Gut microbiome disturbances of altricial Blue and Great tit nestlings are countered by continuous microbial inoculations from parental microbiomes

Running title: Parental effects negate microbiome disruptions in nestlings

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Abstract

Gut microbial communities are complex and heterogeneous and play critical roles for animal hosts. Early-life disruptions to microbiome establishment can negatively impact host fitness and development. However, the consequences of such early-life disruptions are unknown in wild birds. To help fill this gap, after validating the disruptive influence of antibiotic and probiotic treatments on the gut microbiome in adult Great tits (*Parus major*) (efficacy experiment), we investigated the effect of continuous early-life gut microbiome disruptions on the establishment and development of gut communities in wild Great and Blue tit (*Cyanistes caeruleus*) nestlings (field experiment). Despite negative impacts of treatments on microbial alpha and beta diversities in the efficacy experiment, treatment did not affect the composition of nestling microbiomes in the field experiment. Independent of treatment, nestling gut microbiomes of both species grouped by brood, sharing high numbers of bacterial taxa with both the nest environment and their mother. The distance between nests increased inter-brood microbiome dissimilarity, but only in Great tits, indicating species-specific influence of environment on microbiomes. The strong maternal effect, driven by continuous recolonization from the nest environment and vertical transfer of microbes during feeding thus appear to provide resilience towards early-life disruptions in nestling gut microbiomes.

Key Words: antibiotics, probiotics, brood feeding, vertical transmission**,** environmental microbiomes, *Parus major*, *Cyanistes caeruleus*

Introduction

Complex and heterogeneous gut microbial communities affect vertebrate host physiology, development and behavior, with ramifications for host ecology and evolution [1–5]. Early-life establishment of a functioning consortium of gut symbionts is critical for microbiome structure

and function later in life [6–8]. Consequently, disruptions to early-life microbiome assembly processes can negatively impact host fitness and health by altering immune system development, increasing the probability of autoimmune diseases, and reducing resistance to parasitic infections [3, 9–12]. Oviparous birds (class Aves) acquire their initial gut symbionts after hatching, although the sterile nature of eggs and the transfer of maternal microbiota during egg formation is still controversial [13]. Post-hatching, parental and nest microbiomes [14, 15] along with diet [7, 16–18] and habitat [19–23] are thought to be the major factors shaping avian gut microbiomes.

The drivers and trajectory of establishment of the avian gut microbiomes in early life could rely on developmental patterns, from precocial to altricial species. Precocial chicks leave the nest area and feed independently shortly after hatching, whereas altricial chicks spend the brooding period within the nest and are fed directly by the parents [24]. Thus, the gut microbiome establishment of precocial chicks tends to be strongly influenced by the feeding environment, resulting in similar microbiome structures within and between broods [25]. In contrast, gut microbiomes of altricial species tend to be more similar within than between broods [26–28], possibly influenced by mutually non-exclusive vertical transmission of bacteria from parents during feeding events [15, 17] and environmental transfer of microbiomes from food items and the nest [6, 29, 30].

Gut microbiome disruptions have been carried out for decades in the poultry industry by applying antibiotics [31, 32]. In recent years, probiotics [33] have been used as growth promoters, where treatments lead to an increase in weight gain and feed efficiency [34]. Comparisons of treatment outcomes between poultry and wild birds are difficult, as living environments, diets and microbial communities of chickens have been selected by humans for

decades [5]. Nevertheless, the limited number of studies that have investigated the effect of antibiotic treatments on wild chick microbiomes have demonstrated an increase in growth rate (in Magellanic penguins *Spheniscus magellanicus* [35]), together with a higher food conversion efficiency (in House sparrows *Paser domesticus* [36]) similar to in poultry. However, our understanding of the resilience of wild bird gut microbiomes to disruptions at the developmental stage is still limited. Such knowledge is important to understand the stability of host-microbe associations and consequences of microbiome disruptions in a current global environment where anthropogenic stresses continuously influence wild bird microbiomes [37–40].

As a step toward filling this gap, we explored the resilience of nestling gut microbiome to disruptions induced by antibiotics or probiotics during the brooding period in two sympatric altricial passerine (order Passeriformes) species: the Blue tit (BT: *Cyanistes caeruleus*) and the Great tit (GT: *Parus major*). First, we confirmed the influence of antibiotics and probiotics on gut microbiomes of adult birds (efficacy experiment). Then, we applied antibiotics and probiotics to nestlings in the wild and characterized the cloacal microbiomes throughout the brooding period with MiSeq amplicon sequencing of the bacterial 16s rRNA gene. We hypothesized that applying antibiotic or probiotic treatments would disrupt gut microbiomes, and that treated chicks would harbor less diverse and compositionally different microbiomes compared to control and non-treated chicks. However, if the microbiome transfer from the parents or the nest environment outweighs the disruptions caused by treatments, we expect the microbiomes of the chicks to differ less between treatments, prevailing the brood effect [14, 41].

Materials and methods

Study species

BTs and GTs are cavity-nesting passerines that readily accept nest-boxes for breeding [42]. This facilitates sample collection and, to some extent, homogenization of pre-treatment breeding parameters [43]. Both species are dimorphic and monogamous, with females building the nest, and laying and incubating the eggs, while both parents feed the brood [42, 44, 45]. BTs and GTs differ in body size [42] and therefore prey selection [46, 47], which is expected to affect gut microbiomes [23, 48].

Testing the effects of antibiotics and probiotics (efficacy experiment)

The use of antibiotics alters the gut microbial communities of passerine birds compared to controls [36]. To confirm that our treatments influence gut microbiomes of tits, we conducted an efficacy experiment investigating the effect of commonly used broad-spectrum antibiotic Doxycycline (Doxygal 50mg/g) and probiotic *Lactobacillus fermentum* CCM7158 (Propigeon plv.) on ten GT adults, five per treatment. Experimental birds were mist-netted during winter 2020 (December-January) in Branišov forest (48º58'48"N, 14º25'23"E) in České Budějovice (Czech Republic). Birds were ringed, sexed, weighed (Table S1), and transported into a breeding room at the Faculty of Sciences, University of South Bohemia, České Budějovice (day 0). GTs were housed in individual cages, given fresh water (including vitamins (Acidomid exot®) three times per week) and fed a standard daily diet following a well-established protocol [18]. We surface-sterilized diet content with a UV lamp for 20 min before feeding it to the birds in order to minimize microbial input.

We handled the experimental GTs once per day in the early morning. Treatment started on day 1 and continued for three subsequent days (days 1-3). We supplied 0.5 mg of Doxygal or 6.7 mg of *Lactobacillus* probiotic per gram of body weight based on the weight at mist-netting,

following product instructions, diluted in 0.25 ml of water for an adequate oral administration with a syringe. Birds were weighed (electronic scale 0.01 g) and cloacal swabs were taken (minitip Flocked Swab FLOQSwab® 501CS01) from day 1 (initial non-treated microbiome) to day 4, and later on day 8. Additionally, we collected two samples of diets to investigate the potential diet-associated microbial transfer. The handling of GTs and food was strictly done wearing nitrile gloves (cleaned with 70% ethanol between birds). After taking the last sample on day 8, we released GTs back to their original mist-netting location. We preserved swabs in 2 ml sterile vials filled with 100 µl of RNAlater® at -80ºC until the DNA extractions.

Manipulation of nestlings and sample collection (Field experiment)

The field experiment was conducted in a nest-box population in Branišov forest. All nest-boxes were inspected during the last week of April 2020. From that day on, BT and GT clutches at the incubation stage were checked daily until hatching. The first ten nest-boxes of each species that successfully hatched were assigned to the experiment (hatching date for each nestling = day 1) (Fig. 1). The first six hatchlings in each nest (average number of hatchlings per nest ± SD: BT = 11.3 ±1.25; GT = 8.9 ± 0.99) were randomly assigned in duplets to three treatments: antibiotic, probiotic, and control (day 1). The rest of the chicks in each nest-box remained untreated. We color-marked them with nontoxic pens for individual identification, took a cloacal swab and weighed them. In the field experiment we followed a slightly modified 16-day protocol compared with the efficacy experiment (Fig. 1). We changed the experimental procedure by administering the treatment every third day instead of daily, to avoid frequent disturbances in the nest. Control hatchlings were provided with water only (Table S2). Breeding parents were captured when entering their nest-boxes to feed the brood (day 10) by blocking the entrance of the nest-box. We sexed them based on plumage coloration and took a cloacal swab. Given the small size of the nestlings' cloaca, all swabs were lubricated by

144 immersing them in a vial filled with 2 ml of ultrapure water (IWA 20 IOL) just before use. We
145 used separate vials per nest and visit and collected a water sample to control for potential
146 microbiome transfer. Bird handling and storage of the cloacal samples was similar to the
147 efficacy experiment.
148
149 *DNA extractions and MiSeq amplicon sequencing*
150 DNA from cloacal swabs, food samples (from the efficacy experiment) and water samples used
151 to lubricate swabs (field experiment) were extracted using Qiagen DNeasy blood and tissue
152 kit® (Hilden, Germany) following an already validated protocol [49]. The presence of bacterial
153 DNA in samples was validated using primers (SA511 and SB701) targeting bacterial 16S
154 rRNA gene and samples were sequenced on an Illumina MiSeq platform at the Microbiome
155 Core at University of Michigan.
156
157 *Data analyses*
158 MiSeq amplicon sequences were cleaned and aligned using the DADA2 pipeline [50] within
159 QIIME2 [51]. Sequences were clustered into amplicon sequence variants (ASVs) at 100%
160 similarity and assigned to taxonomy using the SILVA 132 bacterial database [52]. All
161 chimeric, archaeal, mitochondrial and chloroplast sequences were removed following the
162 QIIME pipeline. We detected contamination in the water samples used to lubricate the cloacal
163 swabs. These sequences were consistently found across samples and were removed from the
164 full dataset. A rooted bacterial phylogeny was acquired using the *align-to-tree-mafft-fasttree*
165 command in QIIME2. We also removed samples with less than 3,000 sequences from further
166 analyses. Subsequent analyses were conducted separately for the efficacy and the field
167 experiment.
168

Each dataset was rarefied using the sample with the smallest number of sequences (efficacy experiment: 5,158 and field experiment: 3,037) to correct for differences in sequencing depths using *rarefy_even_depth* function in the phyloseq package [53] (Tables S3 and S4) and subsequent analyses were conducted in R 4.0 [54]. Using the *diversity* function in the microbiome package [55], we calculated multiple alpha diversity matrices: observed ASV richness, Shannon's diversity index, and relative dominance (relative abundance of the most abundant bacterial taxa). We further calculated Faith's phylogenetic diversity of microbial communities using the picante package [56].

The effect of experimental treatments on body mass and alpha diversities in the efficacy experiment was assessed by building linear mixed-effect models (LMMs) using the lme4 package [57]. We used treatment (antibiotics or probiotics), day of experiment (quadratic term via *poly* function), sex (male or female), and the interaction between treatment and day of experiment as fixed explanatory variables. Bird identity was used as a random intercept variable.

In the field experiment, we conducted separate analyses for BT and GT chicks. We investigated body mass and alpha diversities from day 1 to 16, by building models containing treatment (control, antibiotics or probiotics) and the day of the experiment (quadratic term via *poly* function) as fixed explanatory variables. We added brood identity as a variable with random intercept because of its grouping nature and modelled the repeatability of chick microbiome sampling with random slopes within day of experiment. We assessed tarsus length, as a proxy for size, at day 16, between all chick groups by building a LMM using the experimental category as a fixed factor (untreated, control, antibiotics, probiotics), and brood identity as a random variable. We additionally compared alpha diversity indexes between all chick groups,

194 adults and the nest microbial environment at day 16 (last day of experiment) using group as a
195 fixed factor (untreated, control, antibiotics, probiotics, male, female and nest) and brood
196 identity as a random variable. We also conducted analyses comparing alpha diversity indexes
197 between chicks (pulling together the three experimental treatments) and nests at day 1 and at
198 day 16 using group (nest and chick) and the day of the experiment as fixed factors, including
199 their interaction, and brood identity as a random variable. Continuous explanatory variables
200 were centered and, when necessary, the data were log or square root transformed (see Tables
201 S5 and S6). We used the *r.squaredGLMM* function from MuMIn package [58] to compute R^2
202 values [59].
203
204 To investigate bacterial community structures (beta diversities) we used Bray-Curtis and
205 weighted UniFrac (accounting for bacterial phylogeny) distances and visualized using non-
206 matric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) plots. The
207 influence of different treatment types and sample types were assessed using permutational
208 multivariate analyses of variance (PERMANOVAs) with the *adonis2* function in the vegan
209 package [60] with the "by" parameter set to "margin" to assess the marginal effect of the tested
210 variables. Pairwise differences in microbial communities were investigated using the
211 pairwiseAdonis wrapper package [61]. To investigate the effect of treatments on associations
212 between microbes in the efficacy experiment, we calculated microbial co-occurrence networks
213 with the *trans_network* function in microeco package [62] using the SparCC method from the
214 SpiecEasi package [63]. We filtered out ASVs with abundances below 1% from the data set
215 and used 100 SparCC simulations. The network properties were calculated with the igraph
216 package [64] and visualized using Gephi [65].
217

Similarly to alpha diversities, field experiment beta diversities were analyzed separately by host species, for manipulated chicks and for final day samples. To tease apart the parental transmission of microbiomes to chicks, we analyzed microbiomes between adults and 16-day old chicks (treated and untreated). We first evaluated the parental and environmental transfer of ASVs through characterizing shared and unique ASVs between adults (males and females), chicks, and nest environment using *UpSet* plots in the UpSetR package [66]. Secondly, we investigated the influence of parental and nest microbiomes on core microbiomes (consistent bacterial taxa) of nestlings using the *core* function in the microbiome package [55]. We assigned an ASV to the core if the ASV was found in abundances of a minimum of 0.001% across >50% of the samples in the same treatment group. We also examined the transfer of microbiomes from the nest through comparing the nest microbiomes with chicks on day 1 (the day a chick hatched), under the assumption that recently hatched chicks do not strongly influence nest microbiomes, but reversely, the nest environment may influence the bacterial communities in the chicks. We conducted similar analyses between chick and nest microbiomes of day 16 to investigate whether chick microbiomes converge similar to nest microbiomes at the end of the brooding period. We further investigated the influence of the distance between nests on microbiome similarity of chicks using a *mantel test* in the vegan package [60], to evaluate whether the proximity of nests (i.e., similar environmental conditions and diet availability) influence chick microbiomes. We used nest-box GPS coordinates (Table S7) to calculate the distances between them, using the *st_distance* function from the sf package [67]. For this analysis we averaged the chick microbiomes from the final day from each nest.

Results

Notable disruptions to adult gut microbiomes in the efficacy experiment

In the efficacy experiment, 47 of the 50 samples passed the quality filtering steps and these samples contained overall 1 799 504 (average ± SD = 38 287 ± 25 372) bacterial sequences. These sequences were assigned to 4 886 ASVs. GTs lost body mass after 8 days in captivity (average ± SD = 12.6 g ± 4.97%, n = 10), which is expected from the adaptation to laboratory conditions [68, 69]. Mass loss was only associated with the number of days since the beginning of the experiment (estimate ± SE = -2.71 ± 0.282, t = 9.623, p <0.001), independent of treatment (estimate ± SE = -0.05 ± 0.270, t = -0.196, p = 0.850) (Fig. 2A). Observed ASV richness and Faith's phylogenetic diversity declined similarly in both antibiotic and probiotic treated individuals during the experiment (Figs. 2B, S1, and Table S3) while we did not detect any temporal pattern in Shannon's diversity index or relative dominance (Fig. S1, Table S3).

At the phylum level, Proteobacteria dominated the microbiomes (50.2%) followed by Bacteroidetes (17.2%), Tenericutes (27.5%), Actinobacteria (9.6%) and Firmicutes (5.2%) (Fig. S2). In both antibiotic and probiotic treated individuals, the relative abundance of Proteobacteria (Antibiotic: Initial = 56.2%, last day = 38.7%; Probiotic: Initial = 53.2%, last day = 25.3%) and Bacteroidetes (Antibiotic: Initial = 23.5%, last day = 12.5%; Probiotic: Initial = 22.8%, last day = 2.9%) decreased during the treatment time, while Tenericutes increased notably (Antibiotic: Initial = 4.3%, last day = 28.4%; Probiotic: Initial = 1.3%, last day = 60.7%) (Fig. S2). In the probiotic treatment, the relative abundance of Firmicutes had increased from the first to the last day, potentially influenced by the inoculation of lactic-acid bacteria (Fig. S2). Microbial community compositions measured with either distance matrices were not significantly different between sampling days for both treatment groups (antibiotic $_{(Bray\text{-}Curtis)}$: F = 1.054, R^2 = 0.1898, p = 0.2516, antibiotic $_{(UniFrac)}$: F = 1.227, R^2 = 0.2143, p = 0.1139, probiotic $_{(Bray\text{-}Curtis)}$: F = 1.196, R^2 = 0.2012, p = 0.1316, probiotic $_{(UniFrac)}$: F = 1.036, R^2 = 0.1791, p = 0.3719; Fig. 2C). However, microbiomes on day 1 exhibited reduced interspecific

variation compared to the last day in both treatment groups (Fig 2C), indicating that antibiotic and probiotic treatments increase variability in microbiomes between individuals.

Microbial network analyses confirmed the effects of antibiotic and probiotic treatment on changing co-occurrence patterns of ASVs between days 1 and 8 (Fig. 1D). The number of ASVs in networks reduced during the period, while the proportion of negative associations between ASVs increased in both groups (Fig. 1D). Overall, this indicates that the influence of antibiotic and probiotic treatments on alpha and beta diversities of microbiomes lead to notable changes in structure of microbial networks.

We observed the presence of a few food-borne ASVs in the gut communities (Fig. S3A and B), but these ASVs were abundant, as community composition of food microbiomes were similar to some of the gut microbiomes (Fig. S3C). Removal of these food-borne microbes from the dataset did not influence overall community compositions or effects of antibiotic or probiotic treatment, so we retained them in the dataset.

Treatment does not affect gut microbiomes of manipulated chicks in the wild

In the field manipulation experiment, from chicks, we acquired 4 356 709 bacterial sequences from BTs (n = 203, average ± SD: 21 461 ± 10 158) and 6 179 421 sequences from GTs (n = 257, average ± SD: 24 044 ± 11 794), and these sequences were assigned to 14 309 ASVs in BT and 18 796 ASVs in GT samples. Alpha diversity indexes of microbiomes increased overall during chick development in both species (except for the relative dominance index) with major changes occurring between day 1 and day 7 (Figs. 3, S4 and Table S4). Alpha diversity indexes showed a clear negative quadratic effect, stabilizing between day 10 and 16, following the decrease in body growth rate (Figs. 3 and S4). We did not find a clear effect of treatment on

292 the diversity matrices in BTs, but GTs showed a trend for higher observed ASV richness and
293 Faith's phylogenetic diversity in antibiotic treated chicks than in controls (Fig. 3, Table S4,
294 and S5).
295
296 The increase in body mass during development was similar in antibiotic and probiotic treated
297 chicks as well as controls for both species (Fig. 3, Table S6). However, on day 16, GT probiotic
298 treated chicks tended to be larger than untreated (estimate ± SE = 0.27 ± 0.140, t-value = 1.933,
299 $p = 0.057$) and antibiotic treated chicks (Table S7).
300
301 The microbiomes of manipulated chicks were dominated by Proteobacteria (BT: 37.6%, GT:
302 38.1%), Firmicutes (BT: 19.1%, GT: 19.3%), Actinobacteria (BT: 18.1%, GT: 18.0%) and
303 Bacteroidetes (BT: 17.8%, GT: 15.8%) bacterial phyla and the relative abundance of these
304 phyla did not differ between days nor treatments (Fig. S5). The composition of microbiomes
305 (beta diversity) was strongly affected by brood identity and sampling day (Fig. 4, Table 1),
306 irrespective of the distance matrix used. Antibiotic or probiotic treatments did not strongly
307 influence microbiome compositions in developing chicks (Table 1).
308
309 *Maternal microbial transfer is important for structuring chick microbiomes*
310 From adults, we acquired 283 044 sequences in BTs (males (n = 6): 21 265 ± 7 832; females
311 (n = 8): 19 432 ± 10 328) and 272 345 sequences in GTs (males (n = 10): 19 342 ± 8 496;
312 females (n = 4): 19 729 ± 8 496). Overall, the phylogenetic diversity of microbiomes did not
313 differ between chicks at day 16 and adults in both species (Fig. 5A, and S6, Table S8). GT
314 adult male microbiomes showed a lower bacterial richness and Shannon's diversity index,
315 compared to chicks (Fig. 5A, and S6, Table S8). For adult BTs, we only found a lower
316 Shannon's diversity index of both males and females than chicks (Fig. 5A, and S6, Table S8).

Of the bacterial ASVs shared between adults and chicks, females shared a higher number of unique ASVs with chicks (BT: 96, GT: 120) than males (BT: 29, GT: 74), indicating a strong effect of maternal microbiome transfer to chicks (Fig. 5B). Overall, core microbiomes were small in all groups, with chicks harboring a larger core microbiome than adults (Fig. 5C). The smaller core microbiomes in adults could be driven by environmental and dietary impacts on microbial variation and/or that fewer adults were examined than chicks [18, 70]. Despite small core microbiomes, chicks shared more core taxa with females (BT: 3 ASVs and GT: 5 ASVs) than males (1 ASV in both BT and GT) (Fig. 5C), underscoring the stronger maternal than paternal effect.

Adult microbiome compositions differed from the microbiomes of chicks on the last sampling day (Fig. S7A). Relative abundance of major bacterial phyla, such as Proteobacteria, Actinobacteria and Bacteroidetes, were comparable between adults and chicks (Fig. S7A). However, in both bird species, adult birds harbored a larger relative proportion of Tenericutes (BT females: 31.8%; males: 35.8%, chicks: 0.4%; GT females: 26.8%; males: 26.6%, chicks: 1.2%), and a lower relative proportion of Firmicutes (BT females: 8.9%; males: 2.5%, chicks: 11.4%; GT females: 6.6%; males: 3.5%, chicks: 25.4%) compared to all chicks on the last day (Fig. S7A).

Microbiome composition was significant different between treatment groups ($PERMANOVA_{10,000\ permutations}$: BT: $F_6 = 1.408$, $R^2 = 0.1166$, $p < 0.0001$ and GT: $F_6 = 1.728$, $R^2 = 0.0975$, $p < 0.0001$) (Fig. S7B and C). The pair-wise comparisons confirmed the reduced influence of paternal microbiomes on the composition of chick microbiomes, as we observed differences in microbial community composition between males and chicks (Table S9). Female

342 microbiome composition did not differ from manipulated chicks but did differ from untreated
343 chicks (Table S9). This suggests that disruptions to the microbiomes increased transfer of
344 maternal microbes to treated chicks. Taken together, these results indicate that maternal
345 transfer of microbes to developing chicks counter disturbances to developing microbiomes, but
346 that only a subset of maternal microbes establish in chick guts.
347
348 *Environmental transfer of microbiomes*
349 From the nest environment, we acquired 337 615 (n = 10; average ± SD = 33 762 ± 15 502)
350 bacterial sequences from BTs and 323 275 (n = 10; 32 328 ± 13 041) from GTs on day 1, and
351 200 844 sequences from BTs (n = 8; 25 106 ± 4 372) and 252 088 (n = 10; 25 209 ± 6 478)
352 from GTs on the day 16. Bacterial richness, Shannon's diversity and phylogenetic diversity of
353 nest microbiomes did not differ between the sampling times for GTs, but BT nests increased
354 in bacterial richness and phylogenetic diversity over time (Figs. 6, S8A and B, and Table S10).
355 Nest beta diversity did not differ between days 1 and 16 for neither bird species (Fig. S8C).
356
357 Alpha diversities were significantly higher in nest microbiomes than chick gut microbiomes
358 on day 1 (Fig. 6A and B and Table S10) and on day 16, except for phylogenetic diversity in
359 GTs (Fig. 6A and B and Table S10). We observed increased microbial diversity in chicks
360 towards the end of the brooding period compared to the hatching day. Microbial diversity in
361 chicks on day 16 (just before fledging) became similar to their nests. The microbiome
362 composition of BTs was significantly different between nests and one day old chicks
363 ($PERMANOVA_{10,000\ permutations}$: $F_1 = 1.581$, $R^2 = 0.0307$, $p = 0.0013$) (Fig. 6C). This was not
364 the case for GTs ($PERMANOVA_{10,000\ permutations}$: $F_1 = 1.078$, $R^2 = 0.0211$, $p = 0.2812$) (Fig.
365 6D). Despite the significant difference between community composition of day 1 chicks and

366 nest microbiomes in BTs, the visual inspection of ordination plots indicated that this is driven
367 by higher variability in chick microbiomes than nest microbiomes (Fig. 6C).
368
369 The similarity in average chick microbiome composition on day 1 (per brood) was not
370 associated with distance between nests (Fig. 6E). This was retained in BTs until the last day of
371 sampling (day 16) but there was a strong positive correlation between nest distance and gut
372 microbiome dissimilarity in GTs on day 16 (Fig. 6F), suggesting that the effect of environment
373 varies between species.
374
375 **Discussion**
376 Here we investigated the influence of continuous disruption on the establishment of the gut
377 microbiomes during development in two altricial wild bird species. Despite the influence of
378 antibiotic and probiotic treatments on gut microbiomes of adult birds, treatments did not
379 negatively impact the diversity and composition of the gut microbiomes of nestlings during
380 development. Consistent with previous studies [14, 26–28, 41], we observed a strong brood
381 effect, driven by the continuous transfer of microbes from the parents and the potential transfer
382 of microbes from the environment (e.g., nest and diet associated microbes). This underlines the
383 importance of environmental and parental transfers, including rescue after disruption, for
384 microbiomes in chicks with plausible influence on both their health and fitness later in life.
385
386 The strong within-brood similarity in chick gut microbiomes underlines the importance of
387 parents in shaping offspring gut communities during the brooding period, through direct (e.g.,
388 feeding events [71, 72]) and indirect (e.g., accumulation of parental microbes in the nest itself
389 [14, 15]) microbial transfer. Parents usually take turns feeding the brood (10 to 40 individual
390 feeding events per hour [46, 71–73]), with similar frequencies in males and females [74, 75].

391 This should ensure a continuous inoculation of parental microbes to chicks, leading to strong
392 convergence of offspring gut microbiome within a brood, while counteracting disruptions to
393 gut microbiomes during development. However, despite bi-parental care, we observed a
394 stronger effect of maternal than paternal microbiomes on chick gut microbiomes (see also
395 [27]). Females spend more time than males during nest building [42, 76], egg laying [77–79],
396 incubation [80–82], and brooding [42, 83, 84], which could lead to a maternally biased
397 shedding of microbes that can be acquired by the chicks. Indirect transfer of maternal microbes
398 via nest environment has also been shown in Zebra finch chicks (*Taeniopygia guttata*) [15].
399 Similar mechanisms are likely in both tit species we studied, as we observed comparably stable
400 microbiomes within nests during the brooding period (Figs. 6A, B and S8), higher similarity
401 of maternal and nest microbiomes than paternal and nest microbiomes (Fig. 5B and Table S8),
402 and higher levels of microbiome sharing and convergence between chicks and nests during the
403 brooding period (Fig. 5B).
404
405 The direct and indirect transfer of maternal microbiomes is likely essential for naturally
406 developing chick microbiomes, as they may lose some gut symbionts due to diet and habitat
407 changes, and during infections with natural pathogens or ones associated with anthropogenic
408 activities [16, 23, 38, 85]. Skewed maternal microbial transfer may reduce competition between
409 parental microbial symbionts sharing the same niches within offspring guts, with potential
410 deleterious effects to chicks [86, 87]. However, male microbial symbionts are not completely
411 lost during generational transmission, indicating that colonization of males does not necessarily
412 mean a dead end for microbes. This may thus reflect a bet-hedging situation for optimal access
413 to important symbionts, secured through biparental transfer. However, it appears more likely
414 that the maternal-biased microbial transfer is derived mainly from the dominant role of females
415 during the breeding period, implying that species with more equal biparental contribution

throughout the nest building, incubation and brooding periods should also exhibit more equal transfer of parental microbes to the next generation.

In GTs, nests located further from each other were more different in gut microbiome structure at the end of the brooding period, highlighting the joint impact of the microhabitat and diet on gut microbiomes. Surprisingly, we did not detect such an association in BTs. Previous work has shown that differences in habitat composition influence wild bird gut microbiomes [23], specifically, the microbiome similarity between prey and predator (caterpillar – tit) is higher when the prey is captured closer to the nest-box [17]. Our observed interspecific differences could originate from differential foraging behaviors or habitat quality of the proximal environment. Tits usually forage within a 25 m radius of the nest-box and increase travelling distances when resources are scarce [88]. GTs are larger and dominant over BTs in competing for nest-boxes [89, 90], which may lead GTs to select nest-boxes in higher-quality habitat patches compared to BTs. Consequently, GTs could forage closer to the nest, which reinforces the association between gut microbiomes and nest-box location. As a result, BT nests might be located in lower-quality habitat patches associated with longer foraging distances [73, 91], reducing the strength of the gut microbiome–location association. Alternatively, or in conjunction with habitat quality, GT is a more generalist species than BT [47, 92], and may be able to exploit multiple food resources near the nest. The more specialist BTs would have to forage further away if their preferred prey is scarce nearby, leading to a reduced influence of nest location on chick gut microbiomes. Overall, this indicates that nest location can also influence the gut microbiome composition of developing chicks, but this effect may depend on prey preference and foraging behavior of the species.

Conclusions

Disruptions to early-life establishment of gut microbiomes can have negative consequences for the development and fitness of animal hosts. Our gut microbiome manipulation study in natural environments highlights the resilience, yielded by parental feeding and environmental acquisition of microbes from nests, to disruptions of gut microbiomes during early life in two altricial bird species. Despite the counteracting effects of continuous transfer of maternal gut microbiomes during chick development, the influence of nest and diet-associated microbes indicate that chick microbiomes are still vulnerable to the introduction of new bacterial symbionts during brooding. If pathogenic, these newly arriving symbionts occupying niches opened-up by gut disruptions may hinder natural host microbial associations, affecting host development and compromising health. Altogether, our findings indicate that maternal-driven transfer of microbial symbionts is important for the establishment and stability of chick microbiomes, potentially affecting long-term associations between avian hosts and their gut symbionts.

Ethics declaration

All the necessary permits were obtained for this experimental project: licence no. 1004 issued by the National Museum in Prague to capture wild birds, licence no. 43873/2019-MZE-18134 issued by the Czech Ministry of Agriculture to house wild birds and licence no. MZP/2020/630/1544 granted by the Czech Ministry of Environment to conduct behavioural experiments with wild birds.

Availability of data and material

Microbiome sequences are submitted to Sequence Read Archive database in GenBank (Efficacy experiment: PRJNA800248, Field experiment: PRJNA800611), and accession numbers of samples are available in Zenodo (doi: 10.5281/zenodo.6174091).

466

467 ***Competing interests***

468 The authors declare that they have no competing interests.

469

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477

478 ***Author contributions***

479 The study was conceived by K.S., K.B. and D.D-M., the efficacy and field experiment were
480 carried out by I.F. and D.D-M.; I.K. carried out the laboratory work and K.B. and D.D-M.
481 analyzed the data. K.B and D.D-M. led the writing of the manuscript, with inputs from I.F and
482 I.K and critical contributions from K.A.J., M.P, and K.S.

483

484 ***References***

485 1. Heijtz RD, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, et al. Normal gut
486 microbiota modulates brain development and behavior. *Proc Natl Acad Sci USA* 2011; **108**:
487 3047–3052.

488 2. Alberdi A, Aizpurua O, Bohmann K, Zepeda-Mendoza ML, Gilbert MTP. Do
489 vertebrate gut metagenomes confer rapid ecological adaptation? *Trends Ecol Evol* 2016; **31**:
490 689–699.

491 3. Macke E, Tasiemski A, Massol F, Callens M, Decaestecker E. Life history and eco-
492 evolutionary dynamics in light of the gut microbiota. *Oikos* 2017; **126**: 508–531.
493 4. Davidson GL, Raulo A, Knowles SCL. Identifying microbiome-mediated behaviour
494 in wild vertebrates. *Trends Ecol Evol* 2020; **35**: 972–980.
495 5. Bodawatta KH, Hird SM, Grond K, Poulsen M, Jønsson KA. Avian gut microbiomes
496 taking flight. *Trends Microbiol* 2022; **30**: 268–280.
497 6. Jacob S, Parthuisot N, Vallat A, Ramon-Portugal F, Helfenstein F, Heeb P.
498 Microbiome affects egg carotenoid investment, nestling development and adult oxidative
499 costs of reproduction in Great tits. *Funct Ecol* 2015; **29**: 1048–1058.
500 7. Davidson GL, Wiley N, Cooke AC, Johnson CN, Fouhy F, Reichert MS, et al. Diet
501 induces parallel changes to the gut microbiota and problem solving performance in a wild
502 bird. *Sci Rep* 2020; **10**: 20783.
503 8. Velando A, Noguera JC, Aira M, Domínguez J. Gut microbiome and telomere length
504 in gull hatchlings. *Biol Lett* 2021; **17**: 20210398.
505 9. Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in
506 early life shapes the immune system. *Science* 2016; **352**: 539–544.
507 10. Simon K, Verwoolde MB, Zhang J, Smidt H, de Vries Reilingh G, Kemp B, et al.
508 Long-term effects of early life microbiota disturbance on adaptive immunity in laying hens.
509 *Poultry Science* 2016; **95**: 1543–1554.
510 11. Knutie SA, Wilkinson CL, Kohl KD, Rohr JR. Early-life disruption of amphibian
511 microbiota decreases later-life resistance to parasites. *Nat Commun* 2017; **8**: 86.
512 12. Kirschman LJ, Khadjinova A, Ireland K, Milligan-Myhre KC. Early life disruption of
513 the microbiota affects organ development and cytokine gene expression in Threespine
514 Stickleback. *Integr Comp Biol* 2020; icaa136.
515 13. Trevelline BK, MacLeod KJ, Knutie SA, Langkilde T, Kohl KD. *In ovo* microbial

516 communities: a potential mechanism for the initial acquisition of gut microbiota among
517 oviparous birds and lizards. *Biol Lett* 2018; **14**: 20180225.
518 14. Teyssier A, Lens L, Matthysen E, White J. Dynamics of gut microbiota diversity
519 during the early development of an avian host: Evidence from a cross-foster experiment.
520 *Front Microbiol* 2018; **9**: 1524.
521 15. Chen C-Y, Chen C-K, Chen Y-Y, Fang A, Shaw GT-W, Hung C-M, et al. Maternal
522 gut microbes shape the early-life assembly of gut microbiota in passerine chicks via nests.
523 *Microbiome* 2020; **8**: 129.
524 16. Teyssier A, Matthysen E, Hudin NS, de Neve L, White J, Lens L. Diet contributes to
525 urban-induced alterations in gut microbiota: experimental evidence from a wild passerine.
526 *Proc R Soc B* 2020; **287**: 20192182.
527 17. Dion-Phénix H, Charmantier A, de Franceschi C, Bourret G, Kembel SW, Réale D.
528 Bacterial microbiota similarity between predators and prey in a blue tit trophic network.
529 *ISME J* 2021; **15**: 1098–1107.
530 18. Bodawatta KH, Freiberga I, Puzejova K, Sam K, Poulsen M, Jønsson KA. Flexibility
531 and resilience of Great Tit (*Parus major*) gut microbiomes to changing diets. *Anim*
532 *microbiome* 2021; **3**: 20.
533 19. Hird SM, Carstens BC, Cardiff SW, Dittmann DL, Brumfield RT. Sampling locality
534 is more detectable than taxonomy or ecology in the gut microbiota of the brood-parasitic
535 Brown-headed Cowbird (*Molothrus ater*). *PeerJ* 2014; **2**: e321.
536 20. Grond K, Santo Domingo JW, Lanctot RB, Jumpponen A, Bentzen RL, Boldenow
537 ML, et al. Composition and drivers of gut microbial communities in arctic-breeding
538 shorebirds. *Front Microbiol* 2019; **10**: 2258.
539 21. Loo WT, García-Loor J, Dudaniec RY, Kleindorfer S, Cavanaugh CM. Host
540 phylogeny, diet, and habitat differentiate the gut microbiomes of Darwin's finches on Santa

541 Cruz Island. *Sci Rep* 2019; **9**: 18781.

542 22. Herder EA, Spence AR, Tingley MW, Hird SM. Elevation correlates with significant
543 changes in relative abundance in hummingbird fecal microbiota, but composition changes
544 little. *Front Ecol Evol* 2021; **8**: 597756.

545 23. Drobniak SM, Cichoń M, Janas K, Barczyk J, Gustafsson L, Zagalska-Neubauer M.
546 Habitat shapes diversity of gut microbiomes in a wild population of blue tits *Cyanistes*
547 *caeruleus*. *J Avian Biol* 2021; jav.02829.

548 24. Starck JM, Ricklefs RE. Patterns of development: The Altricial-Precocial spectrum.
549 In: Starck JM, Ricklefs RE (eds). *Avian growth and development. Evolution within the*
550 *altricial-precocial spectrum*. 1998. Oxford University Press, New York, pp 2–30.

551 25. Grond K, Lanctot RB, Jumpponen A, Sandercock BK. Recruitment and establishment
552 of the gut microbiome in arctic shorebirds. *FEMS Microbiol Ecol* 2017; **93**: fix142.

553 26. Benskin CMcWH, Rhodes G, Pickup RW, Mainwaring MC, Wilson K, Hartley IR.
554 Life history correlates of fecal bacterial species richness in a wild population of the blue tit
555 *Cyanistes caeruleus*. *Ecol Evol* 2015; **5**: 821–835.

556 27. Kreisinger J, Kropáčková L, Petrželková A, Adámková M, Tomášek O, Martin J-F, et
557 al. Temporal stability and the effect of transgenerational transfer on fecal microbiota structure
558 in a long distance migratory bird. *Front Microbiol* 2017; **8**: 50.

559 28. Davidson GL, Somers SE, Wiley N, Johnson CN, Reichert MS, Ross RP, et al. A
560 time-lagged association between the gut microbiome, nestling weight and nestling survival in
561 wild Great Tits. *J Anim Ecol* 2021; **90**: 989–1003.

562 29. Goodenough AE, Stallwood B, Dandy S, Nicholson TE, Stubbs H, Coker DG. Like
563 mother like nest: similarity in microbial communities of adult female Pied Flycatchers and
564 their nests. *J Ornithol* 2017; **158**: 233–244.

565 30. Devaynes A, Antunes A, Bedford A, Ashton P. Progression in the bacterial load

566 during the breeding season in nest boxes occupied by the Blue Tit and its potential impact on
567 hatching or fledging success. *J Ornithol* 2018; **159**: 1009–1017.
568 31. Moore PR, Evenson A, Luckey TD, McCoy E, Elvehjem CA, Hart EB. Use of
569 sulfasuxidine, streptothricin, and streptomycin in nutritional studies with the chick. *J Biol*
570 *Chem* 1946; **165**: 437–441.
571 32. Jukes TH, Williams WL. Nutritional effects of antibiotics. *Pharmacol Rev* 1953; **5**:
572 381–420.
573 33. Reuben RC, Roy PC, Sarkar SL, Alam R-U, Jahid IK. Isolation, characterization, and
574 assessment of lactic acid bacteria toward their selection as poultry probiotics. *BMC Microbiol*
575 2019; **19**: 253.
576 34. Dumonceaux TJ, Hill JE, Hemmingsen SM, Van Kessel AG. Characterization of
577 intestinal microbiota and response to dietary Virginiamycin supplementation in the broiler
578 chicken. *Appl Environ Microbiol* 2006; **72**: 2815–2823.
579 35. Potti J, Moreno J, Yorio P, Briones V, García-Borboroglu P, Villar S, et al. Bacteria
580 divert resources from growth for Magellanic Penguin chicks: Bacteria affect penguin chick
581 growth. *Ecol Lett* 2002; **5**: 709–714.
582 36. Kohl KD, Brun A, Bordenstein SR, Caviedes-Vidal E, Karasov WH. Gut microbes
583 limit growth in house sparrow nestlings (*Passer domesticus*) but not through limitations in
584 digestive capacity. *Integr Zool* 2018; **13**: 139–151.
585 37. Knutie SA. Food supplementation affects gut microbiota and immunological
586 resistance to parasites in a wild bird species. *J Appl Ecol* 2020; **57**: 536–547.
587 38. Murray MH, Lankau EW, Kidd AD, Welch CN, Ellison T, Adams HC, et al. Gut
588 microbiome shifts with urbanization and potentially facilitates a zoonotic pathogen in a
589 wading bird. *PLoS ONE* 2020; **15**: e0220926.
590 39. Berlow M, Phillips JN, Derryberry EP. Effects of urbanization and landscape on gut

591 microbiomes in White-Crowned Sparrows. *Microb Ecol* 2021; **81**: 253–266.

592 40. Berlow M, Wada H, Derryberry EP. Experimental exposure to noise alters gut

593 microbiota in a captive songbird. *Microb Ecol* 2021; https://doi.org/10.1007/s00248-021-

594 01924-3.

595 41. Lucas FS, Heeb P. Environmental factors shape cloacal bacterial assemblages in

596 Great Tit *Parus major* and Blue Tit *P. caeruleus* nestlings. *J Avian Biol* 2005; **36**: 510–516.

597 42. Perrins CM. British tits. 1979. HarperCollins, London.

598 43. Møller AP, Adriaensen F. Variation in clutch size in relation to nest size in birds. *Ecol*

599 *Evol* 2014; **4**: 3583–3595.

600 44. Gibb J. The breeding biology of the Great and Blue Titmice. *Ibis* 1950; **92**: 507–539.

601 45. Perrins CM. Tits and their caterpillar food supply. *Ibis* 1991; **133**: 49–54.

602 46. García-Navas V, Ferrer ES, Sanz JJ. Prey choice, provisioning behaviour, and effects

603 of early nutrition on nestling phenotype of titmice. *Écoscience* 2013; **20**: 9–18.

604 47. Barrientos R, Bueno-Enciso J, Sanz JJ. Hatching asynchrony vs. foraging efficiency:

605 the response to food availability in specialist vs. generalist tit species. *Sci Rep* 2016; **6**:

606 37750.

607 48. Bodawatta KH, Klečková I, Klečka J, Pužejová K, Koane B, Poulsen M, et al.

608 Specific gut bacterial responses to natural diets of tropical birds. *Sci Rep* 2022; **12**: 713.

609 49. Bodawatta KH, Puzejova K, Sam K, Poulsen M, Jønsson KA. Cloacal swabs and

610 alcohol bird specimens are good proxies for compositional analyses of gut microbial

611 communities of Great Tits (*Parus major*). *Anim microbiome* 2020; **2**: 9.

612 50. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2:

613 High-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016; **13**: 581–

614 583.

615 51. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al.

616 Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.
617 *Nat Biotechnol* 2019; **37**: 852–857.
618 52. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA
619 ribosomal RNA gene database project: improved data processing and web-based tools.
620 *Nucleic Acids Res* 2012; **41**: 590–596.
621 53. McMurdie PJ, Holmes S. phyloseq: An R package for reproducible interactive
622 analysis and graphics of microbiome census data. *PLoS ONE* 2013; **8**: e61217.
623 54. R Core Team. R: A language and environment for statistical computing. 2021. R
624 Foundation for Statistical Computing, Viena, Austria.
625 55. Lahti L, Shetty S. Tools for microbiome analysis in R. 2017.
626 56. Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD, et al.
627 Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 2010; **26**: 1463–
628 1464.
629 57. Bates D, Maechler M, Bolker BM, Walker S. Fitting linear mixed-effects models
630 using lme4. *J Stat Softw* 2015; **67**: 1–48.
631 58. Bartón K. MuMIn: multi-model inference. 2015. R package.
632 59. Nakagawa S, Schielzeth H. A general and simple method for obtaining R2 from
633 generalized linear mixed-effects models. *Methods Ecol Evol* 2013; **4**: 133–142.
634 60. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan:
635 Community Ecology Package. 2020.
636 61. Arbizu PM. pairwiseAdonis: Pairwise multilevel comparison using adonis. 2018.
637 62. Liu C, Cui Y, Li X, Yao M. *microeco* : an R package for data mining in microbial
638 community ecology. *FEMS Microbiol Ecol* 2021; **97**: fiaa255.
639 63. Kurtz ZD, Müller CL, Miraldi ER, Littman DR, Blaser MJ, Bonneau RA. Sparse and
640 Compositionally Robust Inference of Microbial Ecological Networks. *PLoS Comput Biol*

641 2015; **11**: e1004226.

642 64. Csardi G, Nepusz T. The igraph software package for complex network research. *Int J*
643 *Complex Syst* 2006; **1695**: 1–9.

644 65. Bastian M, Heymann S, Jacomy M. Gephi: an open source software for exploring and
645 manipulating networks. 2009.

646 66. Conway JR, Lex A, Gehlenborg N. UpSetR: an R package for the visualization of
647 intersecting sets and their properties. *Bioinformatics* 2017; **33**: 2938–2940.

648 67. Pebesma E. Simple Features for R: Standardized Support for Spatial Vector Data. *The*
649 *R Journal* 2018; **10**: 439.

650 68. Krams I, Vrublevska J, Cirule D, Kivleniece I, Krama T, Rantala MJ, et al. Stress,
651 behaviour and immunity in wild caught wintering Great Tits (*Parus major*). *Ethology* 2013;
652 10.

653 69. Fischer CP, Wright-Lichter J, Romero LM. Chronic stress and the introduction to
654 captivity: How wild house sparrows (*Passer domesticus*) adjust to laboratory conditions. *Gen*
655 *Comp Endocrinol* 2018; **259**: 85–92.

656 70. Bodawatta KH, Koane B, Maiah G, Sam K, Poulsen M, Jønsson KA. Species-specific
657 but not phylosymbiotic gut microbiomes of New Guinean 2 passerines are shaped by diet and
658 flight-associated gut modifications. *Proc R Soc Lond B* 2021; **288**: 20210446.

659 71. Hinde CA, Kilner RM. Negotiations within the family over the supply of parental
660 care. *Proc R Soc B* 2007; **274**: 53–60.

661 72. Wilkin TA, King LE, Sheldon BC. Habitat quality, nestling diet, and provisioning
662 behaviour in great tits *Parus major*. *J Avian Biol* 2009; **40**: 135–145.

663 73. Tremblay I, Thomas D, Blondel J, Perret P, Lambrechts MM. The effect of habitat
664 quality on foraging patterns, provisioning rate and nestling growth in Corsican Blue Tits
665 *Parus caeruleus*. *Ibis* 2004; **147**: 17–24.

666 74. Dickens M, Berridge D, Hartley IR. Biparental care and offspring begging strategies:
667 hungry nestling blue tits move towards the father. *Anim Behav* 2008; **75**: 167–174.
668 75. Santema P, Schlicht E, Kempenaers B. Testing the conditional cooperation model:
669 what can we learn from parents taking turns when feeding offspring? *Front Ecol Evol* 2019;
670 **7**: 94.
671 76. Mainwaring MC. Causes and consequences of intraspecific variation in nesting
672 behaviors: Insights from Blue Tits and Great Tits. *Front Ecol Evol* 2017; **5**: 39.
673 77. Pendlebury CJ, Bryant DM. Night-time behaviour of egg-laying tits. *Ibis* 2005; **147**:
674 342–345.
675 78. Lord AM, Mccleery RH, Cresswell W. Incubation prior to clutch completion
676 accelerates embryonic development and so hatch date for eggs laid earlier in a clutch in the
677 Great Tit *Parus major*. *J Avian Biol* 2011; **42**: 187–191.
678 79. Diez-Méndez D, Sanz JJ, Barba E. Impacts of ambient temperature and clutch size on
679 incubation behaviour onset in a female-only incubator songbird. *Ibis* 2021; **163**: 1056–1071.
680 80. Nilsson J-Å. Time-dependent reproductive decisions in the blue tit. *Oikos* 2000; **88**:
681 351–361.
682 81. Bambini G, Schlicht E, Kempenaers B. Patterns of female nest attendance and male
683 feeding throughout the incubation period in Blue Tits *Cyanistes caeruleus*. *Ibis* 2019; **161**:
684 50–65.
685 82. Diez-Méndez D, Cooper CB, Sanz JJ, Verdejo J, Barba E. Deconstructing incubation
686 behaviour in response to ambient temperature over different timescales. *J Avian Biol* 2021;
687 jav.02781.
688 83. Rodríguez S, Barba E. Nestling growth is impaired by heat stress: an experimental
689 study in a mediterranean Great Tit population. *Zool Stud* 2016; **55**: 40.
690 84. Andreasson F, Nord A, Nilsson J-Å. Brood size constrains the development of

691 endothermy in blue tits. *J Exp Biol* 2016; **219**: 2212–2219.

692 85. Hird SM, Ganz H, Eisen JA, Boyce WM. The cloacal microbiome of five wild duck
693 species varies by species and influenza A virus infection status. *mSphere* 2018; **3**: e00382-18.

694 86. Constable GWA, Fagan B, Law R. Maternal transmission as a symbiont sieve, and the
695 absence of lactation in male mammals. 2022. https://doi.org/10.1101/2022.01.10.475639.

696 87. Frank SA. Host–symbiont conflict over the mixing of symbiotic lineages. *Proc R Soc*
697 *Lond B* 1996; **263**: 339–344.

698 88. Naef-Daenzer B. Patch time allocation and patch sampling by foraging great and blue
699 tits. *Anim Behav* 2000; **59**: 989–999.

700 89. Minot EO, Perrins CM. Interspecific interference competition - nest sites for blue and
701 great tits. *J Anim Ecol* 1986; **55**: 331–350.

702 90. Kempenaers B, Dhondt AA. Competition between Blue and Great tit for roosting sites
703 in winter: an aviary experiment. *Ornis Scand* 1991; **22**: 73–75.

704 91. Stauss MJ, Burkhardt JF, Tomiuk J. Foraging flight distances as a measure of parental
705 effort in blue tits *Parus caeruleus* differ with environmental conditions. *J Avian Biol* 2005;
706 **36**: 47–56.

707 92. Bańbura J, Lambrechts MM, Blondel J, Perret P, Cartan-Son M. Food handling time
708 of blue tits chicks: constraints and adaptation to different prey types. *J Avian Biol* 1999; **30**:
709 263–270.

710

711 **Table 1.** Influence of nest, treatment day and treatment on the composition of gut microbial
712 communities in the manipulated chicks based on permutational multivariate analyses of
713 variance (PERMNOVAs) tests. Analyses were conducted through measuring community
714 composition using both Bray-Curtis and weighted UniFrac distances.
715

Species	**Distance matrix**	**Variable**	**F_{df}**	**R^2**	**p**
Great tit	Bray-Curtis	Nest	12.28_{9}	0.2976	<0.001
		Day	4.491_{4}	0.0484	<0.001
		Treatment	0.8659_{2}	0.0047	0.838
	Weighted UniFrac	Nest	2.594_{9}	0.0843	<0.001
		Day	2.739_{4}	0.0396	<0.001
		Treatment	0.7775_{2}	0.0056	0.911
Blue tit	Bray-Curtis	Nest	7.461_{9}	0.2517	<0.001
		Day	2.542_{4}	0.0381	<0.001
		Treatment	0.8697_{2}	0.0065	0.887
	Weighted UniFrac	Nest	1.886_{9}	0.0797	<0.001
		Day	1.789_{4}	0.0381	<0.001
		Treatment	0.7986_{2}	0.0075	0.884

716
717

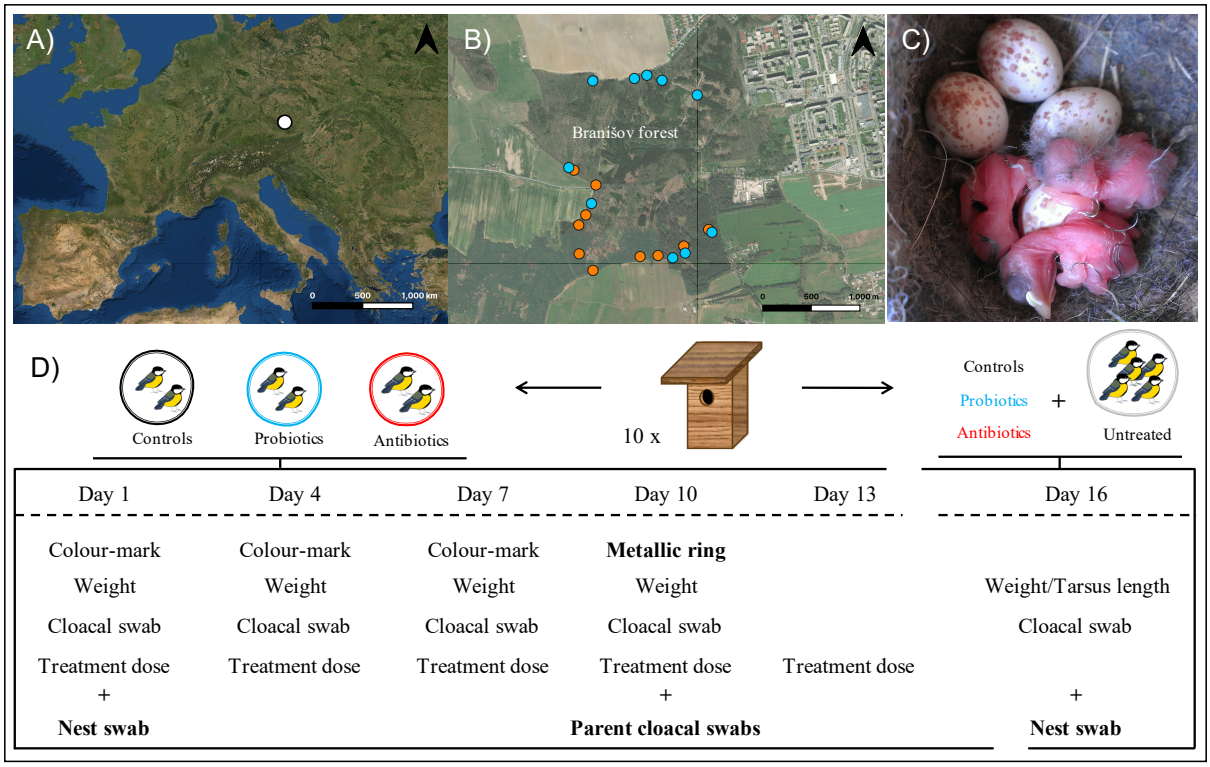


Fig. 1. Location of the study site and schematic representation of the data collection in the field experiment. (A) The location of the study site (white dot), (B) the distribution of the experimental nest-boxes (blue dots refer to experimental Blue tit nests, orange dots to experimental Great tits nests), (C) first Great tit hatchlings in a nest, and (D) the experimental procedure on chicks in both Great and Blue tit nests (illustrations only represent Great tits). Day 1 = nestling hatching day.

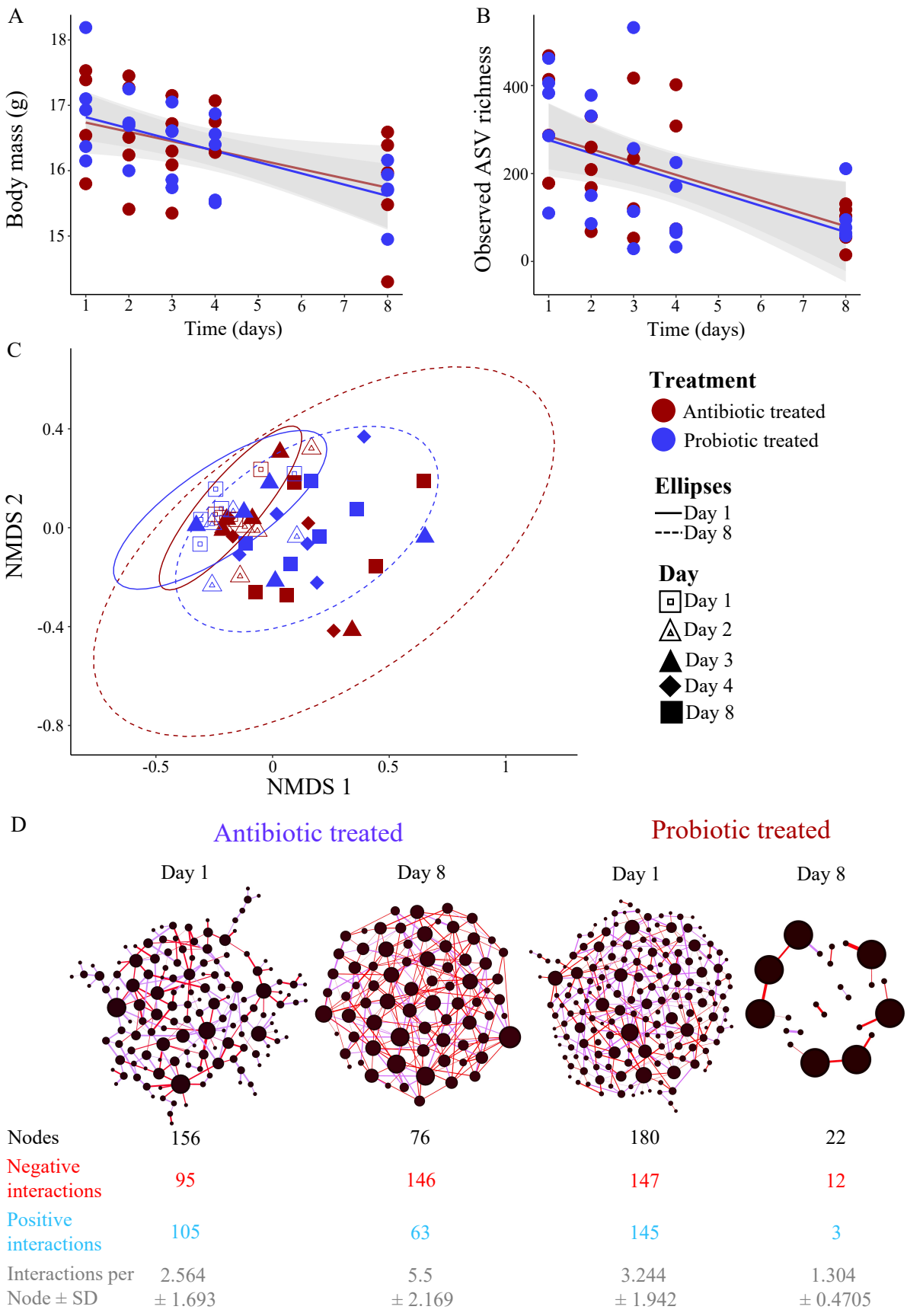
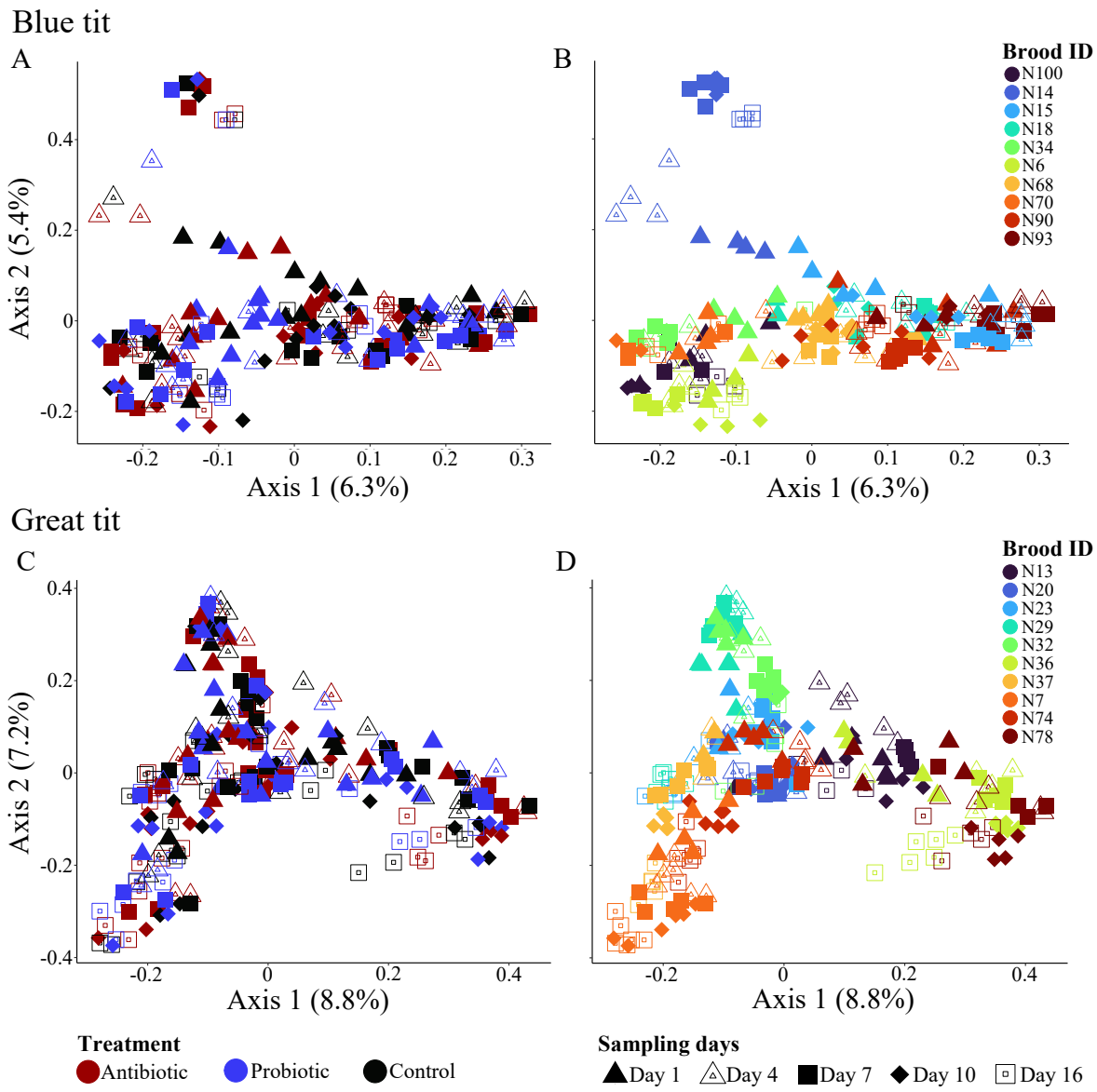


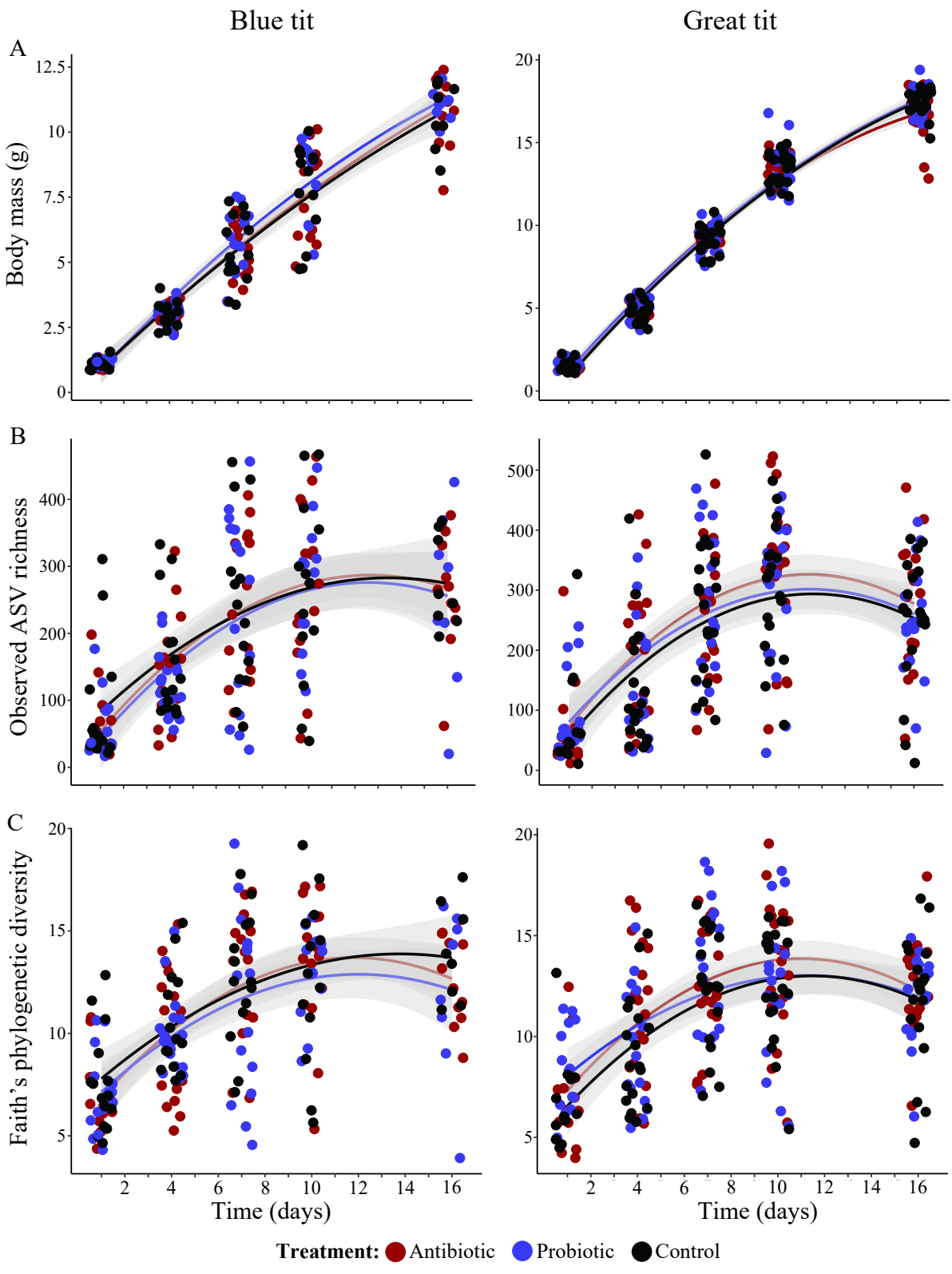
Fig. 2. Antibiotic and probiotic treatments influence host body mass, and alpha and beta

diversities of microbiomes during the efficacy experiment. (A) Decline in body mass during the efficacy experiment in both antibiotic and probiotic treated individuals. Standard errors for regression lines are indicated with grey shaded area. (B) Observed ASV richness of gut microbial communities decreased at the end of the treatment period compared to initial microbiomes. (C) Non-metric multidimensional scaling plot (NMDS) of the gut microbial communities of antibiotic and probiotic treated individuals (stress = 0.243). Individual microbiome variation was lower at the beginning of the experiment (Day 1: solid-line ellipse) in both treatment groups compared to the microbial variation at the end of the treatment (Day 8: dashed-line ellipse). Ellipses represent 95% confident intervals of the data. (D) Microbial co-occurrence networks of initial microbiomes (Day 1) and at the last sampling day (Day 8) after the treatments. Nodes represent individual ASVs, and size correspond to the degree of the ASV (number of interactions each ASV have with other ASVs) in each network. Edges represent whether association are positive (blue) or negative (red). Network attributes are given below each network.



743

744 **Fig. 3. Growth rate and gut microbial alpha diversities did not differ between chicks in**
745 **different treatment groups.** Body mass (A), observed ASV richness (B), and Faith's
746 phylogenetic diversity (C) of microbiomes of manipulated chicks during the sampling period.
747 Gray areas around the trend lines represent standard errors. Data points are colored according
748 to the treatment.



749

750 **Fig. 4. Nest environment has a stronger effect than antibiotic/probiotic treatments on**
751 **shaping the gut microbiomes of developing chicks.** Microbial communities of manipulated
752 chicks of Great (A, B) and Blue (C, D) tits. Individuals in A and C are colored according to the

753 treatment, while individuals in B and D are colored according to nest. Shapes indicate day of
754 sampling across all four plots.
755

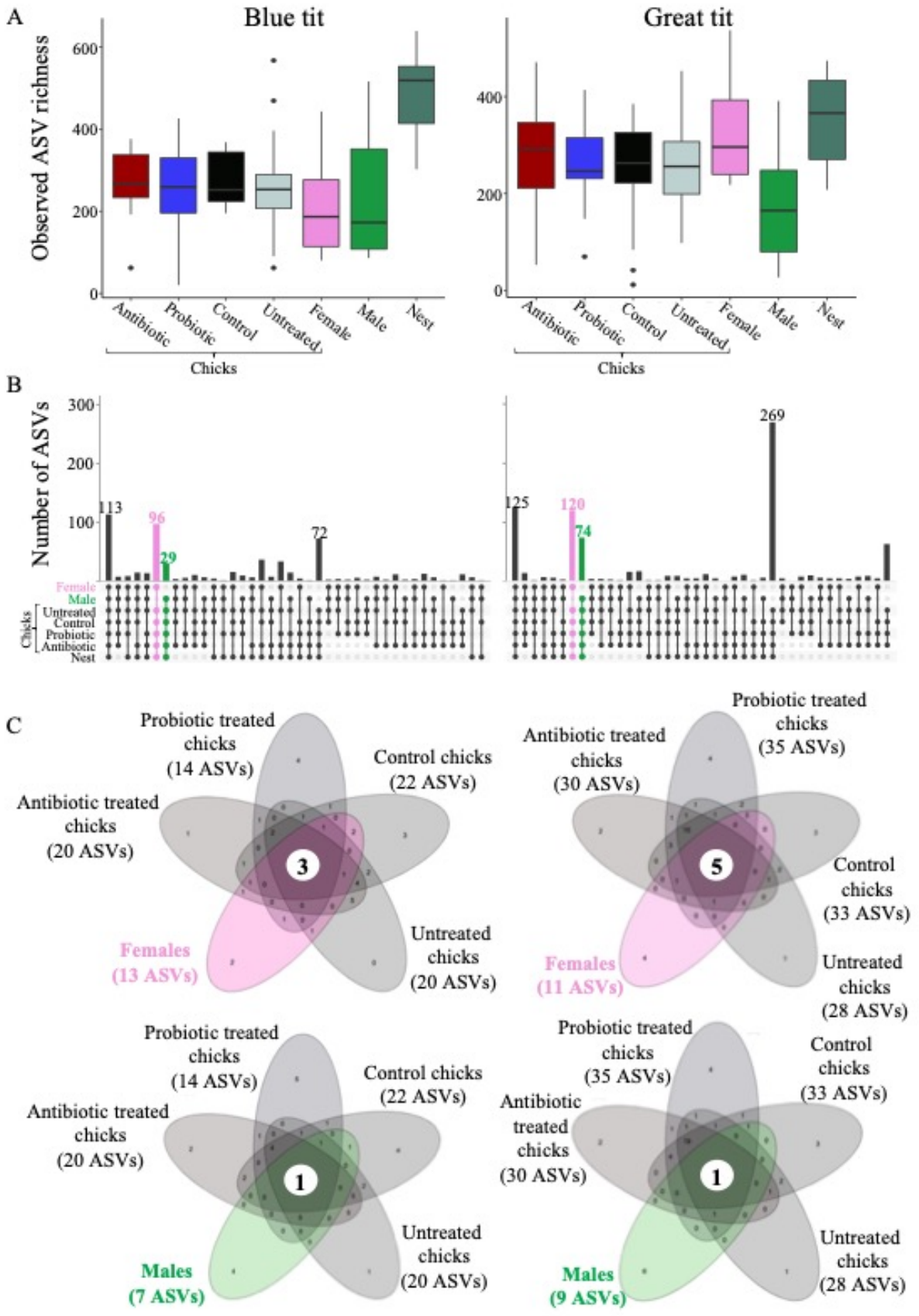
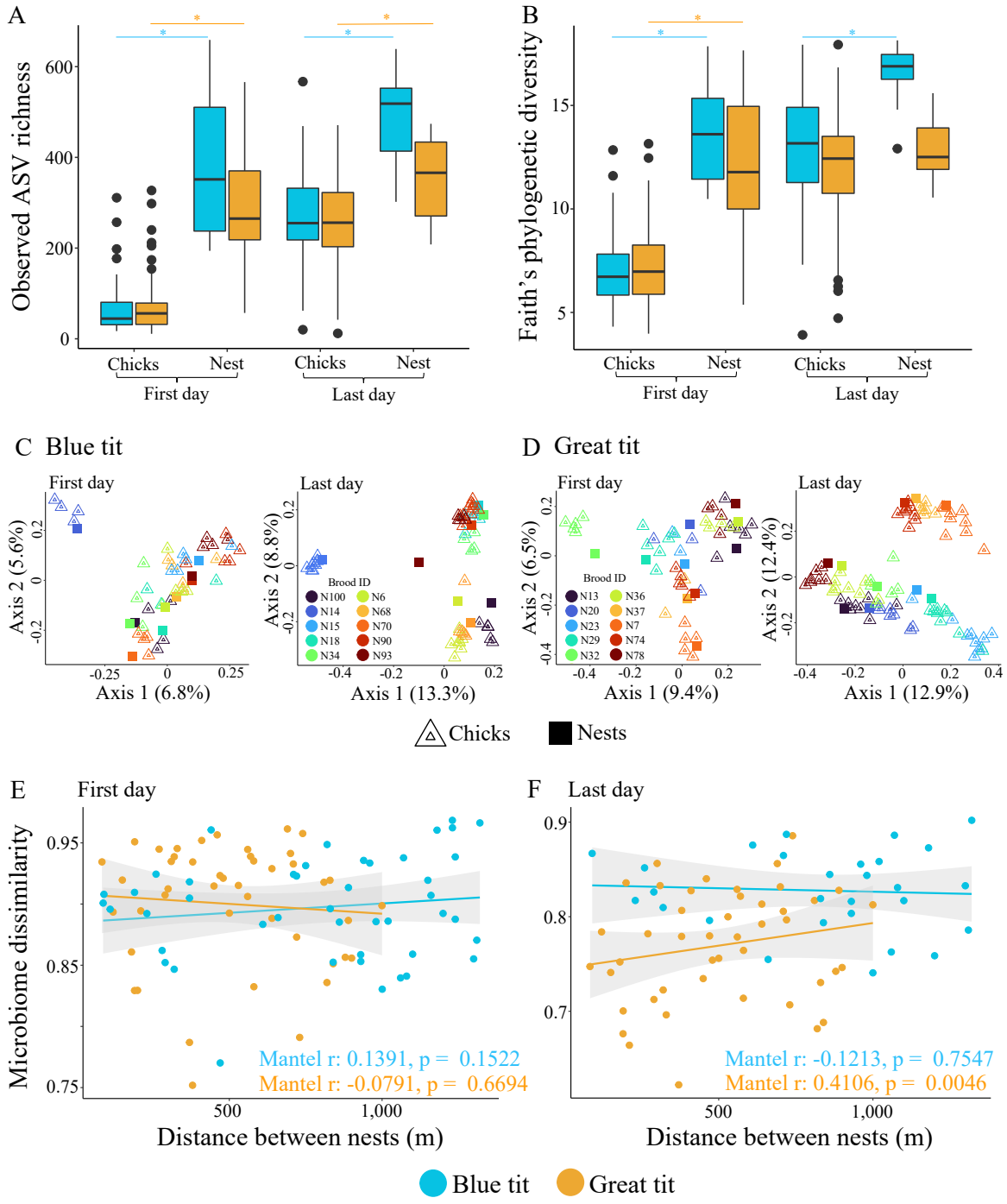


Fig. 5. Alpha diversities of parent microbiomes did not differ from chicks, but maternal

microbiomes contributed notably to the composition of chick microbiomes. (A) Box plots depicting the observed ASV richness in chicks and adults. (B) Upset plots showing the number of shared and unique ASVs found between chicks and adults. Unique ASVs found between chicks and female or male are indicated with colored bars. (C) Flower plots depicting the shared core microbiome between chicks and adults.



764

765 **Fig. 6. Nest microbiomes tend to influence cloacal microbiomes of chicks.** Observed ASV
766 richness (A) and Faith's phylogenetic diversity (B) of chick and nest microbiomes during first
767 and last sampling days. Statistical differences are indicated with asterisks. Ordination plot
768 depicting the compositional differences (measured with Bray-Curtis distances) in chick and
769 nest microbiomes during first and last sampling days of Blue (C) and Great (D) tits. Association

770 between distance among nests and average chick microbiome per nest in first (E) and last (F)

771 sampling days. Mantel test statistics are given within each graph.