverka M, Zakostelska Z, Klimesova K, Hudcovic T, Hansen AK. Patterns of early gut colonization shape future immune responses of the host. PloS one. 2012; 7:3:e34043.

[19] Cox LM, Yamanishi S, Sohn J, Alekseyenko AV, Leung JM, Cho I, Blaser MJ et al. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell. 2014; 158:4:705-721.

[20] Grond K, Lanctot RB, Jumpponen A, Sandercock BK. Recruitment and establishment of the gut microbiome in arctic shorebirds. FEMS Microbiology Ecology. 2017; 93:9.

[21] Knutie SA, Shea LA, Kupselaitis M, Wilkinson CL, Kohl KD & Rohr JR. Early-life diet affects host microbiota and later-life defenses against parasites in frogs. Integrative and Comparative Biology. 2017; 57:4:732-742.

[22] Videvall E, Song SJ, Bensch HM, Strandh M, Engelbrecht A, Serfontein N, Cornwallis CK et al. Early-life gut dysbiosis linked to juvenile mortality in ostriches. Microbiome. 2020; 8:1:1-13.

[23] Carmody RN, Gerber GK, Luevano Jr JM, Gatti DM, Somes L, Svenson KL & Turnbaugh PJ. Diet dominates host genotype in shaping the murine gut microbiota. Cell host & microbe. 2015; 17:1:72-84.

[24] Hooper LV, Littman DR & Macpherson AJ. Interactions between the microbiota and the immune system. Science. 2012; 336:6086:1268-1273.

[25] Teyssier A, Matthysen E, Hudin NS, De Neve L, White J & Lens L. Diet contributes to urban-induced alterations in gut microbiota: experimental evidence from a wild passerine. Proceedings of the Royal Society B. 2020; 287:1920:20192182

[26] Bodawatta KH, Freiberga I, Puzejova K, Sam K, Poulsen M & Jønsson KA. Flexibility and resilience of great tit (Parus major) gut microbiomes to changing diets. Animal microbiome. 2021a;3:1:1-14.

[27] Góngora E, Elliott KH & Whyte L. Gut microbiome is affected by inter-sexual and inter-seasonal variation in diet for thick-billed murres (Uria lomvia). Scientific reports. 2021; 11:1:1-12.

[28] Schmiedová L, Tomášek O, Pinkasová H, Albrecht T & Kreisinger J. Variation in diet composition and its relation to gut microbiota in a passerine bird. Scientific reports. 2022; 12:1:1-13.

[29] Datta FU, Nolana JV, Rowe JB & Gray GD. Protein supplementation improves the performance of parasitised sheep fed a straw-based diet. International Journal for Parasitology. 1998; 28:8:1269-1278.

[30] Cotter SC, Simpson SJ, Raubenheimer D & Wilson K. Macronutrient balance mediates trade-offs between immune function and life history traits. Functional Ecology. 2011; 25:1:186-198.

[31] Linden M, Gustafsson L & Part T. Selection on fledging mass in the collared flycatcher and the great tit. Ecology. 1992; 73:1:336-343.

[31] Spor A, Koren O & Ley R. Unravelling the effects of the environment and host genotype on the gut microbiome. Nature Reviews Microbiology. 2011; 9:4:279-290

[33] Bodawatta KH, Hird SM, Grond K, Poulsen M & Jønsson KA. Avian gut microbiomes taking flight. Trends in microbiology. 2021c.

[34] Waite DW & Taylor M. Exploring the avian gut microbiota: current trends and future directions. Frontiers in microbiology. 2015; 6:673.

[35] Palmer C, Bik EM, DiGiulio DB, Relman DA & Brown PO. Development of the human infant intestinal microbiota. PLoS biol. 2007; 5:7:e177.

[36] Chen X, Xu J, Ren E, Su Y & Zhu W. Co-occurrence of early gut colonization in neonatal piglets with microbiota in the maternal and surrounding delivery environments. Anaerobe. 2018; 49:30-40.

[37] Kalbermatter C, Fernandez Trigo N, Christensen S & Ganal-Vonarburg SC. Maternal microbiota, early life colonization and breast milk drive immune development in the newborn. Frontiers in immunology. 2021; 12:1768.

[38] Kohl KD. Diversity and function of the avian gut microbiota. Journal of Comparative Physiology B. 2012; 182:5:591-602.

[39] Trevelline BK, MacLeod KJ, Knutie SA, Langkilde T, Kohl KD. In ovo microbial communities: a potential mechanism for the initial acquisition of gut microbiota among oviparous birds and lizards. Biology Letters. 2018; 14:7:4

[40] Ruiz-Rodriguez M, Lucas FS, Heeb P & Soler JJ. Differences in intestinal microbiota between avian brood parasites and their hosts. Biological Journal of the Linnean Society. 2009; 96:2:406-414.

[41] Benskin CMH, Rhodes G, Pickup RW, Wilson K & Hartley IR. Diversity and temporal stability of bacterial communities in a model passerine bird, the zebra finch. Molecular ecology. 2010;19.24:5531-5544.

[42] Lee CY, Peralta-Sánchez JM, Martínez-Bueno M, Møller AP, Rabelo-Ruiz M, Zamora-Muñoz C & Soler JJ. The gut microbiota of brood parasite and host nestlings

reared within the same environment: disentangling genetic and environmental effects. The ISME Journal. 2020; 14:11:2691-2702.

[43] Lucas FS & Heeb P. Environmental factors shape cloacal bacterial assemblages in great tit Parus major and blue tit P. caeruleus nestlings. *Journal of Avian Biology*. 2005; *36*:6:510-516.

[44] Hird SM, Carstens BC, Cardiff S, Diffmann DL & Brumfield RT. Sampling locality is more detectable than taxonomy or ecology in the gut microbiota of the brood-parasitic Brown-headed Cowbird (Molothrus ater). Peerj 2014.

[45] van Veelen HPJ, Salles JF & Tieleman BI. Multi-level comparisons of cloacal, skin, feather and nest-associated microbiota suggest considerable influence of horizontal acquisition on the microbiota assembly of sympatric woodlarks and skylarks. Microbiome. 2017; 5:1:1-17.

[46] Devaynes A, Antunes A, Bedford A & Ashton P. Bacterial species richness at three stages of the breeding season in Cyanistes caeruleus (blue tit). Acta Oecologica. 2018; 92:123-130.

[47] Ding J, Dai R, Yang L, He C, Xu K, Liu S et al. Inheritance and establishment of gut microbiota in chickens. Frontiers in Microbiology. 2017; 8:1967: doi: 10.3389/fmicb.2017.01967

[48] Preest MR, Folk DG & Beuchat CA. Decomposition of nitrogenous compounds by intestinal bacteria in hummingbirds. The Auk. 2003; 120:4:1091-1101.

[49] Godoy-Vitorino F, Leal SJ, Díaz WA, Rosales J, Goldfarb KC, García-Amado MA, Domínguez-Bello MG et al. Differences in crop bacterial community structure between hoatzins from different geographical locations. Research in Microbiology. 2012; 163:3:211-220.

[50] Mendoza MLZ, Roggenbuck M, Vargas KM, Hansen LH, Brunak S, Gilbert MTP & Sicheritz-Pontén T. Protective role of the vulture facial skin and gut microbiomes aid adaptation to scavenging. Acta Veterinaria Scandinavica. 2018; 60:1:1-19.

[51] Fuirst M, Veit RR, Hahn M, Dheilly N & Thorne LH. Effects of urbanization on the foraging ecology and microbiota of the generalist seabird Larus argentatus. PLoS One. 2018; 13:12:e0209200.

[52] Teyssier A, Lens L, Matthysen E & White J. Dynamics of gut microbiota diversity during the early development of an avian host: evidence from a cross-foster experiment. Frontiers in microbiology. 2018; 9:1524

[53] Pettifor RA, Perrins CM & McCleery RH. Individual optimization of clutch size in great tits. Nature. 1988; 336:6195:160-162.

[54] Wright J, Both C, Cotton PA & Bryant D. Quality vs. quantity: energetic and nutritional trade-offs in parental provisioning strategies. Journal of Animal Ecology. 1998; 620-634.

[55] Badyaev AV & Ghalambor CK. Evolution of life histories along elevational gradients: Trade-off between parental care and fecundity. Ecology. 2001; 82:10:2948-2960.

[56] Naef-Daenzer B & Keller LF. The foraging performance of great and blue tits (Parus major and P. caeruleus) in relation to caterpillar development, and its consequences for nestling growth and fledging weight. Journal of Animal Ecology. 1999, 68: 708–718.

[57] de Kogel CH. Long-term effects of brood size manipulation on morphological development and sex-specific mortality of offspring. Journal of Animal Ecology. 1997; 167-178.

[58] Pettifor RA, Perrins CM & McCleery RH. The individual optimization of fitness: variation in reproductive output, including clutch size, mean nestling mass and offspring recruitment, in manipulated broods of great tits Parus major. Journal of Animal Ecology. 2001; 70:1:62-79.

[59] Smith HG, Källander H & Nilsson JA. The trade-off between offspring number and quality in the great tit Parus major. The Journal of Animal Ecology. 1989; 383-401.

[60] Simon K, Verwoolde MB, Zhang J, Smidt H, de Vries Reilingh G, Kemp B & Lammers A. Long-term effects of early life microbiota disturbance on adaptive immunity in laying hens. Poultry science. 2016; 95:7:1543-1554.

[61] Davidson GL, Somers SE, Wiley N, Johnson CN, Reichert MS, Ross RP, Quinn JL et al. A time-lagged association between the gut microbiome, nestling weight and nestling survival in wild great tits. Journal of Animal Ecology. 2021; 90:4:989-1003.

[62] Kabir SM. The role of probiotics in the poultry industry. International Journal of Molecular Sciences. 2009; *10*:8:3531-3546.

[63] Maki JJ, Klima CL, Sylte MJ & Looft T. The microbial pecking order: utilization of intestinal microbiota for poultry health. *Microorganisms*. 2019; *7*:10:376.

[64] Du X, Xiang Y, Lou F, Tu P, Zhang X, Hu X, Xiao Y et al. Microbial community and short-chain fatty acid mapping in the intestinal tract of quail. Animals. 2020; 10:6:1006.

[65] Den Besten G, Van Eunen K, Groen AK, Venema K, Reijngoud DJ & Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of lipid research. 2013; 54:9:2325-2340.

[66] Harrison XA, Blount JD, Inger R, Norris DR & Bearhop S. Carry-over effects as drivers of fitness differences in animals. Journal of Animal Ecology. 2011; 80:1, 4-18.

[67] Leonard ML, Horn AG, Gozna A & Ramen S. Brood size and begging intensity in nestling birds. Behavioral Ecology. 2000; 11:2:196-201.

[68] Nicolaus M, Michler SP, Ubels R, Van Der Velde M, Komdeur J, Both C & Tinbergen JM. Sex-specific effects of altered competition on nestling growth and survival: an experimental manipulation of brood size and sex ratio. Journal of Animal Ecology. 2009; 78:2:414-426.

[69] Perrins CM. Populationfluctuations and clutch size inthe Great tit,Parus majorL. Journal of Animal Ecology. 1965; 34:601–647

[70] Smith HG, Källander H & Nilsson JÅ. Effect of experimentally altered brood size on frequency and timing of second clutches in the great tit. The Auk. 1987; 104:4:700-706.

[71] Hõrak P, Tegelmann L, Ots I & Møller AP. Immune function and survival of great tit nestlings in relation to growth conditions. Oecologia. 1999; 121:3:316-322.

[72] Sanz JJ & Tinbergen JM. Energy expenditure, nestling age, and brood size: an experimental study of parental behavior in the great tit Parus major. Behavioral Ecology. 1999; 10:5:598-606.

[73] Tinbergen JM & Verhulst S. A fixed energetic ceiling to parental effort in the great tit? Journal of Animal Ecology. 2000; 69:2:323-334.

[74] Neuenschwander S, Brinkhof MW, Kölliker M & Richner H. Brood size, sibling competition, and the cost of begging in great tits (Parus major). Behavioral Ecology. 2003; 14:4:457-462.

[75] Tinbergen JM & Sanz JJ. Strong evidence for selection for larger brood size in a great tit population. Behavioral Ecology. 2004; 15:4:525-533.

[76] Cossin-Sevrin N, Hsu BY, Marciau C, Viblanc VA, Ruuskanen S & Stier A. Effect of prenatal glucocorticoids and thyroid hormones on developmental plasticity of mitochondrial aerobic metabolism, growth and survival: an experimental test in wild great tits. Journal of Experimental Biology. 2022; 225:9:jeb243414.

[77] Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Knight R. et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal. 2012; 6:8:1621-1624.

[78] Parada AE, Needham DM & Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environmental Microbiology. 2016; 18:5:1403–1414

[79] de Muinck EJ, Trosvik P, Gilfillan GD, Hov JR & Sundaram AY. A novel ultra high-throughput 16S rRNA gene amplicon sequencing library preparation method for the Illumina HiSeq platform. Microbiome. 2017; 5:1:1-15.

[80] Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. Journal*. 2011; *17*:1:10-12.

[81] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA & Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nature methods. 2016; 13:7:581-583.

[82] Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Glöckner FO et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research. 2012; 41:D1:D590-D596.

[83] Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., & Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018; 6:1:1-14.

[84] McMurdie PJ & Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one. 2013; 8:4:e61217.

[85] Lahti L & Sudarshan Shetty et al. Tools for microbiome analysis in R. 2017;<http://microbiome.github.com/microbiome>

[86] Wickham, H. (2016). Data analysis. In *ggplot2* (pp. 189-201). Springer, Cham.

[87] Wright ES. Using DECIPHER v2. 0 to analyze big biological sequence data in R. R Journal. 2016; 8:1.

[88] Schliep KP. phangorn: phylogenetic analysis in R. Bioinformatics. 2011; 27:4: 592-593.

[89] Paradis E, Blomberg S, Bolker B, Brown J, Claude J, Cuong HS, Didier G et al. Package 'ape'. Analyses of phylogenetics and evolution, version. 2019; 2(4).

[90] Yu G, Smith DK, Zhu H, Guan Y & Lam TTY. ggtree: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods in Ecology and Evolution. 2017; 8:1:28-36.

[91] Bates D, Maechler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software. 2015; 67(1), 1-48;doi:10.18637/jss.v067.i01.

[92] Monrós JS, Belda EJ, Barba E. Post-fledging survival of individual great tits: the effect of hatching date and fledging mass. Oikos. 2002; Dec;99:3:481-8.

[93] Fox J, Weisberg S, Adler D, Bates D, Baud-Bovy G, Ellison S, Monette G et al. Package 'car'. Vienna: R Foundation for Statistical Computing. 2012; 16.

[94] Hartig F & Hartig MF. Package 'DHARMa'. *R package*. 2017.

[95] Bray JR & Curtis JT. An ordination of the upland forest communities of southern Wisconsin. Ecological monographs.1957; 27:4:326-349.

[96] Lozupone C & Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Applied and environmental microbiology. 2005; 71:12:8228-8235.

[97] Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara RB, Oksanen MJ etl al. Package 'vegan'. Community ecology package, version. 2013; 2:9:1-295.

[98] Moreno J, Cowie RJ, Sanz JJ & Williams RS. Differential response by males and females to brood manipulations in the pied flycatcher: energy expenditure and nestling diet. Journal of Animal Ecology. 1995; 721-732.

[99] Askenmo C. Effects of addition and removal of nestlings on nestling weight, nestling survival, and female weight loss in the Pied Flycatcher Ficedula hypoleuca (Pallas). Ornis Scandinavica. 1977;1-8.

[100] Nur N. The consequences of brood size for breeding blue tits II. Nestling weight, offspring survival and optimal brood size. The Journal of Animal Ecology. 1984; 497-517.

[101] Sanz JJ. Clutch size manipulation in the pied flycatcher: effects on nestling growth, parental care and moult. Journal of Avian Biology. 1998; 157-162.

[102] Burness GP, McClelland GB, Wardrop SL & Hochachka PW. Effect of brood size manipulation on offspring physiology: an experiment with passerine birds. Journal of Experimental Biology. 2000; 203:22:3513-3520.

[103] Naef-Daenzer L, Naef-Daenzer B & Nager RG. Prey selection and foraging performance of breeding Great Tits Parus major in relation to food availability. Journal of Avian Biology. 2000; 31:2:206-214.

[104] Rytkönen S & Orell M. Great tits, Parus major, lay too many eggs:experimental evidence in mid-boreal habitats. Oikos. 2001; 93: 439 – 450

[105] Gebhardt-Henrich SG & Van Noordwijk AJ. The genetical ecology of nestling growth in the great tit. Environmental influences on the expression of genetic variances during growth. Functional ecology. 1994; 469-476.

[106] van der Most PJ, de Jong B, Parmentier HK & Verhulst S. Trade-off between growth and immune function: a meta-analysis of selection experiments. Functional Ecology. 2011; 25:1:74-80.

[107] Rauw WM. Immune response from a resource allocation perspective. Frontiers in genetics. 2012; 3:267.

[108] Naef-Daenzer B & Keller LF. The foraging performance of great and blue tits (Parus major and P. caeruleus) in relation to caterpillar development, and its consequences for nestling growth and fledging weight. Journal of Animal Ecology. 1999; 6:84:708-718.

[109] Tinbergen JM & Dietz MW. Parental energy expenditure during brood rearing in the great tit (Parus major) in relation to body mass, temperature, food availability and clutch size. Functional ecology. 1994; 563-572.

[110] Ilmonen P, Hasselquist D, Langefors Å & Wiehn J. Stress, immunocompetence and leukocyte profiles of pied flycatchers in relation to brood size manipulation. Oecologia. 2003; 136:1:148-154.

[111] Kohl KD, Brun A, Caviedes-Vidal E & Karasov WH. (2019). Age-related changes in the gut microbiota of wild House Sparrow nestlings. Ibis. 2019; 161:1:184-191.

[112] Waite DW & Taylor MW. Characterizing the avian gut microbiota: membership, driving influences, and potential function. Frontiers in microbiology. 2014; 5:223.

[113] Davidson GL, Wiley N, Cooke AC, Johnson CN, Fouhy F, Reichert MS, Quinn JL et al. Diet induces parallel changes to the gut microbiota and problemsolving performance in a wild bird. Scientific reports. 2020; 10:1:1-13.

[114] Murray MH, Lankau EW, Kidd AD, Welch, CN, Ellison T, Adams HC, Hernandez SM et al. Gut microbiome shifts with urbanization and potentially facilitates a zoonotic pathogen in a wading bird. PLoS One. 2020; 15:3:e0220926.

[115] Bodawatta KH, Koane B, Maiah G, Sam K, Poulsen M & Jønsson KA. Species-specific but not phylosymbiotic gut microbiomes of New Guinean passerine birds are shaped by diet and flight-associated gut modifications. Proceedings of the Royal Society B. 2021b;288:1949:20210446.

[116] Bodawatta KH, Klečková I, Klečka J, Pužejová K, Koane B, Poulsen M, Sam K et al. Specific gut bacterial responses to natural diets of tropical birds. Scientific reports. 2022; 12:1:1-15.

[117] Alberdi A, Aizpurua O, Bohmann K, Zepeda-Mendoza ML & Gilbert MTP. Do vertebrate gut metagenomes confer rapid ecological adaptation? Trends in Ecology & Evolution 2016; 31:9:689-699.

[118] Wilkin TA, King LE & Sheldon BC. Habitat quality, nestling diet, and provisioning behaviour in great tits Parus major. Journal of Avian Biology. 2009; 40:2:135-145.

[119] Rytkönen S, Vesterinen EJ, Westerduin C, Leviäkangas T, Vatka E, Mutanen M, Orell M et al. From feces to data: A metabarcoding method for analyzing consumed and available prey in a bird-insect food web. Ecology and evolution. 2019; 9:1:631-639.

[120] Drobniak SM, Cichoń M, Janas K, Barczyk J, Gustafsson L & Zagalska-Neubauer M. Habitat shapes diversity of gut microbiomes in a wild population of blue tits Cyanistes caeruleus. Journal of Avian Biology. 2022;2:e02829.

[121] Fleischer R, Risely A, Hoeck PE, Keller LF & Sommer S. Mechanisms governing avian phylosymbiosis: Genetic dissimilarity based on neutral and MHC regions exhibits little relationship with gut microbiome distributions of Galápagos mockingbirds. Ecology and evolution. 2020; 10:23:13345-13354.

[122] Hanning I, Diaz-Sanchez S. The functionality of the gastrointestinal microbiome in non-human animals. Microbiome. 2015; 3:5: doi: 10.1186/s40168-015-0113-6

[123] Doak DF, Bigger D, Harding EK, Marvier MA, O'malley RE & Thomson D. The statistical inevitability of stability-diversity relationships in community ecology. The American Naturalist. 1998; 151:3:264-276.

[124] Naef-Daenzer B, Widmer F & Nuber M. Differential post-fledging survival of great and coal tits in relation to their condition and fledging date. Journal of Animal ecology. 2001; 70:5:730-738.

[125] Nilsson JA & Svensson M. Sibling competition affects nestling growth strategies in marsh tits. Journal of Animal Ecology. 1996; 825-836.

[126] Magrath RD. Nestling weight and juvenile survival in the blackbird, Turdus merula. The Journal of Animal Ecology. 1991; 335-351.

[127] Ringsby TH, Sæther BE & Solberg EJ. Factors affecting juvenile survival in house sparrow Passer domesticus. Journal of Avian Biology. 1998; 241-247.

[128] Both C, Visser ME & Verboven N. Density–dependent recruitment rates in great tits: the importance of being heavier. Proceedings of the Royal Society of London. Series B: Biological Sciences. 1999; 266:1418:465-469.

[129] Cleasby IR, Nakagawa S, Gillespie DO & Burke T. The influence of sex and body size on nestling survival and recruitment in the house sparrow. Biological Journal of the Linnean Society. 2010; 101:3:680-688.

[130] Tinbergen JM & Boerlijst MC. Nestling weight and survival in individual great tits (Parus major). The Journal of Animal Ecology. 1990; 1113-1127.