JYU DISSERTATIONS 816

Martta Liukkonen

How Fit is Your Gut?

Disentangling the Associations between the Gut Microbiome, the Environment and Host Performance in Wild Birds

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

JYU DISSERTATIONS 816

Martta Liukkonen

How Fit is Your Gut?

Disentangling the Associations between the Gut Microbiome, the Environment and Host Performance in Wild Birds

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi Ylistönrinteen auditoriossa YAA303
syyskuun 6. päivänä 2024 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Science of the University of Jyväskylä,
in Ylistönrinne, auditorium YAA303, on September 6, 2024, at 12 o'clock.

JYVÄSKYLÄN YLIOPISTO
UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2024

Editors
Jari Haimi
Department of Biological and Environmental Science, University of Jyväskylä
Päivi Vuorio
Open Science Centre, University of Jyväskylä

Cover picture by Anni K.

ISBN 978-952-86-0264-4
ISSN 2489-9003

Permanent link to this publication: http://urn.fi/URN:ISBN:978-952-86-0264-4

ABSTRACT

Liukkonen, Martta
How fit is your gut? Disentangling the associations between the gut microbiome, the environment and host performance in wild birds
Jyväskylä: University of Jyväskylä, 2024, 80 p. + original articles
(JYU Dissertations
ISSN 2489-9003; 816)
ISBN 978-952-86-0264-4 (PDF)
Yhteenveto: Suolistomikrobiomin, ympäristötekijöiden ja yksilön menestyksen yhteys luonnonvaraisilla linnuilla
Diss.

The gut microbiome is a complex community of microorganisms that inhabit the host's gastrointestinal tract, and it influences host physiology and health. The gut microbiome has been studied widely with captive species and humans, whereas research with wild host taxa is slowly increasing. The gut microbiome is influenced by both intrinsic and extrinsic factors and thus, it is vital to understand how gut microbiomes evolve and function in wild environments. Birds provide a great study system due to their wide dispersal, unique life history traits and oviparous reproduction. However, majority of existing knowledge about bird gut microbiomes comes from domestic bird research, which cannot be generalized to wild birds. Here, experimental methods and long-term monitoring data are used to study the associations between the gut microbiome, the environment and individual performance in a natural setting. Nestling birds are used to investigate whether the early-life environment contributes to nestling gut microbiome variation and performance. Adult birds are used to study whether environmental and population-level factors associate with gut microbiome variation at a large biogeographical scale, and whether gut microbiome variation associates with individual reproductive success and survival. The results show that environmental variation contributes to differences in gut microbiome diversity and composition in adults. In nestlings, the nest of rearing explains part of the observed variation in gut microbiome diversity. Moreover, variation in the gut microbiome associates with reproductive success, which is the ultimate measure of fitness. This association between reproductive success and gut microbiome variation is particularly strong in male birds and thus, suggests that gut microbiome may have sex-specific effects on individuals. Overall, the results indicate that environmental variation contributes to variation in the gut microbiome and overall performance of wild birds.

Keywords: Avian gut microbiome; environmental associations; *Ficedula albicollis*; gut microbiome; *Parus major*; phenotypic variation.

Martta Liukkonen, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

TIIVISTELMÄ

Liukkonen, Martta
Suolistomikrobiomin, ympäristötekijöiden ja yksilön menestyksen yhteys luonnonvaraisilla linnuilla
Jyväskylä: Jyväskylän yliopisto, 2024, 80 s. + alkuperäiset artikkelit
(JYU Dissertations
ISSN 2489-9003; 816)
ISBN 978-952-86-0264-4 (PDF)
Yhteenveto: Suolistomikrobiomin, ympäristötekijöiden ja yksilön menestyksen yhteys luonnonvaraisilla linnuilla
Diss.

Suolistomikrobiomi on mikroskooppisten organismien muodostama eliöyhteisö, joka elää isäntäeliön suolistossa ja se vaikuttaa isäntäeliön fysiologiaan ja terveyteen. Suolistomikrobiomin merkitystä isäntäeliölle on tutkittu paljon laboratorio-oloissa ja ihmistutkimuksissa, kun taas luonnonympäristössä tapahtuvien tutkimusten määrä on vasta kasvussa. Linnut ovat erinomaisia tutkimusorganismeja, koska ne ovat levittäytyneet laajalti ympäri maapalloa, kykenevät lentämään ja lisääntyvät munimalla. Lintujen suolistomikrobiomit ovat hyvin erilaisia verrattuna esimerkiksi nisäkkäiden suolistomikrobiomeihin, ja esimerkiksi ympäristötekijät kuten elinympäristö ja ruokavalio vaikuttavat huomattavan paljon lintujen suolistomikrobiomiin. Suurin osa olemassa olevasta lintujen suolistomikrobiomitutkimuksesta on kuitenkin tehty vankeudessa kasvatetuilla lajeilla ja siipikarjalla, eikä näitä tutkimustuloksia voi yleistää luonnonympäristöissä eläviin lintulajeihin. Tässä väitöskirjatyössä tutkitaan lintujen suolistomikrobiomin monimuotoisuuden mahdollisia syitä ja seurauksia luonnonympäristöissä. Väitöskirjan osatöissä tutkitaan sitä, 1) onko ympäristötekijöillä yhteys suolistomikrobiomin monimuotoisuuteen, 2) miten varhainen kasvuympäristö vaikuttaa pesäpoikasten suolistomikrobiomiin ja selviytymiseen ja 3) onko suolistomikrobiomin ja lisääntymismenestyksen välillä yhteyttä. Väitöskirjan osatöissä hyödynnetään kokeellisia tutkimusmenetelmiä sekä pitkäaikaisseuranta-aineistoa. Tulokset osoittavat, että ympäristötekijöiden vaihtelu korreloi suolistomikrobiomin diversiteetin ja koostumuksen kanssa. Lisäksi suolistomikrobiomin vaihtelu korreloi lisääntymismenestyksen kanssa, joka on yksilön kelpoisuuden päämittari. Kaiken kaikkiaan tulokset viittaavat siihen, että ympäristön vaihtelu vaikuttaa suolistomikrobiomin monimuotoisuuteen, joka taas kytkeytyy yksilön menestymiseen ja lisääntymiseen.

Avainsanat: Adaptaatio; lintumikrobiomi; sepelsieppo; sopeutuminen; suolistomikrobiomi; talitiainen; ympäristövaikutukset.

Martta Liukkonen, Jyväskylän yliopisto, Bio- ja ympäristötieteiden laitos PL 35, 40014 Jyväskylän yliopisto

Author's address Martta Liukkonen, M.Sc.
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
martta.a.liukkonen@jyu.fi

Supervisors Associate Professor Suvi Ruuskanen, Ph.D
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
suvi.k.ruuskanen@jyu.fi

Dr. Kirsten Grond, Ph.D
Department of Biological Sciences
3211 Providence Drive
CPSB 101
University of Alaska Anchorage, AK
99508, USA
kgrond@alaska.edu

Reviewers Associate Professor Rose Thorogood, Ph.D
Helsinki Institute of Life Science
PL 65, 00014
University of Helsinki
rose.thorogood@helsinki.fi

Assistant Professor Brian Trevelline, Ph.D
Biological Sciences
256 Cunningham Hall
Kent State University, OH
44242, USA
btrevell@kent.edu

Opponent Professor David S. Richardson, Ph.D
01.29 Biology
School of Biological Sciences
University of East Anglia
david.richardson@uea.ac.uk

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The PhD thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-III.

I Liukkonen M., Muriel, J., Martínez-Padilla J., Nord A., Pakanen V-M., Rosivall B., Tilgar V., van Oers K., Grond K. & Ruuskanen S. 2024. Seasonal and environmental factors contribute to the variation in the gut microbiome: a large-scale study of a small bird**.** *Journal of Animal Ecology* 00: 1-18.

II Liukkonen M., Hukkanen M., Cossin-Sevrin N., Stier A., Vesterinen E., Grond K., & Ruuskanen S. 2023. No evidence for associations between brood size, gut microbiome diversity and survival in great tit (*Parus major*) nestlings. *Animal microbiome* 5:19.

III Liukkonen M., Gustafsson L., Grond K. & Ruuskanen S. 2024. Gut microbiome diversity associates with estimated lifetime and annual reproductive success in male but not female collared flycatchers. Manuscript.

Table of author contributions to the original publications

	I	II	III
Original idea	SR	SR, AS	SR, ML
Study design	SR, ML	SR, AS	SR, ML
Fecal sample collection	ML, NCS, MH, JM, JMP, AN, VMP, BR, VT, KVO	MH, NCS, AS, SR	LG
Environmental data collection	ML, JM, JMP, AN, VMP, BR, VT, KVO	MH, NCS, AS, SR	LG
Sample processing	ML	ML	ML
Data analysis	ML	ML, KG	ML
Writing original manuscript	ML	ML	ML
Approving final manuscript	ML, JM, JMP, AN, VMP, BR, VT, KVO, KG, SR	ML, MH, NCS, AS, EV, KG, SR	ML, LG, KG, SR

NCS = Nina Cossin-Sevrin, KG = Kirsten Grond, LG = Lars Gustafsson, MH = Mikaela Hukkanen, ML = Martta Liukkonen, JMP = Jesús Martínez-Padilla, JM = Jaime Muriel, AN = Andreas Nord, VMP = Veli-Matti Pakanen, BR = Balázs Rosivall, SR = Suvi Ruuskanen, AS = Antoine Stier, VT = Vallo Tilgar, KVO = Kees van Oers, EV = Eero Vesterinen

"We have no need of other worlds. We need mirrors. We don't know what to do with other worlds. A single world, our own, suffices us; but we can't accept it for what it is."

- From the book "Solaris" by Stanislaw Lem

1 INTRODUCTION

1.1 A multitude of factors contribute to organism's phenotype

Earth is home to a vast number of species inhabiting wildly varying habitats. These species have adapted to survive in their habitats, and while some species inhabit a large biogeographical range, some are highly specialized to very specific habitats. Phenotype i.e., the traits or characteristics of an organism, which are defined by individual genotype and the environment (Bull 1987) is at the core of adaptation. Phenotype includes morphology and physiology, development, and behaviour and enables the individual to survive in its chosen habitat (Willmore *et al.* 2007). Some individuals may have a phenotype that enables them to better match the environment and thus, be more likely to reproduce and pass their genes on to the next generation (i.e., evolution by natural selection). While morphology and physiology, development, and behaviour as factors contributing to phenotypic variation have been studied rather extensively (e.g., Wagner and Altenberg 1996, Mitchell-Olds *et al.* 2007, Geiler-Samerotte *et al*. 2013, Forsman 2014, Fox *et al.* 2019, Xue *et al.* 2019), the role of host colonizing and symbiotic microorganisms is still a new field. These microorganisms and their potential role in host phenotypic variation are gathering research interest across different biology disciplines such as microbiology and ecology.

1.2 The gut microbiome is an inseparable part of host phenotype

Multicellular organisms are often colonized by microorganisms that can be neutral, beneficial, or pathogenic to the host (McFall-Ngai *et al.* 2013). Together with the host organism these microorganisms form host-microbe interactions that can be considered an inseparable single unit of selection, the holobiont

(Zilber-Rosenberg and Rosenberg 2008, Bordenstein and Theis 2015, Theis *et al.* 2016, Rosenberg and Zilber-Rosenberg 2018). Particularly, the gastrointestinal tract (hereafter, GI tract) and the microorganisms colonizing it have been the focus of thousands of scientific articles most of which are focused on humans (Sekirov *et al.* 2010, Clemente *et al.* 2012, Sommer and Bäckhed 2013, Thursby and Juge 2017). In many species the GI tract is colonized by a vast number of microorganisms such as bacteria, archaea, fungi, viruses / phage, and protozoa (Sommer and Bäckhed 2013). Within the host, the bacteria, and the other before-mentioned microorganisms in the GI tract and all their genomes combined form the gut microbiome (Zilber-Rosenberg and Rosenberg 2008, Rosenberg and Zilber-Rosenberg 2018, Roughgarden *et al.* 2018,). The gut microbiome is strongly shaped by not only phylogeny but also the environment outside the host. Several host-mediated interactions such as social environment and physical contact with surrounding organisms can influence the structure of the gut microbiome (Miller *et al.* 2018, Sarkar *et al.* 2020).

A vast amount of medical and laboratory animal studies emphasize the gut microbiome's importance in host metabolism, immune system functioning and behaviour (Sekirov *et al.* 2010, Tilg and Kaser 2011, Jašarević *et al.* 2016). The gut microbiome benefits the host by protecting against pathogens (Kamada *et al.* 2013a, b, Pickard *et al.* 2017), regulating immune system functioning (Round and Mazmanian 2009, Hooper *et al.* 2012) and influencing energy metabolism (Besten *et al.* 2013). The gut microbiome can affect the metabolome (i.e., small molecule compounds) of the host (Moriya *et al.* 2017, Nagata *et al.* 2019) and influence gene expression and the function of cells and tissue (Mathewson *et al.* 2016, Rastelli *et al.* 2019, Nichols and Davenport 2021). For example, the gut microbiome can improve cold tolerance by altering metabolic pathways that influence energy homeostasis (Chevalier *et al.* 2015) and prepare the organism for hibernation by increasing fat deposition (Sommer *et al.* 2016). Additionally, multiple human studies have showed that there is a link between the host phenotype and the gut microbiome; the gut microbiome underlies individual metabolic phenotypes and can regulate obesity, inflammatory bowel diseases and the likelihood of depression (Li *et al.* 2008, Holmes *et al.* 2012, Blekhman *et al.* 2015, Stevens *et al.* 2021). It could be said that the gut microbiome is vital to the host because it can influence host phenotype and performance. Moreover, it could influence host ecology and even evolution via selection on phenotypes that are better adapted to the prevailing environmental conditions (Zilber-Rosenberg and Rosenberg 2008, Bordenstein and Theis 2015, Rosenberg and Zilber-Rosenberg 2018).

While there is evidence for between and within individual variation in the gut microbiome in both laboratory-bred and wild species, evidence for population level differences is only slowly growing (birds: Hird *et al.* 2014; fish: Ma *et al.* 2024; mammals: Pasciullo Boychuck *et al.* 2024). In a recent review by Maritan *et al.* (2024) it was suggested that there is still very little knowledge of the factors that drive variation in these host-gut microbiome interactions. Variation is a prerequisite for local adaptation and evolution and therefore, the individual and population level variation in the gut microbiome and the potential causes and consequences of this variation should be studied

extensively. The concept of extended phenotype (Dawkins 2016) suggests that each organism contributes to its surrounding environment via phenotypic effects (Whitham *et al.* 2006, Dawkins 2016). This same theory could apply to host-gut microbiome interactions because the gut microbiome can influence the environment i.e., the host gut and therefore, host physiology (Mueller and Sachs 2015). Furthermore, as the gut microbiome includes the genomes of all the microorganisms colonizing the host's GI tract, this "extended genotype" may expand the host's phenotypic potential within a population and therefore, influence host evolution (as reviewed in Shapira 2016, Koskella *et al.* 2017, Carthey *et al.* 2018, Rosenberg and Zilber-Rosenberg 2018, Henry *et al.* 2021).

1.3 The causes and consequences of gut microbiome variation

1.3.1 Diversity and composition at the centre of gut microbiome variation

Variation in the gut microbiome is usually defined by two key terms: diversity and composition. Gut microbiome diversity measures the number of different taxa within the gut microbiome. Generally, more diversity in the gut microbiome is considered beneficial for the host as it is more stable and robust especially when encountering variation in the host environment (as reviewed by Lozupone *et al.* 2012). Gut microbiome composition measures the proportions of different taxa within the gut microbiome and how the ratios of these taxa and their presence / absence vary and may aid host performance. For example, taxa belonging to the genera *Clostridium* and *Streptococcus* are important in the synthesis of short-chain fatty acids, which are needed in host energy metabolism and thus, vital in host performance (Besten *et al.* 2013, Maki *et al.* 2019, Du *et al.* 2020). On the contrary, the absence of genera *Lactobacillus* and *Bifidobacteria* can lead to dysfunction in the neural pathways in the host brain and negatively influence host health (Rao *et al.* 2009). Variation in the gut microbiome can have different consequences on the host organism and these consequences can affect cell and tissue functioning, organ functioning and the metabolome of the host just to name a few.

1.3.2 Establishment of the gut microbiome

The gut microbiome is usually established at birth via vertical and horizontal transmission of microorganisms, and it develops rapidly during the development of the host. Mammals acquire their gut microbiota during maternal vaginal birth and therefore, their initial gut microbiome is vertically inherited from the mother (Palmer *et al.* 2007, Bäckhed *et al.* 2015, Ferretti *et al.* 2018). In oviparous vertebrates such as birds the gut microbiome is usually established after hatching and the initial gut microbiome is defined by the hatching environment i.e., the horizontal transmission of environmental microorganisms (Kohl 2012, Grond *et al.* 2017, 2018). Post-birth, the development of the gut

microbiome is influenced by a set of environmental factors such as the initial diet of the newborn (Mackie *et al.* 1999, Fernández *et al.* 2013), social contacts and the surrounding environment (Tung *et al.* 2015, Moeller *et al.* 2016, 2018, Perofsky *et al.* 2017). Priority effects in the gut microbiome i.e., the order at which microorganisms first colonize the GI tract, can influence the community composition of the gut microbiome (Sprockett *et al.* 2018, Debray *et al.* 2022). Current knowledge suggests priority effects influence the further establishment of the gut microbiome by affecting microorganisms that arrive later in host's life (Martínez *et al.* 2018, Furman *et al.* 2020).

Once established the gut microbiome hosts resident microorganisms (Rodríguez *et al.* 2015, but see Hammer *et al.* 2017, 2019). The host and its resident gut microbiome come in contact with various microorganisms that the host encounters within its everyday life. The resident gut microbiome can prevent colonization by these exogenous (or transient) microorganisms e.g., via competitive exclusion and thus, prevent the colonization of potentially pathogenic taxa (Kamada *et al.* 2013a). For example, rich and diverse gut microbiomes can include both functional and functionally redundant bacteria that improve the stability of the gut microbiome and make it more robust when encountering transient and potentially pathogenic bacteria (as reviewed by Lozupone *et al.* 2012, Zhang *et al.* 2016). Large disruptions in early-life such as antibiotic treatment may have a longstanding influence on the host gut microbiome composition because it can change the composition of the resident gut microbiome and enable the colonization of the more transient microorganisms (Segura Munoz *et al.* 2022). Overall, the horizontal and vertical transmission of microorganisms, the establishment of the gut microbiome and the dynamics between resident and transient microbes are a result of several intrinsic and extrinsic factors that are closely intertwined (Trujillo *et al.* 2022).

1.3.3 Intrinsic factors that shape the gut microbiome

The vertebrate gut microbiome is shaped by intrinsic factors that are inherent to the host such as phylogeny, reproduction, physiology and individual health, age, and host-specific diet. The evolutionary background of a host species influences the gut microbiome (Youngblut *et al.* 2019, Mallott and Amato 2021). For example, the gut microbiomes of related mammalian species are more like each other than those of more distantly related species (Ley *et al.* 2008). Reproductive stage can also influence the gut microbiome and this variation can be sex specific. In female eastern black rhinos (*Diceros bicornis michaeli*) reproductive hormone concentrations that vary across the reproductive cycle have been shown to correlate with specific bacterial taxa within the gut microbiome (Antwis *et al.* 2019). Previous research has shown that both gut microbiome diversity and composition can be age specific (Jia *et al.* 2018, Adriansjach *et al.* 2020, Burnham *et al.* 2023). Additionally, host-specific diet can be considered an intrinsic factor, which can influence gut microbiome variation. These species-specific dietary preferences can range from omnivorous to very specialized diets, which can shape the gut microbial communities (Youngblut *et al.* 2019). The effect of diet on

gut microbiomes have been observed in many species including mammals (David *et al.* 2014, Carmody *et al.* 2015, Martínez-Mota *et al.* 2020, Trujillo *et al.* 2022, Teullet *et al.* 2023), birds (Hird *et al.* 2015, Davidson *et al.* 2020, Teyssier *et al.* 2020, Bodawatta *et al.* 2021, Baiz *et al.* 2023), and insects (Engel and Moran 2013, Pérez-Cobas *et al.* 2015, Luo *et al.* 2021).

1.3.4 Extrinsic factors that shape the gut microbiome

Extrinsic factors include a variety of environmental conditions that are not host specific but are defined by the local environment. These factors include habitat, season, local food resources, and social interactions. Host species inhabiting different types of habitats usually exhibit habitat-dependent variation in their gut microbiomes. For example, several bird studies in which same-species populations inhabit rural or urban environments show distinct differences in host gut microbiomes (Phillips *et al.* 2018, Gadau *et al.* 2019, Murray *et al.* 2020). Studies have also found season specific variation in the gut microbiomes of many vertebrate species (Davenport *et al.* 2014, Maurice *et al.* 2015, Ren *et al.* 2017, Xiao *et al.* 2019, Baniel *et al.* 2021, Góngora *et al.* 2021). Both habitat and season are closely connected to factors such as local food resources, temperature, and precipitation all of which can influence variation in the gut microbiome. Seasonal variation in food item diversity or habitat can contribute to gut microbiome richness because gut microbiomes are known to reflect especially diet and habitat diversity (Muegge *et al.* 2011, Kartzinel *et al.* 2019). For example, variation in snow coverage and temperature between winter and summer can limit or alter dietary preferences and available dietary items (Goodson *et al.* 1991, Thompson *et al.* 2015). Specialist species that rely on fewer food items may have a lowered gut microbiome diversity than the more omnivorous species because fewer microbes are required to digest a narrow range of food items (Crooks and Van Vuren 1995, Pasciullo Boychuck *et al.* 2024). In wild wood mice (*Apodemus sylvaticus*) seasonal change in local diet led to a strong shift in the gut microbiome communities (Maurice *et al.* 2015). Similar association has been found in wild redfronted lemurs (*Eulemur rufifrons*) (Murillo *et al.* 2022), wild geladas (*Theropithecus gelada*) (Baniel *et al.* 2021), the avivorous great evening bat (*Ia io*) (Gong *et al.* 2021), and thick-billed murres (*Uria lomvia*) (Góngora *et al.* 2021). Furthermore, the two season-dependent factors, temperature and precipitation have been found to associate with variation in the gut microbiome. For example, the gut microbiomes of amphibian species are influenced by temperature (Kohl and Yahn 2016, Fontaine *et al.* 2018), and similar associations have been found between the gut microbiome and rainfall in primates (Hicks *et al.* 2018, Orkin *et al.* 2019, Baniel *et al.* 2021).

Additionally, social interactions whether communal living or direct physical contact can influence the gut microbiome composition (Tung *et al.* 2015, Antwis *et al.* 2018). The contribution of both intrinsic and extrinsic factors (Fig. 1) on gut microbiome variation can be seen in same species populations (I, II, III). However, the magnitude of contribution varies across species and especially wild gut microbiomes are still largely underexplored when compared to the

number of studies done with laboratory animals and humans (Hird 2017, Grond *et al.* 2018, Woodhams *et al.* 2020).

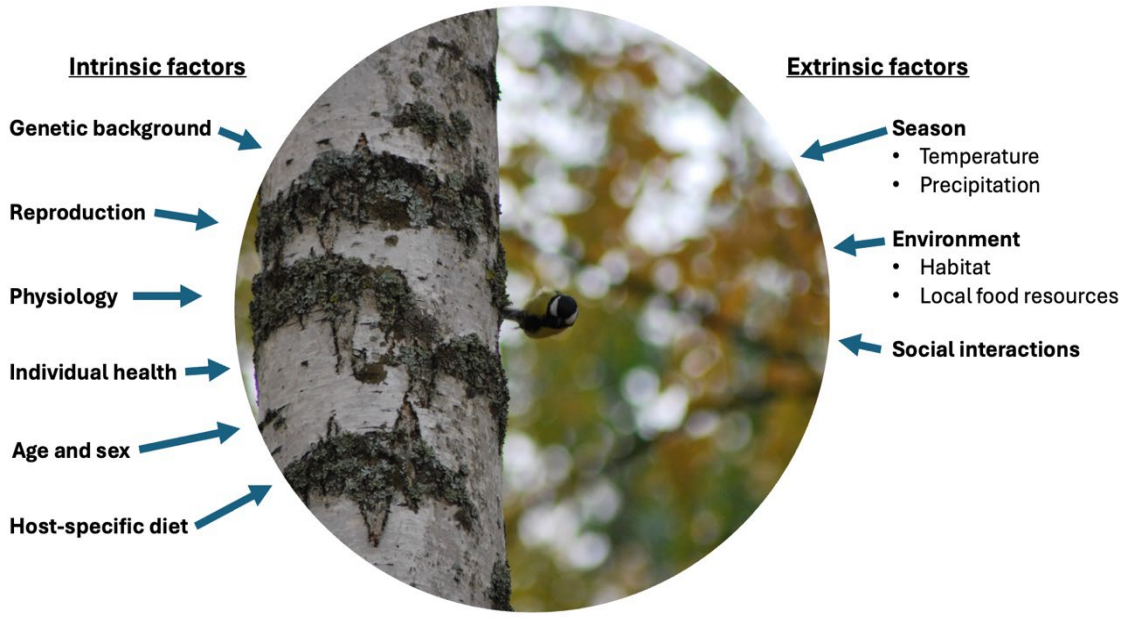

FIGURE 1 The intrinsic and extrinsic factors influencing the gut microbiome.

1.3.5 Consequences of gut microbiome variation on the host

Many studies conducted in laboratory or captive conditions have found that gut microbiomes are consequential to host phenotypes. In fruit flies (*Drosophila melanogaster*) a reciprocal gut microbiome transplantation resulted in changes in male mating duration and increased female offspring production (Morimoto *et al.* 2017). In broiler chickens lower gut microbiome diversity led to a lowered egg-laying performance and increased *Firmicutes* abundance led to higher fat deposition (Wang *et al.* 2021). Moreover, the gut microbiome and specifically the abundance of *Lactobacillus* spp. can influence the amount of calcium deposition in the eggshells of egg-laying hens (Jin *et al.* 2024). The gut microbiome can modulate host physiology via metabolic pathways. Members of the gut microbiome such as the *Bifidobacterium* spp., *Lactobacillus* spp. and *Streptococcus* spp. can transform dietary lipids to compounds that are required in physiological functions such as glucose homeostasis, inflammation suppression and resisting bacterial pathogen growth (Brown *et al.* 2023). The transplantation of two distinct gut microbiomes in gnotobiotic mice (*Mus musculus*) resulted in metabolic, epigenetic, and transcriptional differences that affected the host's response to dietary fiber intake and resulted in changes in hepatic gene expression and cecal and blood metabolites (Murga-Garrido *et al.* 2021). Therefore, the gut microbiome also connects to host health because these metabolic pathways influence allergies and obesity related diseases such as cardiovascular diseases and the metabolic syndrome (Tilg and Kaser 2011, Yoon *et al.* 2021). Moreover, disturbance in gut

microbiome development because of e.g., antibiotic exposure can lead to long-term negative health effects on the host and influence host phenotypes (Hansen *et al.* 2012, Cox *et al.* 2014, Ward *et al.* 2019). In laboratory-bred rats early-life disruption in the gut microbiome led to visceral sensitivity and pain in adulthood (O'Mahony *et al.* 2014). In laboratory-bred mice similar early-life disruption led to altered regulation of lipid metabolism, increased adipose tissue and higher risk of metabolic syndrome later in life (Cho *et al*. 2012), and similar results have been found in human studies (Turnbaugh *et al.* 2009, Vrieze *et al.* 2013, Azad *et al.* 2014).

Similar results have been gotten from wild gut microbiome studies. In Cuban tree frog tadpoles (*Osteopilus septentrionalis*) early-life disruption in gut and skin microbiome development led to a higher parasite infestation later in life and thus, reflected on host phenotype. Tadpoles that did not grow in natural pond water but in water that was sterile had decreased gut and skin microbiome diversity and higher numbers of parasite infections in adulthood (Knutie *et al.* 2017). The transplantation of wild mice gut microbiomes to germ-free mice led to significant changes in food foraging behavior and intestinal morphology: mice with a transplantation from a more herbivorous wild mice foraged for a higher protein-carbohydrate ratio diet and had larger intestinal morphology, a likely result from increased bacterial fermentation (Trevelline and Kohl 2022). Ultimately, these results emphasize the gut microbiome's influence on host phenotypes and provide evidence for the gut microbiome's role in wild animal evolutionary biology.

1.4 The avian gut microbiome

1.4.1 Life-history traits shape avian gut microbiomes

The gut microbiome's importance in individual ecology and physiology has been acknowledged, but previous research has mostly focused on humans, laboratory model species such as fruit flies and economically important species (Pascoe *et al.* 2017, Bodawatta *et al.* 2022a, Sun *et al.* 2022). Because wild animals inhabit environments in which they interact intra- and interspecifically throughout their lives and thus, are exposed to a wide range of environmental variation, laboratory results cannot be generalized to wild species (Hird 2017, Grond *et al.* 2018). One class of taxa that is slowly gaining research interest is birds and particularly, wild birds. Currently, most bird gut microbiome studies have been conducted with broiler chickens. Because broiler chickens have been bred for commercial purposes and food production, and are not adapted to natural (i.e., wild) environments, these broiler chicken studies cannot be generalized to wild birds (Grond *et al.* 2018, Sun *et al.* 2022). Moreover, differences between captive and wild birds have been observed in several studies highlighting the need for wild bird studies (Wienemann *et al.* 2011, Salgado-Flores *et al*. 2019, Oliveira *et al.* 2020, Florkowski *et al.* 2023). For example, wild and captive Eurasian capercaillies (*Tetrao urogallus*) differ in both gut microbiome diversity and composition. The

captive capercaillies had distinctly lowered gut microbiome diversity and their gut microbiome composition lacked bacterial taxa that are important in energy metabolism (Wienemann *et al.* 2011). Recent years have seen an increase in gut microbiome research on wild bird species and thus, the understanding of wild avian gut microbiomes is slowly increasing (Grond *et al.* 2018, Woodhams *et al.* 2020).

Birds are a widespread taxon inhabiting every continent. Birds exhibit a vast diversity of species that are adapted to a wide range of environments and lifestyles from solely nectar eating hummingbirds (*Trochilidae*) to the widespread corvids (*Corvidae*) and the flightless kiwi (*Apteryx*). Bird life-history traits are very different when compared to e.g., humans and other mammals both of which have been used widely in gut microbiome research. Birds reproduce by laying eggs, which is different to the vaginal birth of mammals. The eggshell protects the developing embryo from bacterial inoculation prior hatching and the initial bacterial inoculation is largely defined by the hatching environment (Kohl 2012, Grond *et al.* 2019, Ran *et al.* 2021). However, there is an ongoing debate on whether the embryo inside the eggshell is a truly sterile environment and whether there is maternal bacterial inoculation from mother to offspring already during development (Funkhouser and Bordenstein 2013, Trevelline *et al.* 2018, Těšický *et al.* 2024). The bird gut microbiome is established during the nestling stage, and it stabilizes before fledging (Teyssier *et al.* 2018a).

Most birds are capable of powered flight, which likely resulted in shortened gut retention times and increased paracellular absorption. As the energy requirement of powered flight increases with weight, this has led to selection for lower intestinal volume and efficient gut absorption (Caviedes-Vidal *et al.* 2007). This adaptation has been observed in other flying vertebrates as well (Song *et al.* 2020). Additionally, many bird species migrate biannually between breeding and wintering grounds (Alerstam 2003, 2011). Migration often requires long-distance flying, which may require phenotypic adaptation to increase flying performance such as changes to metabolism and atrophication of the GI tract (Piersma 1998, McWilliams and Karasov 2001, Karasov *et al.* 2004). These adaptations would also include the gut microbiome as it is heavily involved in host metabolism. Indeed, a study with migratory shorebirds indicated that shifts in the gut microbiome enable rapid weight gain via fat deposition (Grond *et al.* 2023). Moreover, a lower gut microbiome diversity was found in migrating thrushes (*Catharus* sp.) when compared to their breeding counterparts (Skeen *et al.* 2023). Migratory behavior such as variation in the timing of migration can also reflect to within species variation in gut microbiome, likely a result of variation in body condition and pre-fueling and migratory stopover sites (Thie *et al.* 2022).

1.4.2 Avian gut microbiomes and the environment

The bird gut microbiome varies among species, but some core bacterial taxa can be identified. The phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* dominate the bird gut microbiome (Hird *et al.* 2015, Grond *et al.* 2018). Each phyla includes both beneficial and pathogenic bacterial taxa and some taxa are known

from their commercial use as probiotics (such as the genus *Bifidobacterium*) (Mountzouris *et al.* 2007, Abdel-Moneim *et al.* 2020). The phylum *Firmicutes* includes both beneficial and pathogenic bacterial classes such as *Bacilli* and *Clostridia* (Benskin *et al.* 2009). *Firmicutes* also degrade polysaccharides and are the producers of short-chain fatty acids, which are important molecules in energy metabolism and immune system functioning (Flint *et al.* 2008, Tap *et al.* 2009). However, the functions of many bacterial taxa in the bird gut microbiome are still largely unknown (Grond *et al.* 2018).

Whereas mammal gut microbiomes are largely influenced by phylogeny (i.e., phylosymbiosis), most wild bird gut microbiomes are strongly influenced by extrinsic factors (Grond *et al.* 2018, Bodawatta *et al.* 2022a). Especially the gut microbiome of passerine birds shows large variation across species and is more determined by the environment when compared to non-avian taxa (Hird *et al.* 2014). Previous studies with wild birds have found that variation in the bird gut microbiome associates with season (Góngora *et al.* 2021, Dietz *et al.* 2022), habitat (Teyssier *et al.* 2018b, Loo *et al.* 2019, Berlow *et al.* 2021, Drobniak *et al.* 2022), and diet (Teyssier *et al.* 2020, Góngora *et al.* 2021, Bodawatta *et al.* 2021, 2022b). Extrinsic (and intrinsic) factors are usually intertwined, which means that a factor such as diet is influenced by season or habitat (Góngora *et al.* 2021, Schmiedová *et al.* 2022). For example, Teyssier *et al.* (2020) investigated the impact of diet and habitat on the bird gut microbiome with an experimental cross-feeding study. The results showed that the type of diet (defined by rural and urban environment) influenced the gut microbiome (Teyssier *et al.* 2020). Furthermore, seasonally varying factors such as temperature and precipitation are known to influence changes in poultry; heat stress resulted in significant changes in egg-laying hen gut microbiome composition and the hens' metabolic activity (Zhu *et al.* 2019), and precipitation associated with gut microbiome composition in the scavenging indigenous chicken (Glendinning *et al*. 2024). Because previous knowledge has shown that the avian gut microbiome is heavily influenced by the environment (Song *et al*. 2020, Trevelline *et al*. 2020, Baiz *et al*. 2023), it is important to investigate the environmental drivers of the gut microbiome. As wild birds inhabit natural environments in which they are constantly interacting with other avian and non-avian taxa, it would be beneficial to study multiple explanatory factors when aiming to explain the causes of variation in wild bird gut microbiomes. Also, large scale studies including sampling multiple populations at a wide biogeographical range are needed (and currently nonexistent) because a single bird species can inhabit a wide biogeographical range.

In this PhD thesis, it was investigated whether there are associations between multiple extrinsic and intrinsic factors and the gut microbiome variation in a small wild passerine bird. Birds of the same species were fecal sampled at multiple populations across a large biogeographical range, which enabled to study the possible drivers of seasonal and population level variation in the gut microbiome (I). Overall, it was tested whether there are associations between the gut microbiome variation and factors that are measured at seasonal and population level. By using brood size manipulation and cross-fostering it was possible to determine whether early-life environment associates with gut

microbiome variation and whether this variation may reflect to individual survival later in life (II).

1.4.3 Have the guts to perform better? Gut microbiome connects to individual performance

As the gut microbiome is tightly connected to individual physiology via digestion, immune system, and metabolism, it likely contributes to individual survival as well (Rosshart *et al.* 2017, Sharpton 2018). The gut microbiome's connection with individual survival has been investigated with species such as the water flea (*Daphnia magna*) (Houwenhuyse *et al.* 2021), laboratory-bred mice (Beli *et al.* 2018), and humans (Sims *et al.* 2021). Results from these studies indicate a correlation between the gut microbiome and survival; higher gut microbiome diversity correlated with higher individual survival and survived vs. non-survived individuals had differences in their gut microbiome composition. Similar results have been received from broiler chicken studies in which reduced microbial diversity and changes in gut microbiome composition negatively influenced chicken performance and survival (Le Roy *et al.* 2019, Liang *et al*. 2023). Some studies have also identified specific phyla that associate with bird performance. For example, the phylum *Actinobacteria* hosts the genera *Corynebacterium* and *Mycobacterium*, both of which are known to negatively influence bird health and thus, performance (Potti *et al.* 2002, Witte *et al.* 2010). Curiously though, *Corynebacterium* is also associated with migratory birds as it may enhance fat deposition and immune system functioning during migration (Risely *et al.* 2017, Zhang *et al.* 2021).

However, there are very few studies that investigate whether the gut microbiome associates with survival in wild birds. In wild nestling great tits (*Parus major*), there is some evidence for the association between the gut microbiome and nestling survival (Davidson *et al.* 2021) but the potential causes of this association remain to be studied. In the adult Seychelles' warbler (*Acrocephalus sechellensis*) gut microbiome composition associated with survival to the following breeding season and a higher abundance of pathogenic bacteria correlated with lower survival (Worsley *et al.* 2021). This remains the only study to investigate the association between survival and the gut microbiome in a wild bird species until II and III.

Individual survival is a key determinant of lifetime reproductive success (hereafter, LRS) together with annual reproductive success (hereafter, ARS). Individuals that live longer than their counterparts may have more opportunities to reproduce and thus, may have higher LRS (Murray 2000). Factors contributing to LRS have been studied extensively with birds (e.g, Verhulst *et al.* 1995, Grant and Grant 2000, Jensen *et al.* 2004, Hawn *et al.* 2007, Costanzo *et al.* 2017), but the gut microbiome's association with LRS has not been investigated with any wild species to date. It is likely that variation in the gut microbiome is connected to reproductive success because the gut microbiome regulates reproductive hormone levels (such as estrogen, progesterone, and testosterone) (Hussain *et al.* 2021). Also, a disruption in the gut microbiome such as decreased diversity and

a decrease in *Firmicutes* abundance can influence reproductive success via energy metabolism (Flint *et al.* 2008, Tap *et al.* 2009). Reproduction is energetically expensive and decreased gut microbiome diversity and changes in composition may result in lowered energy metabolism and possible energy deficiency in the host. This energy deficiency can lead to lower chances of reproductive success (Ben-Yosef *et al.* 2008, Morimoto *et al.* 2017).

Studies with captive bred mammals have found that the gut microbiome varies across the breeding season and that this variation associates with reproductive hormone concentrations (Antwis *et al.* 2019, Burnham *et al.* 2023). In broiler breeders, gut microbiome transplantation increased both gut microbiome diversity and egg-laying rate and was connected to increased hormone secretion and ovarian function (Cao *et al.* 2023). In wild yellow-legged gulls (*Larus michahellis*) glucocorticoid hormone levels associated with gut microbiome composition and influences the abundances of both pathogenic and beneficial taxa (Noguera *et al.* 2018). Glucocorticoid hormone levels increase in challenging and stressful situations and can reduce reproductive success (Vitousek *et al.* 2018). Moreover, the breeding status (fertile vs. sterile) of the crested ibis (*Nipponia nippon*) correlated with the gut microbiome; the phyla *Proteobacteria* was significantly higher in sterile than fertile ibises and could potentially indicate problems in reproduction in the crested ibis (Ran *et al.* 2021). These studies suggest that there is an association between variation in the gut microbiome and reproductive success in birds as well and therefore, merit further investigation. It is vital to understand whether the gut microbiome associates with individual LRS because it determines what traits (such as the gut microbiome) are passed on to the next generation (selection).

In this PhD thesis, the associations between gut microbiome variation and reproductive success in adult birds were investigated (III). Long-term monitoring data was used to investigate whether the gut microbiome variation associates with individual survival and estimated LRS, which is the core of Darwinian fitness.

1.5 Study systems

1.5.1 The great tit

The great tit, a well-known ecological model species, is a small passerine bird that nests in different kinds of habitats and uses both natural and artificial cavities as nests. It has a broad distribution range, and it inhabits vast areas around Europe, northern Africa, and parts of Asia. The great tit does partial migrations e.g., when searching for food and new territories, but it does not migrate seasonally (Krebs 1971, Kvist *et al.* 1999). Because the great tit inhabits a vast biogeographical area, it is subjected to a wide range of environmental conditions (Gosler 1993). The great tit is an omnivorous species and uses a wide variety of food items from seeds and nuts to insects and in particular,

lepidopteran larvae (Krebs *et al.* 1977). It lays 6-12 eggs on average in its nest cup and the large variation in clutch size makes it a suitable model for brood size manipulation experiments. The nest is lined with mostly plant material such as mosses, lichen, and animal hair and feathers (Perrins 1979, Alabrudzińska *et al.* 2003, Mainwaring *et al.* 2012, Deeming and Mainwaring 2015). The great tit can adjust its clutch size based on food availability: larger broods require more feeding from the parents (Sanz 1999). The timing of breeding varies based on the geographic location. For example, great tits inhabiting Central Europe breed earlier than their more northern counterparts (Rytkönen and Orell 2001). Because the great tit successfully nests in artificial cavities as well, it breeds in human provided nest boxes and therefore, can be monitored and studied in the wild.

The great tit has been used as a model species in some gut microbiome studies. The gut microbiome of great tit nestlings develops rapidly between days 8 and 15 post-hatch and is shaped by the rearing environment (Teyssier *et al.* 2018a). Also, tree species diversity, forest fragmentation and distance from the edge of the forest have also been shown to contribute to nestling gut microbiome thus, highlighting the significance of the environment on nestling gut microbiome (Goossens *et al.* 2022, Somers *et al.* 2023). Similar environmental effect was found in juvenile great tits inhabiting nest boxes in urban and rural environments: higher gut microbiome diversity was observed in birds that lived in environments with higher tree cover density and more heterogenous environment (Maraci *et al.* 2022b). Moreover, the great tit gut microbiome can change because of a new diet (Bodawatta *et al.* 2021) and these changes may reflect to individual behavior in food item selection (Davidson *et al.* 2020). However, it is not known whether differences in early-life environment result in variation in the gut microbiome and whether these reflect to nestling survival to fledging (a proxy for short-term survival) or juvenile survival (II). Moreover, large scale studies investigating the gut microbiomes of populations spread across a wide biogeographical range are also lacking (I).

1.5.2 The collared flycatcher

The collared flycatcher (*Ficedula albicollis*) is a known model species in the fields of ecology and genetics (Ellegren *et al.* 2012). It is a small migratory passerine bird, and it breeds in parts of Europe and overwinters in central Africa. It nests in nest boxes and thus, breeding birds can be monitored throughout the breeding season. It lays an average of 5-7 eggs in a nest that is made of twigs, leaves, and grass. The male birds arrive to their breeding grounds approximately one week before the females in the spring. The males occupy one or more breeding territories, and the females will select the males to breed with. Collared flycatchers are socially monogamous, but extra pair paternity (hereafter, EPP) is common: the socially monogamous male mates with the female in his "own" nest and with extra females. This can result in higher numbers of offspring per male and thus, increase the number of nestlings per male (Rätti *et al.* 1995, Sheldon and Ellegren 1999). Collared flycatchers have a high return rate to its breeding grounds, and it is known for its high site fidelity, which means that it is possible

to get a good estimate of individual survival and lifetime reproductive success (Gustafsson 1985).

The gut microbiome of collared flycatcher has not been studied previously and this is likely the first time the gut microbiome of the collared flycatcher has been characterized. Of the same genus *Ficedula*, the pied flycatcher (*Ficedula hypoleuca*) cloacal, gut, skin and feather microbiomes have been characterized. The adult female pied flycatcher skin and cloacal gut microbiomes resembled their nest environment suggesting that the environment shapes their microbiomes (Goodenough *et al.* 2017). In nestling pied flycatchers, the presence of opportunistic pathogens such as *Enterococcus faecalis* and *E. faecium* in the nestling gut has been linked to growth and development (Moreno *et al.* 2003, González-Braojos *et al.* 2012). Most recently, it was found that the core gut microbiome of five sympatric flycatchers, the roufous-gorgeted flycatcher (*F. strophiata*), the Daurian redstart (*Phoenicurus auroreus*), the rufous-bellied niltava (*Niltava sundara*), the blue-fronted redstart (*P. frontalis)* and the Himalayan bluetail (*Tarsiger rufilatus*) consists of the phyla *Proteobacteria*, *Firmicutes*, *Actinomycetes* and *Bacteroidetes* (Dong *et al.* 2022), which are some of the most common phyla in the gut microbiomes of many insectivorous small passerines (as reviewed in Grond *et al.* 2018, Bodawatta *et al.* 2022). Some wild studies have investigated whether the gut microbiome associates with individual survival, but none have studied whether there is a connection between the gut microbiome and LRS in a wild bird species (III).

1.6 Aims and scope of the thesis

The aim of this PhD thesis is to increase understanding of how the environment associates with variation in wild bird gut microbiomes and whether this variation in the gut microbiome associates with individual survival and fitness. Research investigating the avian gut microbiome has only recently gained growing interest, but still a large majority of the studies focusing on avian gut microbiomes are done with poultry or captive-bred species (as reviewed in Grond *et al.* 2018, Bodawatta *et al.* 2022, Sun *et al.* 2022). Wild avian gut microbiomes are often studied with limited datasets covering one or few populations (such as Phillips *et al.* 2018, Gadau *et al.* 2019, Berlow *et al.* 2021, Drobniak *et al*. 2022). Also, bird studies in which the causes of the gut microbiome diversity and composition are investigated using experimental biology are still limited (Teyssier *et al.* 2018a, Bodawatta *et al.* 2021, Diez-Méndez *et al.* 2023). This PhD thesis investigates the associations between gut microbiome variation and the environment (I, II, III) and gut microbiome variation and individual performance and fitness (II, III) in wild small passerine birds (Fig. 2). In this PhD thesis, the following questions are addressed:

1. Does environmental variation drive differences in the gut microbiome of wild bird populations (I)?
2. Are early-life environment and survival associated with the gut microbiome in a wild bird (II)?
3. Are there associations between the gut microbiome (trait) and reproductive success in a wild bird (III)?

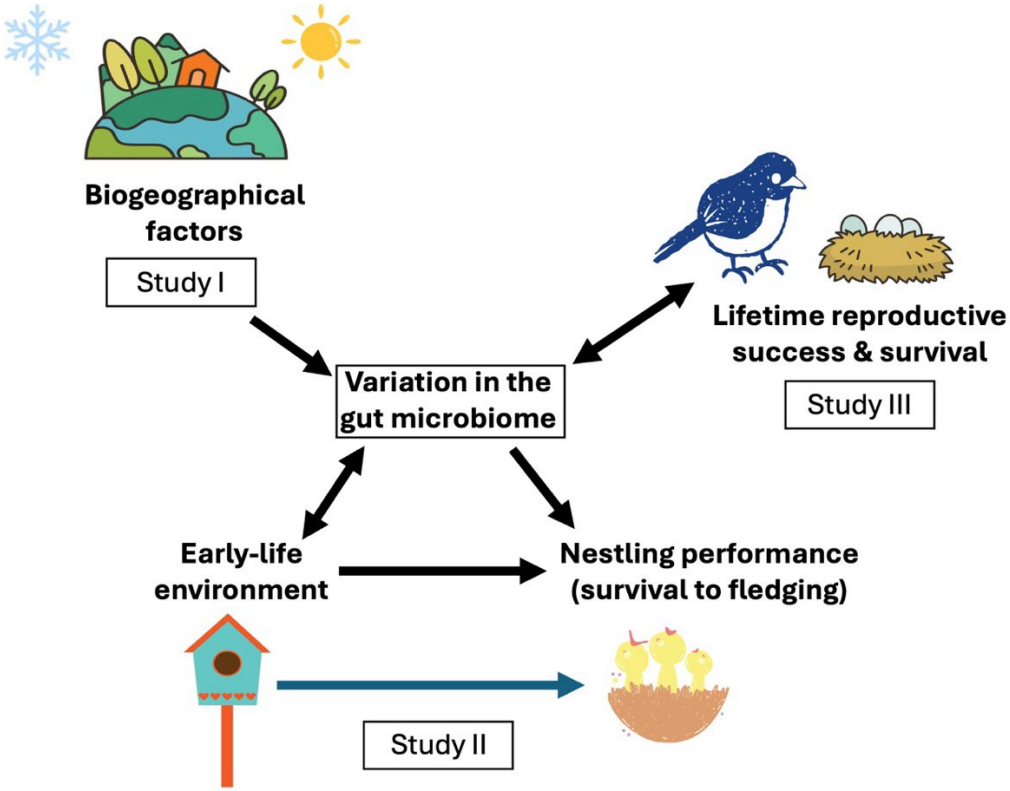

FIGURE 2 A diagram of the three studies and core questions of the PhD thesis. All studies investigate the causes of gut microbiome variation in wild passerine birds. II and III also investigate the consequences of gut microbiome variation on individual performance and fitness.

First, it was predicted that both population and season would associate with gut microbiome variation (I). Populations inhabiting different latitudes are subject to varying abiotic environmental conditions such as differences in snow coverage, rainfall, temperature, and variation in diet (Anderson and Jetz 2005, Williams *et al.* 2015). Therefore, it was predicted that the more southern populations would have higher gut microbiome diversity and to be significantly different from the more northern ones. It was expected that there would be higher gut microbiome diversity during summer than winter due to increased time for foraging and food abundance and diversity (Karr 1976, Cody 1981). It was also predicted that individual and population level factors (latitude, habitat, average temperature, and rainfall) would associate with gut microbiome variation. Second, it was predicted that early-life environment would associate with nestling gut microbiome variation and survival (II). More specifically, the expectation was

that the nestling gut microbiome would be more diverse in smaller broods because there may be more available food for smaller broods and less sibling competition (Leonard *et al.* 2000, Nicolaus *et al.* 2009). Also, it was expected that early-life environment and particularly higher gut microbiome diversity at day 7 post-hatch would associate with higher survival to fledging (i.e., short-term survival) and apparent juvenile survival. Higher gut microbiome diversity is often associated with increased individual health as it increases the stability of the gut (Willing *et al.* 2010, Lozupone *et al.* 2012). Third, it was predicted that gut microbiome diversity would positively correlate with LRS, ARS and survival to the following breeding season (III). Moreover, it was predicted that there would be significant differences in gut microbiome composition based on LRS, ARS and survival to the following breeding season. Differences in the gut microbiome can result in sex-specific effects in reproduction (Morimoto *et al.* 2017) and survival to the following breeding season (Worsley *et al.* 2021). Additionally, some gut bacterial taxa have been found to correlate with increased reproductive success and higher levels of reproductive hormones (Antwis *et al.* 2019).

2 METHODS

2.1 Field work and sample collection

2.1.1 Europe-wide field work and sampling of study I

A total of eight great tit populations located at different parts of Europe were fecal sampled during winter and summer of 2021. First, wild adult great tits of six different populations were sampled during January and February 2021 in Oulu (Finland), Jyväskylä (Finland), Turku (Finland), Tartu (Estonia), Lund (Sweden) and Pilis-Visegrád Mountains (Hungary). Second, wild adult great tits at eight different breeding populations were sampled between May and July 2021 in Oulu, Jyväskylä, Turku, Tartu, Lund, Westerheide (Netherlands), Pilis-Visegrád Mountains, and La Hiruela (Spain) (Fig. 3). During winter, birds were caught near supplementary feeding stations with mist nets. Supplementary feeding was used because catching great tits is nearly impossible without supplementary feeding during winter. During summer, adult birds were caught from their nest boxes as they were breeding birds. Each bird was ringed with an aluminium band for identification. Due to difficult winter conditions (deep snow coverage and colder temperatures than expected) and resulting ecological reasons in 2021, there are no winter samples from Westerheide and La Hiruela populations.

Fecal samples were used in this study and the other studies of this PhD to represent the gut microbiome (here, the colon part of the GI tract). Fecal samples are considered the best available non-invasive way to get a good representation of the bacteria within the colon. Currently, there are no non-invasive ways to get a proper representation of the caecum and ileum, which are the upper parts of the bird GI tract (Videvall *et al.* 2018). To sample the upper parts of the GI tract, it would require euthanizing the birds (Grond *et al.* 2018). To collect fecal samples, each bird was placed in a lined paper bag until defecation (i.e., for

approximately 5-10 minutes). To avoid contamination and destruction of the sample, a rubber covered metal wire grid was placed on top of the sterilized plastic bottom liner following the method by Knutie and Gotanda (Knutie and Gotanda 2018). After defecation, each bird was sexed, weighed and their wing-length was measured. At the end, each bird was released. A total of 285 birds were sampled (population specific sample numbers are indicated in brackets in Fig. 3).

Data of biogeographic and environmental variables was collected onsite (latitude, habitat type, supplementary feeding) and post sample collection (average temperature (°C) and rainfall (mm)). Latitude was population specific and both average temperature and rainfall were sample specific. Average temperature and rainfall were recorded for each bird and was based on daily records from 2 weeks prior to sampling. Habitat types were population specific and categorized as 1) mixed coniferous and deciduous forests and 2) deciduous forests. Mixed coniferous and deciduous forests were dominated by a mix of pine (*Pinus* sp.), spruce (*Picea* sp.) and evergreen deciduous trees such as aspen (*Populus* sp), alder (*Alnus* sp.), willows (*Salix* sp.) and shrubs. Deciduous forests were dominated by the before listed evergreen deciduous trees and shrubs. Populations inhabiting mixed coniferous and deciduous forests were Jyväskylä, Turku, Tartu, Westerheide and La Hiruela. Populations inhabiting deciduous forests were Lund and Pilis-Visegrád Mountains. Samples from the Oulu population were collected from birds inhabiting a deciduous habitat during winter and summer samples from birds inhabiting a mixed coniferous and deciduous habitat because of sampling logistics. These two sampling sites where approximately 4 kilometres apart. As great tits can disperse multiple kilometres between their winter and breeding grounds especially in northern Finland (Orell 1989, Kvist *et al.* 1999), it is likely that the great tits moved between sampling sites between summer and winter in this study as well. Supplementary feeding type was population specific, and the feed was either sunflower seeds or sunflower seeds and peanuts.

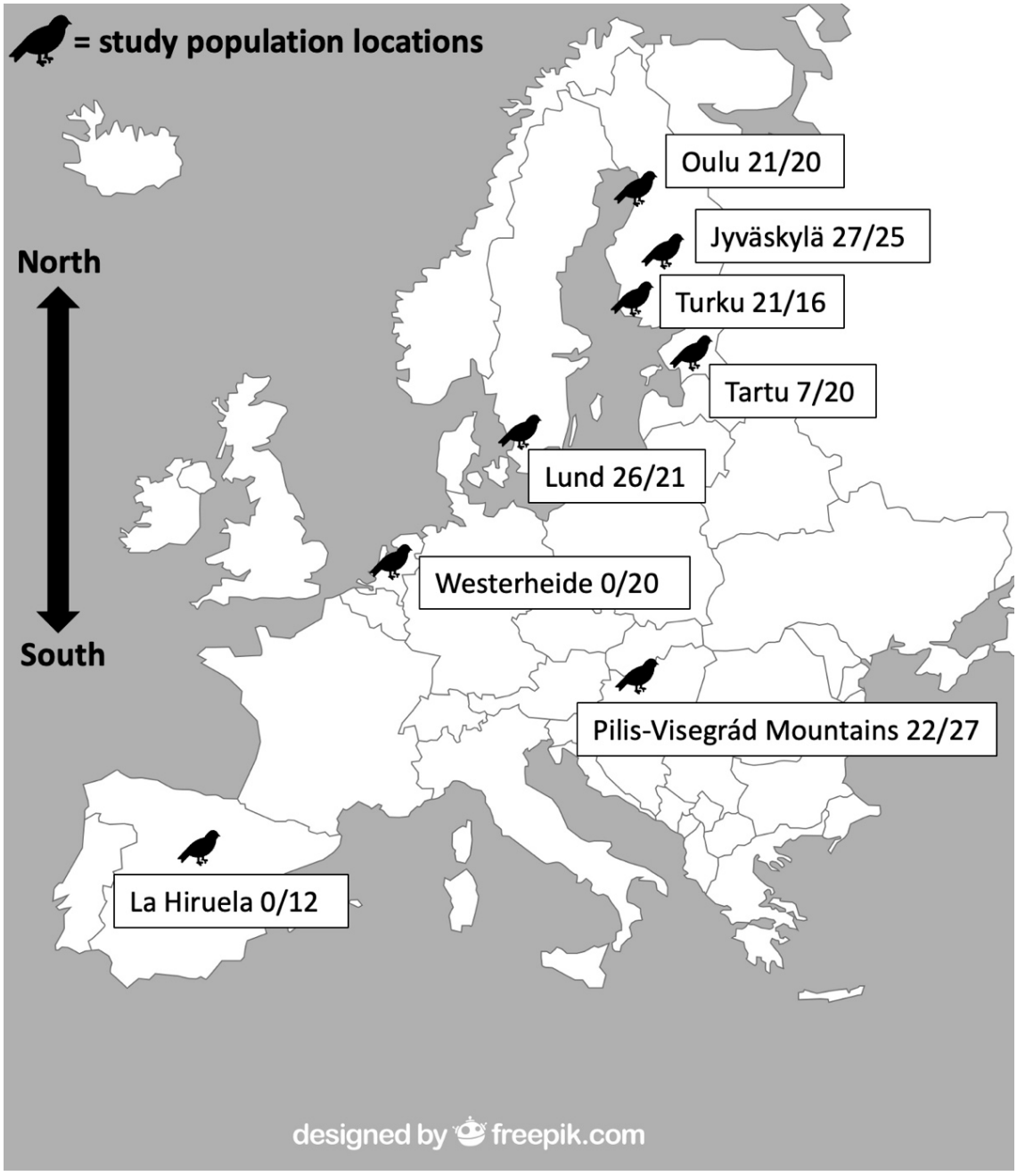

FIGURE 3 A map of the location for each great tit population (I). The numbers in brackets at each location indicate the number of samples from each population (winter/summer).

2.1.2 Experimental manipulation of great tit broods in study II

A brood size manipulation experiment was carried out in a nest box population of great tits in Ruissalo Turku (60°25′59.99″ N 22°09′60.00″ E) during the breeding season between May and July in 2020. The Ruissalo habitat is mostly temperate deciduous forest and meadows, and it has some small patches of coniferous trees. First, great tit broods were monitored weekly and then daily when clutches were close to their estimated hatching date. Second, brood sizes were manipulated two

days after hatching so that 2 nestlings were either added or removed from nests (Fig. 4). The decision to move +/- 2 nestlings was based on previous research in which similar manipulation has been used: changes in great tit brood size can lead to lowered weight in both nestlings and adults (Smith *et al.* 1987, Hõrak *et al.* 1999, Sanz 1999, Tinbergen and Verhulst 2000, Neuenschwander 2003). Great tit nestlings were moved between nest boxes so that there were four treatment groups. In the enlarged group, each brood was increased by two nestlings. In the reduced brood, each brood was decreased by two nestlings. There were also two different control groups: the control broods and the unmanipulated control broods. In the control brood, nestlings were moved between nests, but the original brood size remained the same. In the unmanipulated control brood, no nestlings were moved. This treatment group was used to control for any effects on the nestlings that may be caused by the moving of the nestlings. Potential bias caused by hatching date was avoided by allocating nests evenly to each treatment in any given day. Also, the initial brood size in each treatment group was checked so that all groups had an equal brood size on average: the aim was not to only reduce large broods and enlarge small broods. Nest pairs for reducing or enlarging brood size were selected based on similar hatching date. When there was an uneven number of nests hatching within a day, one or three nests were assigned to the unmanipulated control group. Before any moving of nestlings, nestlings were weighed with a digital scale with a precision of 0.1 g and identified by clipping selected toenails. Then in each nest, the added / removed nestlings were of similar weight to avoid effects that may result from changing the sibling hierarchy of the brood. All nestlings were moved as quickly as possible and transported in a warmed box to limit stressors caused by the moving.

Third, to study the possible effects of the early-life environment on nestling gut microbiome and its association to individual nestling body mass, survival to fledging and apparent juvenile survival, nestlings were fecal sampled seven days after hatching (Fig. 5). Two samples were collected from each nest so that one sample came from an original nestling and another sample from a foster nestling. Fecal samples were collected by gently stimulating the cloaca with the collection tube. Fecal samples were collected straight into sterile 1.5 ml Eppendorf tubes to avoid contamination. Each nestling was weighed (0.1 g) and ringed for individual identification using aluminium bands. Each treatment group had the following number of sampled nestlings: reduced group had 24 nestlings in 16 nests, enlarged group had 23 nestlings in 15 nests, control group had 23 nestlings in 15 nests, and unmanipulated control group had 22 nestlings in 13 nests. Finally, all samples were stored in cool bags onsite and then transported into a -80 °C freezer for storage until DNA extraction.

14 days after hatching, nestlings were weighed, and their wing-length was measured to detect any effects the manipulation may have had on nestling growth. All nests were monitored for fledging success, which was used as an indicator of short-term survival. Also, apparent juvenile survival was monitored approximately 3 months after fledging. Juvenile great tits were caught with mist nets at six different feeding stations in the brood size manipulation nest box areas. These feeding stations had a continuous supply of sunflower seeds and

suet blocks. Capturing was done on three separate days during October-November 2020 for three hours at a time with a total of 69 hours of mist netting. At the end 88 juveniles from the brood size manipulation experiment were caught. These juveniles were weighed, and their wing-length was measured. This catching method provides an estimate of post-fledging apparent juvenile survival, but it can be slightly biased by juvenile dispersal. However, wide dispersal is likely limited given previous results from the same study population (Cossin-Sevrin *et al.* 2022). Unfortunately, sequencing of these juvenile samples to characterize the gut microbiome failed and thus, it was not possible to measure whether there were associations between the gut microbiome of nestlings and the gut microbiome of juveniles.

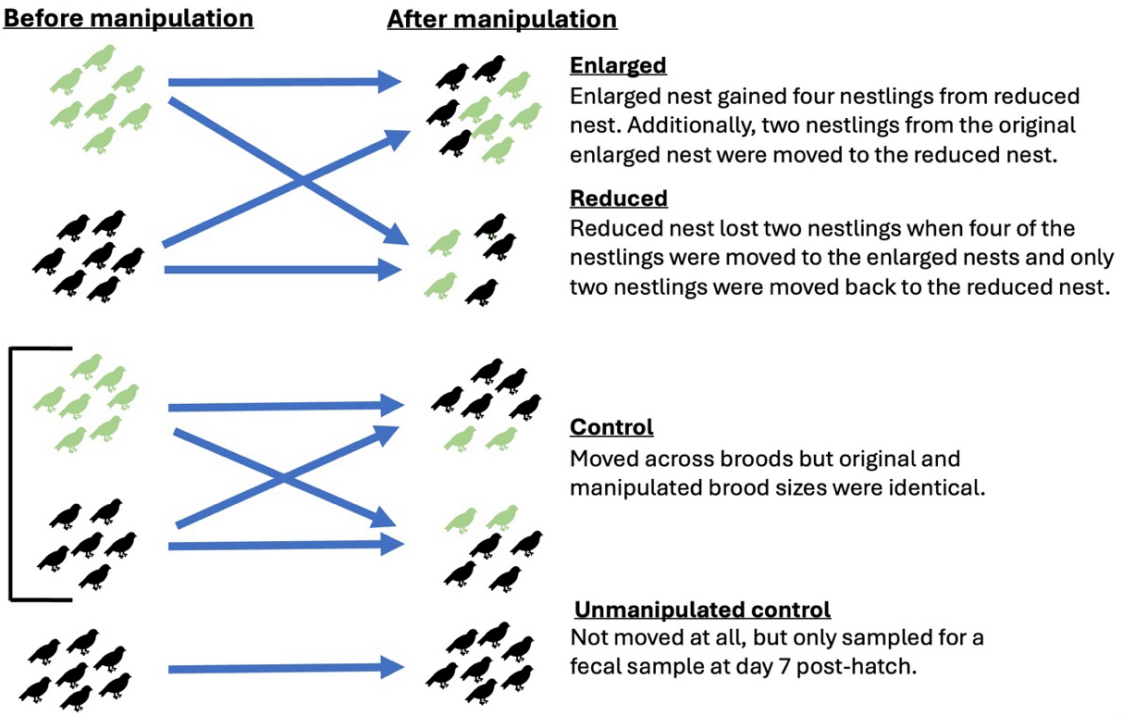

FIGURE 4 A diagram of the brood size manipulation experiment with each treatment group. 2 days after hatching nestlings were moved between nest boxes to either enlarge or reduce original brood size (an example with brood size of seven is given in the diagram). Some nests were control nests ("Control") in which nestlings were moved but brood size remained the same, and some were unmanipulated control nests ("Unmanipulated control") in which no nestlings were moved at all. The original brood size varied between nests. Diagram adapted from Liukkonen *et al.* 2023.

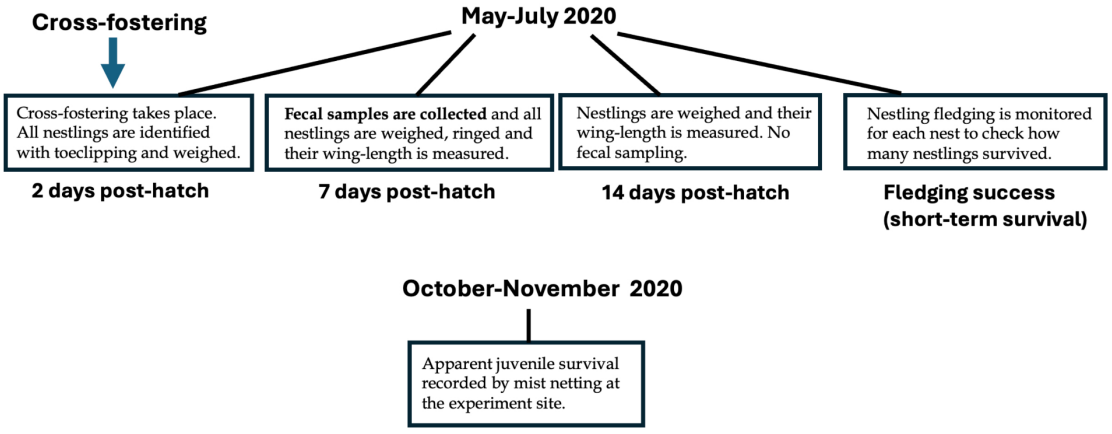

FIGURE 5 A diagram of the brood size manipulation experiment timeline.

2.1.3 Long-term monitoring data and field work of study III

Adult collared flycatchers were caught at 12 different locations on the island of Gotland Island, Sweden (57°03′ N, 18°17′ E) during the breeding season in the summer of 2015. Both female and male birds were caught at the nest boxes with nest box traps to collect fecal samples to represent the gut microbiome. Captured birds were placed inside a paper bag until defecation using the same method as described in I (Knutie and Gotanda 2018). Here, the birds were only fecal sampled once (in 2015). This one-year fecal sample is a proxy of lifetime differences in the gut microbiome across all individuals. After defecation, the birds with no previous identification rings were ringed. Most birds used in this study had been ringed previously on Gotland Island, which has been used as a collared flycatcher study site since the 1970's. All birds were aged based on ringing data, sexed and they were weighed, and their wing and tarsus lengths were measured. Body condition for each bird was calculated based on the residuals of body mass on tarsus length (as done in e.g., Hemborg and Lundberg 1998, Potti 1999, Rosivall *et al.* 2009). A total of 185 birds (females = 122, males = 63) were sampled. The number of nestlings and successful fledging of these nestlings was recorded for each of the adult collared flycatchers that were fecal sampled to measure ARS. Then, this collected data was combined with previous and following years' fledging success details to estimate LRS (as done in Bouwhuis *et al.* 2015, Wysocki *et al.* 2019). Survival to the following breeding season was estimated based on whether the birds that were sampled in 2015 were caught after 2015 on Gotland Island. Collared flycatchers have high site fidelity, and it is common that collared flycatchers that were born on Gotland Island return to breed on Gotland Island (Gustafsson 1985, 1986). Therefore, it is possible to estimate LRS and survival to the following breeding season for these collared flycatchers.

2.2 DNA extraction and sequencing of the 16S rRNA gene

Each of the three studies followed a similar protocol in fecal sample processing and bioinformatics. A more detailed description of study-specific analyses can be found in the two manuscripts and in Liukkonen *et al.* (2023). First, DNA was extracted from each fecal sample with Qiagen QIAamp PowerFecal Pro DNA kit (Qiagen; Germany) following the manufacturer's protocols. In I and III, an incubation in 65 °C for 10 minutes was used prior to lysis step but not in II. Each extraction patch included a negative (RNAse and DNAse free ddH_2O) control to control for contamination during extraction. Second, the extracted DNA samples were amplified with polymerase chain reactions (PCR) in which the hypervariable V4 region in the 16S rRNA gene was targeted. The following primers were used:

- 515F_Parada (5'-GTGYCAGCMGCCGCGGTAA-3') (Parada *et al.* 2016)
- 806R_Apprill (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill *et al.* 2015).

Each PCR in all three studies followed the same PCR protocol (Fig. 6):

PCR 1	PCR 2
1. Initial denaturation at 95 °C for 3 minutes 2. 30 cycles of: 1. 95 °C for 45 seconds 2. 55 °C for 60 seconds 3. 72 °C for 90 seconds 3. 10-minute extension at 72 °C	1. Initial denaturation at 95 °C for 4 minutes 2. 18 cycles of 1. 98 °C for 20 seconds 2. 60 °C for 15 seconds 3. 72 °C for 30 seconds 3. 3-minute extension at 72 °C

FIGURE 6 The two PCR protocols that were used to target the V4 region of the 16S rRNA gene and to attach Illumina barcodes for sample identification.

Third, the PCR products were measured for DNA concentration (Quant-IT PicoGreen dsDNA Assay Kit, ThermoFicher Scientific; Waltham, MA, USA) and quality was checked (TapeStation 4200, Agilent; Santa Clara, CA, USA). Finally, the PCR products were pooled and purified and sequenced with Illumina (Illumina, San Diego, CA, USA) Miseq platform (II) and Illumina Novaseq 6000 platform (I, III).

Raw sequences were processed with the platform provided by CSC – IT Centre for Science (Fig. 7). Raw sequences were quality checked and trimmed,

and then they were assigned for taxonomy. The sequence data was processed using the DADA2 pipeline (Callahan *et al.* 2016). In II, DADA2 pipeline was run with R studio version 4.11.0 (R Core Team). In I and III, QIIME2 (Bolyen *et al.* 2018) with the DADA2 plugin was used to process the sequences. The resulting sequence data was imported into R studio for downstream analyses.

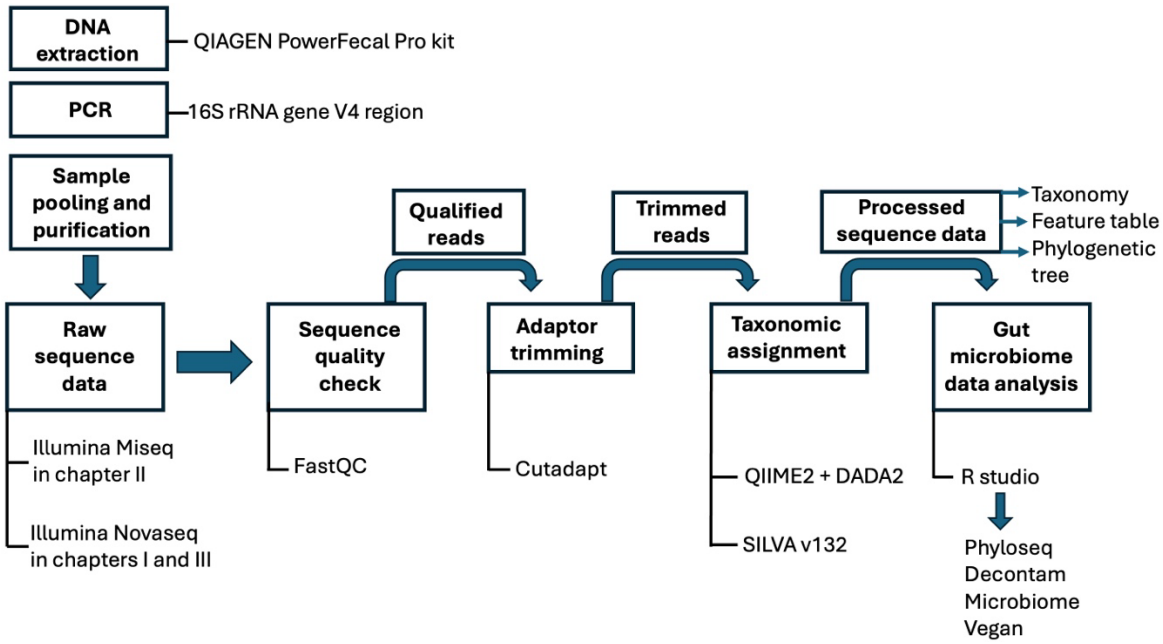

FIGURE 7 A schematic diagram describing the fecal sample processing from DNA extraction to the beginning of downstream analysis.

2.3 Downstream analyses of gut microbiome data

2.3.1 A short introduction to key gut microbiome metrics

At the core of gut microbiome measurements are two terms that measure variation in the gut microbiome: alpha diversity and beta diversity (i.e., composition). Alpha diversity represents the within-sample diversity, and it can be measured with different metrics. For example, observed richness measures the number of unique Amplicon Sequence Variants (hereafter, ASVs) that are found in each sample (i.e., bacterial richness). Each ASV represents a unique (bacterial) taxon. In this PhD thesis, Shannon Diversity Index (hereafter, Shannon) and Chao1 Richness (Chao, 2006; hereafter, Chao1) were used as the core alpha diversity metrics. Shannon considers both the number of observed ASVs and their evenness within the sample, which makes it more robust in case the sample has a lot of rare taxa. Chao1 is an estimation of the observed number of taxa, and it is more sensitive to rare taxa.

Beta diversity measures the similarity or dissimilarity of the gut microbiome between samples, which means that it measures between-sample diversity. For example, beta diversity measures sample dissimilarity i.e., the difference in microbial composition among all samples with the value of 0 representing a complete similarity in composition and the value of 1 representing complete dissimilarity. As with alpha diversity, beta diversity can also be measured with different metrics such as the Bray-Curtis dissimilarity (Bray and Curtis 1957), Unweighted Unique Fraction (hereafter, UniFrac) and weighted UniFrac (Lozupone and Knight 2005) metrics. The Bray-Curtis dissimilarity considers the relative abundance of bacterial communities between samples, which makes it robust to rare taxa and to homogeneity assumption violations (Anderson and Walsh 2013, Schroeder and Jenkins 2018). The Weighted UniFrac metric considers both phylogenetic information and the relative abundance of taxa, whereas the Unweighted UniFrac considers the presence and absence of taxa. In this PhD thesis, the aim was to disentangle the potential causes and consequences of this variation in the alpha and beta diversities. For example, the potential causes of gut microbiome variation such as early-life environment, habitat, season, and population were investigated in I, II and III. In II and III, the potential consequences of this gut microbiome variation such as associations between the gut microbiome and reproductive success and individual survival were investigated. In this PhD thesis the terms "gut microbiome diversity" is used for alpha diversity and "gut microbiome composition" for beta diversity.

2.3.2 General analyses

After the initial sequence processing was completed (see sub-chapter 2.2), the resulting files were imported into R studio (version 4.3.1 and 4.3.3; R Core Team) and combined into a phyloseq object with the *phyloseq* package (version 1.44.0; McMurdie and Holmes 2013). First, possible contaminants and non-bacterial sequences were removed from the dataset using the *decontam* package (version 1.12; Davis *et al*. 2018). Second, after decontamination the phyloseq object was rarefied to control for variation in sequencing depth between samples (McMurdie and Holmes 2014, Schloss 2024). Third, alpha and beta diversity metrics were calculated. Alpha diversity metric (Shannon and Chao1) was used in statistical models (see statistical analyses specific to each study) to measure how it associates with chosen variables. For each model, Variance Inflation Factors were tested to assess the multicollinearity of the predicting variables with the package *DHARMa* (version 0.4.6; Hartig and Hartig 2017). The chosen predicting variables (e.g., habitat or brood size) were tested with Permutational Multivariate Analysis of Variance (PERMANOVA with 9999 permutations) by using the package *vegan* (version 2.6-4; Oksanen *et al.* 2013). This PERMANOVA result showed how much of the variation in beta diversity was explained (R^2 value, $p < 0.05$) by the chosen predicting variables and how much was left unexplained (residual variance). Finally, a differential abundance analysis (DESeq2) was used to check for differentially abundant bacterial taxa between

groups with the package *DESeq2* (version 1.42.1; Love *et al.* 2014). All downstream analyses were performed in R studio.

2.3.3 Statistical analyses specific to study I

To study how population and season and select biogeographical factors defined by population and season associate with the variation in the gut microbiome, linear mixed effects models with the R package *lme4* (version 1.1-35.3; Bates *et al.* 2015) were used. First, alpha diversity was set as the response variable and population and season as the predicting variables to measure whether these two variables associate with gut microbiome alpha diversity. Second, it was measured how select biogeographical factors (latitude, habitat, average temperature, average rainfall, supplementary feed during winter) associate with gut microbiome alpha diversity. These models were first run for all populations across both winter and summer and then specifically for each season to see specific within-season associations. In these models, population was used as a random effect to control for multiple sampling within a population. Each model was first run with the Shannon metric as response variable and then with the Chao1 metric. The output of each of these models resulted in a list of factors that associate with alpha diversity. To measure which factors contributed to beta diversity, PERMANOVA (Bray-Curtis dissimilarity) with the *adonis2* function of the package *vegan* (version 2.6-4; Oksanen *et al.* 2013) was used. These models followed the alpha diversity models: 1) do population and season associate with beta diversity, and 2) do the biogeographical factors associate with beta diversity. The output of these models showed the proportion (R^2 value) of explained variance by each predicting factor and the variance that was left unexplained (residual variance). The DESeq2 test from the R package *DESeq2* (version 1.42.1; Love *et al.* 2014) was used to investigate whether the populations that were sampled both at winter and summer had differentially abundant taxa between seasons. For more detailed description of these analyses, please see Liukkonen *et al. (*2024).

2.3.4 Statistical analyses specific to study II

To study whether the early-life environment associate with nestling gut microbiome and survival, linear mixed effects and generalized linear models were used. First, alpha diversity (first Shannon, then Chao1) was set as the response variable and brood size manipulation treatment, original brood size, weight on day 7 post-hatch and hatching date as predicting variables to measure whether the treatment associated with nestling gut microbiome alpha diversity. Second, the same model was run but brood size manipulation treatment was replaced with the manipulated brood size (continuous variable) to see whether the size of the brood associated with gut microbiome alpha diversity. Nest of origin and nest of rearing were set as random effects to check the magnitude of explained variance of these factors. Third, two additional analyses were done to check whether 1) the brood size manipulation treatment and 2) actual brood size

influenced nestling body weight on day 7 and day 14 post-hatch as this may indicate effects on nestling performance and furthermore, survival. Fourth, to measure whether alpha diversity associated with survival to fledging (short-term survival) and apparent juvenile survival in Autumn 2020, survival (yes-no) was set as the response variable and alpha diversity, weight on day 7 post-hatch, hatching date and manipulated brood size as the predicting variables. Fifth, for gut microbiome beta diversity, PERMANOVA with the Euclidean distance matrix was used to study whether the predicting variables contributed to variation in gut microbiome composition. Here, the same predicting variables as in the models with alpha diversity above were used. Nest of rearing was set as a blocking factor in the PERMANOVA to control for sampling of foster siblings within a nest. Finally, a DESeq2 test was run to see whether there were differentially abundant bacterial taxa between the treatment groups. For more detailed description of the methods, please see Liukkonen *et al.* (2023).

2.3.5 Statistical analyses specific to study III

In this study, it was studied whether the gut microbiome associates with estimated lifetime reproductive success (hereafter, LRS), annual reproductive success (hereafter, ARS), and survival to the following breeding season. First, linear mixed effects models for LRS / ARS as the response variable and generalized linear mixed effects model for survival to the following breeding season as the response variable were run. In each model, gut microbiome alpha diversity, age, and body condition were set as the predicting variables and area of sampling as the random effect. These models were first run for both sexes and then separately for each sex as the number of females and males was unbalanced (females = 122, males = 63). Second, it was tested whether LRS, ARS and survival contributed to the explained variance in gut microbiome beta diversity with PERMANOVA (Bray-Curtis dissimilarity). These models were first run for both sexes and then separately for each sex so that LRS / ARS / survival to the following breeding season, age, body condition and area were set as factors that may contribute to the explained variance. Finally, the DESeq2 test was done to check whether there are differentially abundant taxa between the birds that survived and the birds that did not survive to the following breeding season. This test was run for both sexes combined and then separately for each sex. For more details regarding the statistical analyses, please see III.

3 RESULTS AND DISCUSSION

3.1 Key results

In this PhD thesis, the associations between gut microbiome variation, the environment and individual performance and fitness in wild passerine birds were investigated. The investigation has produced three manuscripts two of which have already been published in peer-reviewed journals, Journal of Animal Ecology and Animal Microbiome. In this results chapter the key results of this PhD thesis are summarized.

1. Gut microbiome diversity was higher during winter than summer and in birds that inhabited mixed forests compared to those in deciduous forests. Temperature was negatively associated with gut microbiome diversity. Season and temperature were both minorly associated with gut microbiome composition. To conclude, gut microbiome variation associates with season and biogeographical factors but is not population specific (I).
2. Gut microbiome variation had little association with early-life environment and none with survival to fledging. Differences in brood size did not associate with gut microbiome variation or survival to fledging / apparent juvenile survival. Furthermore, even though the nest of rearing indicated higher contribution to gut microbiome diversity than the nest of origin, the difference was not statistically significant. To conclude, the results indicate that there may be other factors that contribute to the nestling gut microbiome variation (II).
3. Gut microbiome diversity associated with both LRS and ARS in male but not female collared flycatchers. There was no association between the gut microbiome and survival to the following breeding season. To conclude,

there could be sex-specific interactions between the gut microbiome and fitness in wild birds (III).

To summarize, these three studies examine the dynamics between the gut microbiome, the environment and individual performance in wild small passerine birds (Fig. 8). The results provide new knowledge regarding within-species gut microbiome variation at a large biogeographical scale and the association between the gut microbiome, individual performance and (lifetime) reproductive success in wild birds.

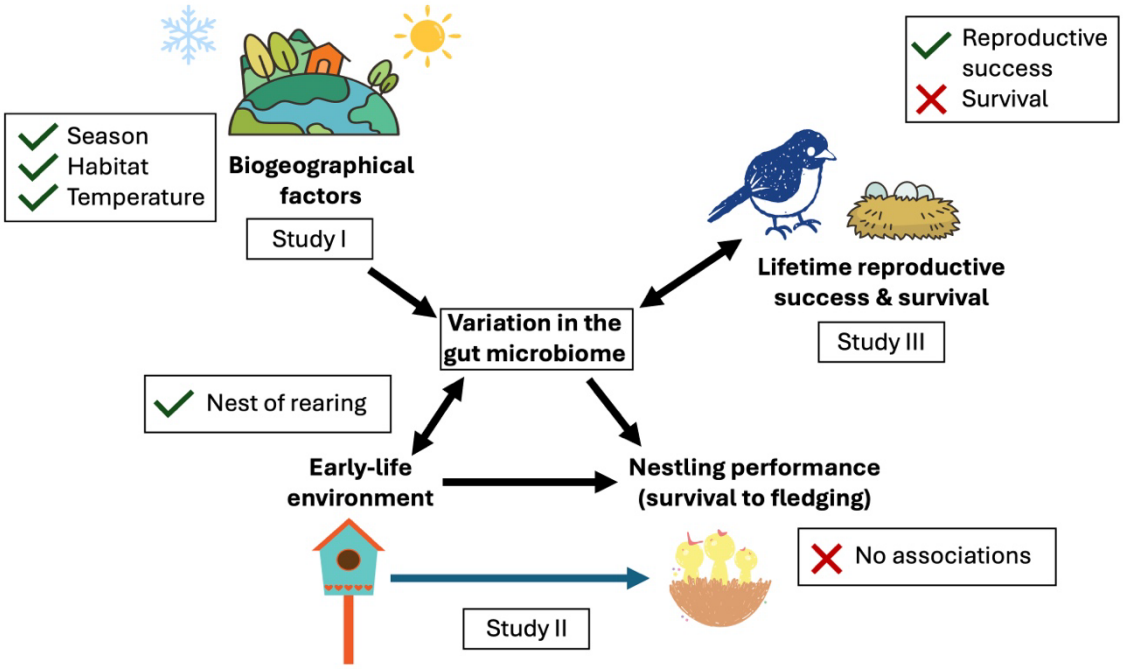

FIGURE 8 An overview of the main results that were found in the studies included in this PhD thesis.

3.2 Seasonal and environmental variation associates with the variation in the gut microbiomes of wild adult great tits (I)

In this study, it was found that wild great tit gut microbiomes differ between summer and winter (I, Fig. 9A). During winter, gut microbiomes were more diverse than during summer and season was also a significant (but minor) factor associating with differences in gut microbiome composition. Additionally, it was found that mixed forest habitats correlate with higher gut microbiome diversity than the deciduous forest habitats during winter (I, Fig. 9B). Finally, temperature associated with both gut microbiome diversity and composition, and results from the diversity analyses showed that lower temperatures associate with higher gut microbiome diversity (I, Fig. 10). These results followed the predictions, yet

concerning season, winter but not summer associated with higher gut microbiome diversity. Contrary to expectations, there was no indication of population level differences in great tit gut microbiomes nor associations between the gut microbiome and latitude, rainfall, type of supplementary winter feed or individual physiological metrics.

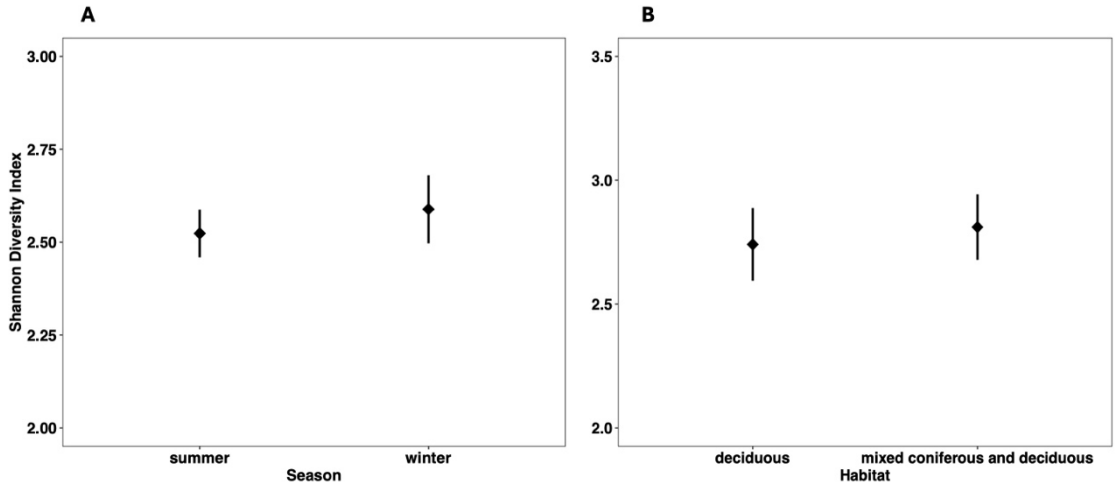

FIGURE 9 Plots showing how the gut microbiome diversity (Shannon) associates with A) season and B) habitat during winter. Overall, diversity was higher during winter than summer and higher in mixed forest habitats during winter. Vertical black bar indicates standard error and black tilted square indicates the mean. Figure taken and adapted from Liukkonen et al. (2024).

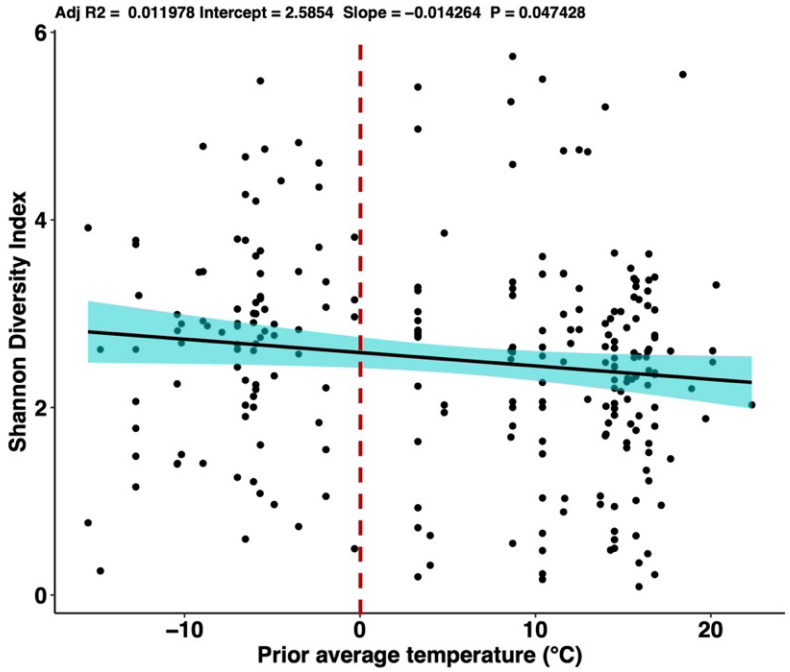

FIGURE 10 The linear model plot showing how gut microbiome diversity (Shannon) associates with average temperature when all samples (both winter and summer) are included in the analysis. Dashed vertical red line indicates zero on the temperature scale and each sample is represented with a black dot. Plot taken from the supplementary files of Liukkonen *et al.* (2024).

Previous studies with wild vertebrate species have found that season is significantly associated with gut microbiome variation (Davenport *et al.* 2014, Ren *et al.* 2017, Xiao *et al.* 2019, Baniel *et al.* 2021, Góngora *et al.* 2021) and the result is in line with these studies. However, the result was opposite to the hypothesis because winter indicated higher gut microbiome diversity than summer. It is possible that the great tits had a more diverse diet during winter than summer. Great tits are omnivorous, and they can use a wider variety of dietary items such as plant material, insects and human provided feed. This diet diversity could be more prevalent during winter than during summer when the birds usually feed mostly on insects such as lepidopteran larvae (Vel'ký *et al.* 2011). Moreover, as the great tits had access to supplementary feed at the winter fecal sampling sites and because supplementary feed was not offered during summer, the seasonal differences may be explained by the availability of supplementary feed. These kinds of dietary effects on bird gut microbiomes have been studied before and diet has been shown to be a significant contributor of gut microbiome diversity (Knutie *et al.* 2019, Teyssier *et al.* 2020, Jones *et al.* 2023). However, I would urge caution with this as this was not experimentally tested and it is only speculation based on previous knowledge.

Gut microbiome diversity, but not composition, associated with habitat during winter. Birds inhabiting mixed forests had higher gut microbiome diversity than their counterparts inhabiting deciduous forests. This result followed the original hypothesis. Mixed forest habitats are usually diverse and the higher the diversity of e.g., trees and other plant species, the higher the diversity of other forest associated species (Ampoorter *et al.* 2020, Tinya *et al.* 2021). Diverse diets have been found to associate with high gut microbiome diversity (Bodawatta *et al.* 2022b) and this is a likely explanation here as well. Moreover, a minor association between average temperature and the gut microbiome was found. Lower temperature associated with higher gut microbiome diversity, which was opposite to the original hypothesis. The association between temperature and the gut microbiome has been observed previously with studies done in captivity (Wang *et al.* 2018, Tian *et al.* 2020, Yang *et al.* 2021, Dietz *et al.* 2022) but are rarely studied in the wild. As an endothermic species, the great tit and its gut microbiome may not be heavily influenced by ambient temperature (Ingala *et al.* 2021). Moreover, it is likely that this temperature result is connected to the result regarding winter (season) associating with higher gut microbiome diversity than summer. The study populations are subjected to a varying range of temperatures throughout the year and this variation could potentially reflect to variation in the gut microbiome as well. Curiously, the gut microbiome composition was only minorly explained by the factors (season and temperature) that were measured in this study. This raises a question on whether there are some underlying mechanisms that contribute to gut microbiome composition but were not identified in this study.

Overall, the results indicate that the great tit gut microbiome reflects variation in seasonal and environmental conditions but are not population specific. Higher diversity can improve the stability of the gut microbiome, which benefits the host; a diverse gut microbiome can be more stable because

functionally similar bacterial taxa can replace one another (Lozupone *et al.* 2012). A more diverse gut microbiome can improve host metabolism and digestion and benefit host nutritional uptake and physiology and therefore, positively contribute to host phenotype (Grond *et al.* 2018).

3.3 Minimal association between the gut microbiome and the early-life environment, but not survival, of wild great tit nestlings (II)

There was variation in the gut microbiome between great tit nestlings, but this variation was not associated with nestling survival to fledging / apparent juvenile survival. Brood size as an early-life factor did not associate with gut microbiome diversity. Gut microbiome composition was not explained by brood size manipulation, hatch date, or weight on day 7 post-hatch. Nest of rearing explained some variation in gut microbiome diversity. These results suggest that the early-life environment has little association with nestling survival and nestling gut microbiome variation. The results are supported by one previous study in which the association between great tit nestlings' performance and gut microbiome was investigated (Davidson *et al.* 2021). However, a large majority of literature shows that the early-life environment correlates with nestling condition and thus, possibly affects nestling performance (Askenmo 1977, Pettifor *et al.* 1988, Sanz 1997, Wright *et al.* 1998, Burness *et al.* 2000, Naef-Daenzer *et al.* 2000, Rytkönen and Orell 2001) and variation in the gut microbiome (Kohl *et al.* 2018, Teyssier *et al.* 2018a).

First, it could be that a larger manipulation (more than + / - 2 nestlings per nest) may have resulted in some differences in the nestlings. However, the + / - 2 nestlings were justified based on previous studies (e.g., Hegner and Wingfield 1987, Hõrak 2003, Parejo and Danchin 2006). Second, it could be that the differences in early-life environment were reflected on the parents instead of the nestlings. The lack of associations between brood size and the gut microbiome could be a result of the parental compensation. No effect was found between brood size and nestling body weight on day 7 post-hatch, which indicates that the parents may have compensated for the differences in brood size. For example, the parents of enlarged nests may have increased their provisioning efforts (Cossin-Sevrin *et al.* 2023). This could increase parental stress and decrease further reproductive success such as making a second clutch (Hegner and Wingfield 1987, Sanz and Tinbergen 1999, Tinbergen and Verhulst 2000, Parejo and Danchin 2006). In III, an association between the adult gut microbiome diversity and LRS / ARS was found, thus indicating that brood size may indeed be reflected on the parents instead of the nestlings.

Third, food quality is a significant factor influencing gut microbiome variation and this has been observed in previous research (Teyssier *et al.* 2020, Góngora *et al.* 2021, Bodawatta *et al.* 2022b, Jones *et al.* 2023). Changes in diet can

be a result of the parents foraging for different types of food items. Birds such as the great tit feed on a wide variety of insects and especially larvae during the breeding season. These food items can vary across the breeding season based on insect and larvae abundances (Nager and van Noordwijk 1995, Naef-Daenzer and Keller 1999, Naef-Daenzer *et al.* 2000, Wilkin *et al.* 2009). Therefore, this variation in diet quality could reflect on the gut microbiome (Davidson *et al.* 2020, Teyssier *et al.* 2020, Góngora *et al.* 2021, Schmiedová *et al.* 2022, Jones *et al.* 2023). Future studies should consider these two aspects and investigate whether these differences in the nestlings' early-life environment were intertwined with the parents (and their gut microbiome) and variation in diet quality.

No association between the gut microbiome variation and survival to fledging / apparent juvenile survival was found. This result is supported by III in which the association between the gut microbiome variation and survival in adult birds was studied. However, the results contrast a previous study by Worsley *et al.* (2021) in which gut microbiome composition was associated with survival to the following breeding season in the Seychelles' warbler. Even though a more diverse gut microbiome is generally considered beneficial for host survival it could be that host health is connected to specific taxa within the gut microbiome instead of diversity in general (Lozupone *et al.* 2012, Zaneveld *et al.* 2017). For example, the functions of specific gut microbiome taxa could associate with host adaptation to variation in the environment (such as early-life environment) (Hanning and Diaz-Sanchez 2015). In the Seychelles' warbler specific gut microbiome taxa were correlated with individual survival (Worsley *et al.* 2021) and there was a link between specific major histocompatibility complex (MHC) genes and gut microbiome variation, which indicates a connection between individual immune system and the gut microbiome (Davies *et al.* 2022). However, these Seychelles' warbler studies were conducted with adult birds. Nestling gut microbiomes are rather flexible when compared to adult gut microbiomes (Grond *et al.* 2017). Great tit nestling gut microbiomes develop rapidly on between 8- and 15-days post-hatch: diversity decreases and the abundance of taxa such as *Firmicutes* increases (Teyssier *et al.* 2018a). The nestlings were sampled at day 7 post-hatch, which is before this 8 to 15-day time window. It is possible but only speculation that a later sampling day could have resulted in a different result regarding survival to fledging and apparent juvenile survival.

These results demonstrate the importance of understanding how early-life environment associates with the gut microbiome. The results indicate that there are unknown factors that contribute to variation in nestling gut microbiomes. I would urge further investigation to 1) understand what these unknown environmental factors may be, 2) how they potentially contribute to nestling gut microbiomes and 3) whether the performance of the parents may mediate variation in early-life environment. Additionally, there may be many unknown factors in the nestlings that may prevent (or aid) certain microbes from colonizing the host gut such as gut physiology.

3.4 Gut microbiome associates with reproductive success in wild adult collared flycatchers (III)

The collared flycatcher has not been used in gut microbiome studies before and these are the first 16S rRNA gene sequencing results of this species. The collared flycatcher gut microbiome was dominated by *Actinobacteria*, *Firmicutes* and *Proteobacteria* (III, Fig. 11).

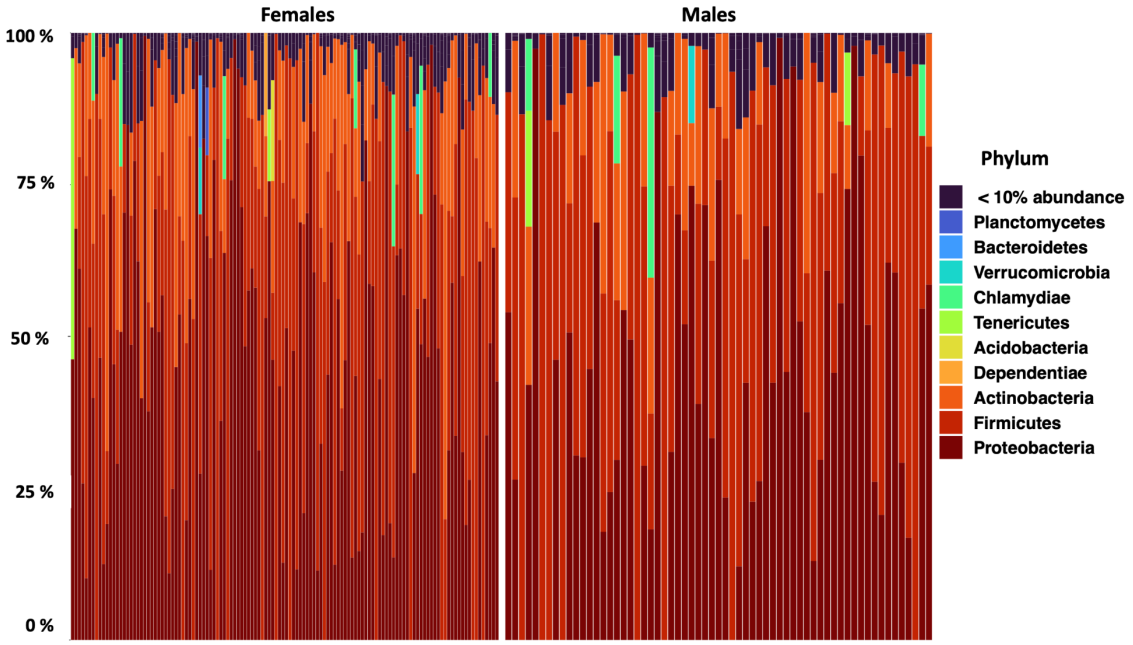

FIGURE 11 A plot of the relative abundance of most common bacterial phyla in the collared flycatcher gut microbiome. Taxa with abundance < 10 % are summed up to one category to improve plot readability. Figure is taken from manuscript III.

The gut microbiome diversity of wild adult collared flycatchers was positively associated with LRS and ARS in males but not females (III, Fig. 12 and Fig. 13). No association between the gut microbiome and survival to the following breeding season was found. Moreover, there was no association between gut microbiome composition and LRS, ARS or survival to the following breeding season. Because high gut microbiome diversity is often linked to better individual quality (Lozupone *et al.* 2012), it is likely that the positive association between gut microbiome diversity and LRS / ARS is a result of differences in individual quality in the collared flycatchers. Generally, wild passerine birds that are in better condition are more likely to produce higher quality offspring (Blomqvist *et al.* 1997, Wendeln and Becker 1999, Jensen *et al.* 2004, Szöllősi *et al.* 2009, Pigeault *et al.* 2020). However, there was no correlation between individual body condition and gut microbiome, yet it could be that other predictors of individual quality may explain the association.

Results of previous studies are inconclusive on whether the gut microbiome is directly associated with body condition (Potti *et al.* 2002, Phillips *et al.* 2018, Teyssier *et al.* 2018a, Videvall *et al.* 2019, Worsley *et al.* 2021). Because the association between gut microbiome diversity and LRS / ARS was only present in males but not females, it suggests that there may be some sex-dependent association between the gut microbiome and reproductive success. These kind of sex-specific effects between the gut microbiome and reproductive success have been found in fruit flies: variation in gut microbiome increased males' mating duration, females' offspring production and daughters', but not sons, gut microbiome (Morimoto *et al.* 2017). Unfortunately, further studies investigating these sex-specific effects are still lacking.

Mammal studies have indicated that testosterone levels positively correlate with gut microbiome diversity (Markle *et al.* 2013, Shin *et al.* 2019) and the gut microbiome may have a regulatory role in testosterone production (Colldén *et al.* 2019). In birds, a gut microbiome study by Escallón *et al.* (2017) found that higher testosterone levels correlated with increase cloacal bacterial diversity, which largely resembles the gut microbiome. High testosterone levels reflect on aggressive behavior and can improve the males' chances of securing a good quality territory and thus, improve breeding success (Szász *et al.* 2019). This could also promote extra pair copulation (hereafter, EPC) and EPP because it increases the males' chances of producing more offspring (Wingfield 1984, Raouf *et al.* 1997, Grear *et al.* 2009). EPC and EPP can increase the number of social contacts per bird and these social contacts could lead to cross infection of cloacal and possibly, gut bacteria (Escallón *et al.* 2019). This is only speculation and requires further investigation, but EPC and EPP could reflect to the higher gut microbiome diversity in this study. There are few studies that have shed a light on EPC and EPP's influence on gut and cloacal gut microbiomes, but their results remain inconclusive (Kreisinger *et al.* 2015, Escallón *et al.* 2019, Prüter *et al.* 2023). The role of EPC and EPP was not investigated in this study but could be incorporated in future studies.

Gut microbiome composition was not associated with ARS, LRS or survival to the following breeding season. This result contrasted with the previous, yet limited and correlative studies that have investigated the relationship between gut microbiome composition and reproductive success (Neuman *et al.* 2015, Antwis *et al.* 2019, Mallott *et al.* 2020, Williams *et al.* 2020, Burnham *et al.* 2023). Of the other measured factors, breeding area was the most significant contributor of differences in gut microbiome composition, which underlines the importance of the environment on gut microbiomes. This is in line with the results of I and previous knowledge in which habitat has been found to be a significant contributor of bird gut microbiomes (Hird *et al.* 2014, Loo *et al.* 2019, Grond *et al.* 2019, Drobniak *et al.* 2022, Maraci *et al.* 2022a).

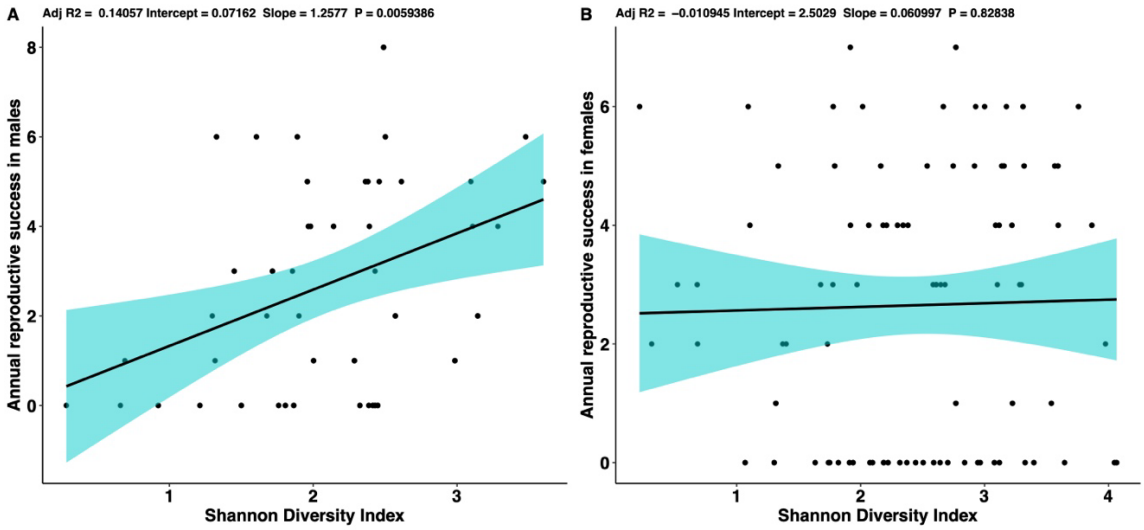

FIGURE 12 A linear model plot of the association between gut microbiome diversity and ARS in A) male and B) female collared flycatchers. Plot is taken from III.

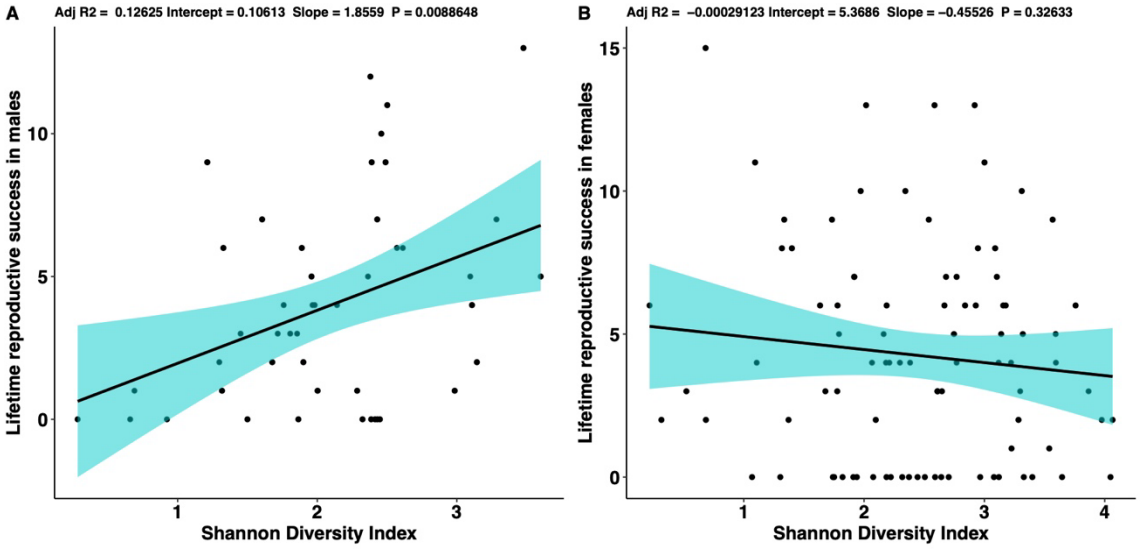

FIGURE 13 A linear model plot of the association between gut microbiome diversity and LRS in A) male and B) female collared flycatchers. Plot is taken from III.

Overall, the results provide brand new information not only of the collared flycatcher gut microbiome but also the relationship between the gut microbiome and reproductive success in a wild bird. Darwinian fitness i.e., the number of offspring an individual produces in its lifetime defines what qualities are passed on to the next generation. Here, individuals with higher LRS and ARS had higher gut microbiome diversity. As the individuals with higher gut microbiome diversity produce more offspring and thus, pass their genes to more offspring than the birds with lower LRS and ARS, selection favors the individuals with higher gut microbiome diversity. Here, it was not studied whether variation in

LRS / ARS or gut microbiome diversity is associated with genetic differences between the birds. This should be investigated in future studies because the host genotype could influence the gut microbiome (as suggested by Lee *et al.* 2020).

It should be noted that as in I and II, most factors contributing to gut microbiome composition were left unexplained. It was also found that in female (but not male) birds LRS and ARS were both associated with hatch date: earlier hatch date indicated a higher LRS / ARS. This was expected as birds that breed earlier are usually more successful breeders. Earlier breeding may enable the females to rear their young more successfully and therefore, increase reproductive success (Verhulst *et al.* 1995, Siikamäki 1998, Verboven and Visser 1998, Halupka *et al.* 2021).

4 CONCLUSIONS AND FUTURE DIRECTIONS

The key results of this PhD thesis suggest that 1) environmental variation is a determinant of wild bird gut microbiome variation, and that 2) gut microbiome diversity associates with reproductive success in especially male birds. It was found that season, winter habitat and temperature all associate with variation in the gut microbiome (I), and that the breeding area contributes to gut microbiome composition (III). It was found that early-life environment explains some of the gut microbiome diversity in nestling great tits, but it does not associate with nestling survival (II). Regarding survival, similar result was found in adult collared flycatchers: gut microbiome variation did not associate with survival to the following breeding season (III). Finally, it was found that variation in the gut microbiome associates with both estimated LRS and ARS and this association was specific to male birds (III). Furthermore, most of the variation in the gut microbiome was left unexplained, which raises the question: what are the other factors that explain the gut microbiome variation? Overall, the results suggest that the gut microbiome is connected to both the environment in which the birds live in and to reproductive success. It might well be that the environment induces variation in the gut microbiome and is a part of individual phenotypic variation and thus, affects individual performance and reproductive success. However, this is pure speculation and these causal effects of gut microbiome on fitness (and whether they exist) require further investigation.

Season significantly associated with gut microbiome variation and birds had higher gut microbiome diversity during winter (I). These results follow previous knowledge in which season has been found to be a significant contributor to gut microbiome variation in birds. However, contrary to expectations it was winter but not summer that showed higher gut microbiome alpha diversity. It is possible that the great tits use a wider variety of dietary items such as insects, plant material and human supplemented feed during winter than during summer (Vel´ký *et al.* 2011). During winter, individuals inhabiting mixed forest habitats harbored a more diverse gut microbiome than their deciduous forest inhabiting counterparts. Temperature was also associated with gut microbiome variation and lower temperatures indicated higher gut microbiome

diversity. The results follow previous studies in which season (Drovetski *et al.* 2019, Góngora *et al.* 2021, Tang *et al.* 2023), habitat (Loo *et al.* 2019, Drobniak *et al.* 2022), and temperature (Ingala *et al.* 2021) have been found to be significant contributors of variation in wild bird gut microbiomes. However, there was little correlation between the gut microbiome variation and early-life environment in wild nestling birds; nest of rearing explained some of the observed variation in gut microbiome diversity, but no other factors had any significant correlation with the gut microbiome (II). The results increase the understanding of the ecological drivers that may shape the wild bird gut microbiomes. They also indicate that the environment may be differentially associated with the gut microbiome variation of nestlings and adults (as found by Somers *et al.* 2023).

Gut microbiome variation was positively correlated with both LRS and ARS in wild birds and further investigation revealed that this pattern is sex specific. As there are no previous studies investigating the gut microbiome's association with reproductive success in wild birds, these results encourage further investigation of the interaction between the gut microbiome and reproductive success. The existing evidence from laboratory mice and human studies has found a link between the gut microbiome and testosterone production, but the mechanisms of this link are still to be investigated (Markle *et al.* 2013, Colldén *et al.* 2019, Shin *et al.* 2019). I would urge researchers to investigate the mechanisms between the gut microbiome, the endocrine system, and the immune system. Multiple studies have found a connection between metabolism, the immune system, and the gut microbiome (e.g., humans: Kau *et al.* 2011, Hooper *et al.* 2012, laboratory mice: Murga-Garrido *et al.* 2021, broiler chickens: Zenner *et al.* 2021) and it is possible that there are complex interactions between the three. Previously, it has been hypothesized that testosterone could decrease immune system functioning, but the results remain inconclusive (described as the Immunocompetence Handicap Hypothesis) (Roberts *et al.* 2004, Rantala *et al.* 2012, but see Nowak et al., 2018). From an ecological point of view this is interesting because an individual with high testosterone level would have high gut microbiome diversity, but also decreased immune system functioning. Therefore, an individual that has high reproductive success may also have an individual "cost" of having this high reproductive success (as discussed in III). Curiously though, there was no correlation between the gut microbiome and individual survival in either nestlings (II) or adults (III). This adds to the existing and mixed knowledge regarding gut microbiome's association with survival in wild birds (Teyssier *et al.* 2018a, Davidson *et al.* 2021, Worsley *et al.* 2021).

Overall, the results of this PhD thesis increase the understanding of host gut microbiome interactions and their importance in wild bird ecology and evolution. As a part of the individual phenotype, the gut microbiome can influence host ecology. Here, gut microbiomes were different based on season, winter habitat and temperature. If this variation was based on adaptive changes that improve individual survival, it could connect to selection. Moreover, individuals with higher gut microbiome diversity had more offspring, which indicates that there may be selection for individuals with high gut microbiome diversity. If there is selection for individuals that harbor higher gut microbiome

diversity, this would be important in e.g., bird conservation. Anthropogenic environmental change poses a severe threat to global biodiversity, and it is not known whether species are able to adapt as fast as needed. Studies in which wild bird gut microbiomes have been studied in rural vs. urban conditions have shown that urbanization and human influence can increase detrimental effects such as pathogen prevalence in wild birds (Murray *et al.* 2020, Teyssier *et al.* 2020). The gut microbiome is considered to enable better host adaptation and survival in a changing environment because these host associated microbes have shorter generational timespans and thus, evolve more rapidly (Alberdi *et al.* 2016, Gould *et al.* 2018).

In this PhD thesis, most of the gut microbiome variation was left unexplained. These results suggest that there are factors that have significant influence on bird gut microbiomes and were not measured here (e.g., diet). Bird gut microbiomes are likely dynamic (as suggested by Grond *et al.* 2019) and it could be that they are more affected by extrinsic factors than e.g., mammals. Future studies could aim to do multiple sampling per individual at different timepoints across the individual's annual cycle to investigate whether the gut microbiome is more dynamic rather than stable. Another possibility is that the unexplained variation in the gut microbiome is only noise. I speculate that this "noise" is transient bacterial taxa that are ingested with diet and from the environment and are passing through the bird gut microbiome but are not in fact a significant or resident part of the gut microbiome. Multiple previous studies have found this same large proportion on unexplained variation in bird and mammal studies (Waite and Taylor 2014, Hird *et al.* 2015, Avena *et al.* 2016, Grond *et al.* 2019), but it is not known what the reason behind it is. As the bird gut retention time is short because of adaptation to powered flight (Caviedes-Vidal *et al.* 2007) it is possible that only a part of the gut microbiome has a significant role in e.g., the host metabolism or immune system functioning. Many bacteria (here, the unexplained variation) could be taxa that are redundant in their functionality and pass through the gut without much significance (Moya and Ferrer 2016).

It must be noted that the results of this PhD thesis are mostly correlative and understanding the potential cause and consequence relationships between the host gut microbiome and its surrounding environment, requires further experimental research. Also, the results measure gut microbiome diversity and composition and the result interpretations are based on these two metrics. The 16S rRNA gene sequencing enables the identification of bacteria in microbiome samples, but it does not tell what the functions of the identified taxa are. For more in-depth understanding of the actual role of the gut microbiome on wild birds, one would need to apply tools such as metagenomic sequencing to support the results of the 16S rRNA gene sequencing. It would be wise to incorporate omics methods such as (shotgun) metagenomics, transcriptomics, and metabolomics (Worsley *et al.* 2024) to understand how this potentially environment-induced variation in the gut microbiome may relate to functional changes in the gut microbiome, and changes in transcription and host metabolism.

Future studies should aim to use experiments in which wild-caught birds are subjected to varying environmental conditions. These kinds of experiments could disentangle the causes of environmental variation on the gut microbiome. As suggested before, sampling the same individuals at different timepoints would enable investigation of the potentially dynamic nature of the gut microbiome. It would be important to combine this longitudinal fecal sampling (for gut microbiome) with endocrinology methods to investigate whether variation in the gut microbiome e.g., before, during and after the breeding season connects to variation in reproductive hormone levels.

To conclude, this PhD thesis adds to the existing knowledge that wild bird gut microbiomes are connected to environmental variation and it sheds a light on the association between the gut microbiome and reproductive success. Phenotypic variation is at the core of evolution; individuals that perform better can reproduce more and pass their genes to the next generation. In this PhD thesis, there was no correlation between the gut microbiome and individual survival. However, the positive correlation between gut microbiome diversity and reproductive success strongly encourages further research because it could be that the gut microbiome underlies some aspect of reproduction and therefore, evolution.

Acknowledgements

For the readers, if you got to this point, I thank you for letting me take you on this journey! If there is one message, I wish you all to take home with you it is that there is so much biodiversity on our beautiful planet and we only understand a small part of it. We must do our very best to protect this biodiversity because without it we cannot exist.

This work was funded and thus, enabled by the Emil Aaltonen Foundation and Societas Pro Fauna et Flora Fennica. I want to thank these two foundations for believing in my work and enabling me to focus on this research project and carry it through.

Reflecting on this nearly a 4-year journey it feels like a few pages of writing "thank you" is not enough. Many people were a part of this project, and their contribution varied from small to great. But still each one of these contributions was significant in the completion of this project. PhD research work is hard, and it requires mental stamina, perseverance and good people (+ "sitting down in front of a computer" muscles!). This PhD is a result of years of hard work with people that collaborated with me, mentored me, and nudged me to the right direction.

First and foremost, my main supervisor Associate Professor Suvi Ruuskanen. Suvi, you mentored me during these past years through both the good and the hard. We began our work together in the middle of covid and did the best we could even if we could not access the lab or sample all the birds we wanted. You were always eager to help and excited about the results that I got. You are an ideal mentor and a supervisor, and I could not have had a better human to help me during this project. You are a true gift to the world of science and bring out the best of this world. I am beyond grateful to have shared this journey with you! Dr. Kirsten Grond, my co-supervisor and bioinformatics & bird gut microbiome wizard. You taught me how to analyze sequence data, which was something I had never done before. You also provided me with tons of code and R support when needed. You always encouraged and cheered me on, which helped me to believe in my work. Even though we did not meet in person, your excitement about birds and their microbiomes was contagious even in Zoom. Thank you!

Thank you to Associate Professor Rose Thorogood and Assistant Professor Brian Trevelline for pre-examining the thesis. It was very helpful to have a fresh pair of eyes to have a look at my work and provide me with good comments on how to improve the thesis. I was a wee bit happy when I learned that you would be my pre-examiners. I find your work very cool and way to look at things interesting. To Professor David S. Richardson, thank you for agreeing to act as my opponent in the defense. I look forward to our discussions and especially your thoughts on the third study of my PhD thesis.

Thank you to all the wonderful Ruuskanen Lab current and former members: Nina, Lis, Liisa, Charli, Mikaela, Sameli, Jaime, Chris, Michal, Natacha, Bin-Yan, Antoine, and Tom! I enjoyed our Friday group meetings – they always

felt like a supportive and kind environment. And a big thank you belongs to all the amazing students, project researchers, lab technicians and ornithologists who helped me: Cassandre, Clemence, Basma, Juho, Katri, Sanniminja, Nelli, Inka, and Jorma thank you for your efforts in the field here in Jyväskylä and at the Konnevesi Research Station! Thank you Jorma for teaching me how to remove a blue tit from the mist net (it was a tough learning curve – blue tits can be vicious). A special thank you to Juho for freezing your butt off when trying to find great tits during winter. To all the co-authors in each study, thank you for your help in the field and ideas in developing each manuscript. Especially reminiscing the first study of this PhD brings up some fun memories of spending time at various bird catching places and sending bird poop across Europe. I think my favorite time was when I went to collect a box of bird poop from Onnibus. The box had a happy and safe journey on the front seat of Onnibus all the way from Oulu to Jyväskylä (thanks Vellu!).

To lab masters Petri Papponen, Sari Viinikainen and Elina Virtanen, thank you for teaching me how to use all the machinery at the labs and finding equipment, when it was needed. To Dr.'s Eva Kallio and Emily Knott, thank you for being in my follow up group and sharing your knowledge and guiding me with wisdom and kindness.

I would not be here without the support of some wonderful people in my life. A huge thank you to Emmi! You were my number one peer support friend and helped me to stay sane during the years of PhD and the too many hours that were spent in the lab. All the avantouinti and tea+cake dates, Kroonisesti Ärhäkkä moments and ranting sessions were needed and very much appreciated. I would have probably lost my mind without our daily chats. Thank you for sharing this PhD journey with me! To Juho, thank you for all the bird facts, photos, bird (and one arctic fox!!! Which made me cry of happiness over the phone) video calls, and sounds since 2017. And the occasional "forest policy sucks" rants. I will always remember when you tried to catch a magpie from my backyard in the Kortepohja F building.

Thank you to the people from the original YAC213.2 office. Sameli, Ilona and Charli it was always a pleasure to share the office with you. You brought humanity and joy to office and lab days (and a lot of the best cat and dog videos). Sameli, blessed be the *pötkötys*, corgis and @tunneviidakko! Charli, thank you for helping me with my coding problems, commenting on my manuscripts and bringing up good discussion points regarding microbiomes and all the coolest birds.

To everyone in our early career scientist support group in 2023-2024, thank you! Our monthly meetings helped me to reflect my values and goals. Our group meetings also provided much needed safe talking space and peer support. I think these meetings were essential during the final steps of my PhD project.

Thank you to Mothers in Science for standing up for mother's rights in STEMM fields. We need mentors, trailblazers, and visibility to change this system and crush the patriarchy. Thank you to each of those researchers and PI's whose lab is vocal and active in diversity, equality, and inclusion. Academia needs more people like you.

To maintain a healthy balance between work and outside of work, I want to thank the local climbing community. Rock climbing is amazing for shutting down the "work noise." Also, to each moment I spent avanto swimming and wandering across Koillismaa, and eastern and northern Lapland. Avanto swimming and staring into the endless wilderness of Lapland give me so much peace and gratitude for the beauty of this planet. I am the happiest when I am surrounded by the rolling hills and endless fells of Lapland listening to European golden plover's melancholic beeping.

Finally, *iso kiitos Annille ja Jonnelle! Innostuitte lintusekoilusta ja luontomeuhkauksesta ja lähditte mukaan, kun halusin etsiä hömötiaisia ja lehtokurppia lähimetsistä.*

Lopuksi lainaus tämän hetken lempikirjastani.

"Mutta ihminen on outo otus, Humanus mysticus: paskoo omaan pesäänsä, sahaa omaa oksaansa, ahtautuu betonikuutioon ja ahdistuu sitten vapaaehtoisesti hypermarkettien päättymättömillä baanoilla kelmeiden keinovalojen ja ainaisen melusaasteen hurratessa kulkua."- Petteri Saario kirjassa *Rakkauskirjeitä luonnolle*

Martta Liukkonen
31.7.2024
Kortepohja, Jyväskylä

YHTEENVETO (RÉSUMÉ IN FINNISH)

Suolistomikrobiomin, ympäristötekijöiden ja yksilön menestyksen yhteys luonnonvaraisilla linnuilla

Eliölajit ovat sopeutuneet elämään erilaisissa ympäristöissä, ja yksilön ominaisuudet määrittelevät sen, miten yksilö menestyy elinympäristössään. Yksilön fenotyyppi eli ilmiasu määräytyy perimän ja ympäristön perusteella. Fenotyyppi muodostuu yksilön rakenteesta, fysiologiasta, kehityksestä ja käyttäytymisestä. Mitä paremmin yksilön fenotyyppi mahdollistaa sopeutumisen elinympäristöön, sitä paremmat mahdollisuudet yksilöllä on lisääntyä ja siirtää ominaisuuksiaan jälkeläisilleen. Fenotyyppisen vaihtelun syitä ja seurauksia on tutkittu runsaasti, mutta eliöyksilössä elävien mikroskooppisten organismien merkitystä fenotyyppiseen vaihteluun ei ole juurikaan tutkittu. Viime vuosina yhä useampi tutkimus on yrittänyt selvittää, miten nämä isäntäyksilön kanssa vuorovaikutuksessa elävät mikrobit mahdollisesti vaikuttavat yksilön fenotyyppiseen vaihteluun.

Suolistomikrobiomi on yksi tunnetuimmista isäntäyksilön ja mikroskooppisten organismien muodostamista kokonaisuuksista. Suolistomikrobiomilla tarkoitetaan isäntäyksilön ruoansulatuselimistöä ja siellä olevia bakteereita, arkkeja, sieniä, yksisoluisia eliöitä, viruksia ja bakteriofageja sekä näiden kaikkien perimää. Suolistomikrobiomin koostumukseen vaikuttaa paitsi isäntäyksilön perimä myös ympäristö, ja tätä koostumusta onkin tutkittu runsaasti ihmisillä ja laboratorioeliöillä. Suolistomikrobiomilla on merkittävä tehtävä yksilön aineenvaihdunnassa, immuunipuolustuksessa ja käyttäytymisessä. Suolistomikrobiomi esimerkiksi säätelee immuunipuolustuksen toimintaa ja suojaa isäntäyksilöä taudinaiheuttajilta. Lisäksi se vaikuttaa isäntäyksilön energia-aineenvaihdunnan säätelyyn, geenien aktiivisuuteen sekä kudosten ja solukoiden toimintaan. Ihmisillä tehdyissä tutkimuksissa on havaittu, että fenotyypin ja suolistomikrobiomin välillä on yhteys. Suolistomikrobiomi vaikuttaa muun muassa yksilön välisiin aineenvaihduntaeroihin, jotka voivat puolestaan olla yhteydessä lihavuuteen, tulehduksellisiin suolistosairauksiin ja masennukseen. Koska suolistomikrobiomin ja fenotyypin välillä on yhteys, suolistomikrobiomilla voi olla merkitystä myös yksilöiden sopeutumiskykyyn ja mahdollisesti evoluutioon.

Suolistomikrobiomi saa alkunsa yksilön syntyessä ja kehittyy voimakkaasti heti syntymän jälkeen ja yksilön kasvaessa. Suolistomikrobiomin kehitykseen vaikuttavat esimerkiksi alatiesynnytys (nisäkkäillä), jonka aikana uusi yksilö altistuu synnytyskanavan mikrobeille sekä syntymäympäristö, sosiaaliset kontaktit ja ruokavalio. Tutkimuksissa on havaittu, että häiriöt suolistomikrobiomin muodostumisvaiheessa syntymän jälkeen voivat vaikuttaa yksilön suolistomikrobiomiin myös myöhemmin elämässä. Esimerkiksi keisarileikkaus tai antibioottihoito voivat aiheuttaa pysyviä muutoksia suolistomikrobiomin koostumuksessa. Yksilön varttuessa yksilö kohtaa jatkuvasti uusia mikrobeita, jotka voivat vaikuttaa suolistomikrobiomiin. Yksilön perimä, lisääntyminen, fysiologia, terveys, ikä ja ruokavalio ovat yksilölle tyypillisiä sisäisiä tekijöitä, jotka vaikuttavat

suolistomikrobiomiin. Lisäksi yksilön ulkopuoliset tekijät, kuten elinympäristö, saatavilla oleva ravinto, sosiaaliset kontaktit ja vuodenajat saattavat vaikuttaa suolistomikrobiomiin. Esimerkiksi vuodenaikaiset vaihtelut elinympäristössä tai ravinnossa voivat heijastua suolistomikrobiomin koostumukseen. Se, missä määrin nämä sisäiset ja ulkoiset tekijät vaikuttavat suolistomikrobiomiin, on mielenkiintoista, koska yhdessä ne voivat vaikuttaa yksilön fenotyyppiin ja kykyyn sopeutua muuttuvaan ympäristöön.

Suurin osa suolistomikrobiomitutkimuksista on tehty joko ihmisillä tai laboratoriossa kasvatetuilla eliöillä. Näiden tutkimusten tuloksia ei kuitenkaan voi yleistää laajasti kaikkiin eliölajeihin, koska erityisesti luonnonympäristöissä elävät lajit ovat jatkuvassa vuorovaikutuksessa lukuisten muiden lajien kanssa. Linnut ovat yksi tällainen eliöryhmä, joiden levinneisyys kattaa jokaisen Maapallon mantereen, ja joiden suolistomikrobiomia ei ole vielä tutkittu yhtä paljon kuin esimerkiksi ihmisten tai laboratorioeliöiden. Koska linnut lisääntyvät munimalla ja ovat pääasiassa lentokykyisiä, eroavat ne huomattavasti esimerkiksi ihmisestä. Aiemmat tutkimukset ovat osoittaneet, että lintujen suolistomikrobiomiin vaikuttavat elinympäristö ja erityisesti ruokavalio. Lisäksi elinkierron vaiheet, kuten kevät- ja syysmuutto, voivat heijastua suolistomikrobiomin koostumukseen. Lintujen suolistomikrobiomia on tutkittu runsaasti taloudellisesti tärkeillä lajeilla (kuten siipikarjalla) sekä yksittäisillä lintulajeilla. Suurin osa tutkimuksista keskittyy kuitenkin vain tietyllä alueella elävään saman lajin populaatioon tai yhteen suolistomikrobiomia selittävään tekijään, joka voi vaikuttaa suolistomikrobiomin vaihteluun ja koostumukseen. Lisäksi linnuilla ei ole juurikaan tutkittu, miten suolistomikrobiomi saattaa vaikuttaa yksilön selviytymiseen ja lisääntymiskykyyn. Sekä selviytyminen että lisääntymiskyky ovat tekijöitä, jotka vaikuttavat siihen, mitkä yksilön ominaisuudet mahdollisesti siirtyvät seuraavalle sukupolvelle.

Tässä väitöskirjatutkimuksessa ja sen kolmessa erillisessä tutkimusartikkelissa selvitettiin 1) mitkä tekijät vaikuttavat suolistomikrobiomin vaihteluun saman lajin eri lintupopulaatioissa laajalla maantieteellisellä alueella, 2) vaikuttaako varhainen kasvuympäristö suolistomikrobiomiin pesäpoikasilla, ja 3) onko suolistomikrobiomin, lisääntymismenestyksen ja yksilön selviytymisen välillä yhteyttä. Ensimmäisessä tutkimuksessa tarkasteltiin suolistomikrobiomin eroja luonnonvaraisissa lintupopulaatioissa, jotka ovat levittäytyneet laajalle maantieteelliselle alueelle. Tutkimuslajina oli talitiainen (*Parus major*) ja sen eri populaatiot Oulussa, Jyväskylässä, Turussa, Tarttossa (Viro), Lundissa (Ruotsi), Westerheidessa (Alankomaat), Pilis-Visegrád vuorilla (Unkari), ja La Hiruelassa (Espanja). Tutkimuksen hypoteesina oli, että suolistomikrobiomin vaihtelu selittyy populaatiolla ja vuodenajalla, ja että maantieteellinen sijainti, elinympäristö, lämpötila ja sademäärä voivat mahdollisesti selittää suolistomikrobiomin vaihtelua ja koostumusta populaatioiden ja yksilöiden välillä. Tutkimuksessa havaittiin, että vuodenaika ja talviajan elinympäristö merkitsevästi selittävät suolistomikrobiomin runsautta. Lisäksi havaittiin, että vuodenaika ja lämpötila ovat yhteydessä suolistomikrobiomin koostumukseen. Suolistomikrobiomissa ei kuitenkaan ollut merkitseviä eroja talitiaispopulaatioiden välillä. Talviaikaan

suolistomikrobiomi oli monipuolisempi ja on mahdollista, että tämä selittyy monipuolisemmalla ravinnonhankinnalla verrattuna kesään ja pesimäkauteen. Yksilöillä, joiden elinympäristö oli sekametsää, oli myös monipuolisempi suolistomikrobiomi kuin yksilöillä, jotka elivät lehtimetsissä. Sekametsissä on usein runsaampi lajisto ja tämä voi heijastua myös sekametsissä asuvien lintujen suolistomikrobiomiin. Onkin mahdollista, että monipuolisempi elinympäristö tukee suolistomikrobiomin monimuotoisuutta.

Toisessa tutkimuksessa selvitettiin, miten varhainen elinympäristö mahdollisesti vaikuttaa talitiaisen poikasten suolistomikrobiomiin ja onko suolistomikrobiomilla merkitystä poikasten selviytymisessä. Tutkimuksessa muokattiin talitiaisen poikueiden kokoa Turun Ruissalossa. Poikuekokoa muokattiin siirtämällä poikasia pesien välillä ja suurentamalla (+ 2) tai pienentämällä (- 2) poikueiden kokoa. Lisäksi poikasten lentoonlähtöä seurattiin, jotta voitiin mitata poikasten selviytymistä. Tuloksen osoittivat, että kasvuympäristö selitti jonkin verran suolistomikrobiomin vaihtelusta. Poikuekoolla ei kuitenkaan ollut vaikutusta suolistomikrobiomiin tai poikasten selviytymiseen. On mahdollista, että poikuekoolla ei ollut merkitystä, koska pesintäkaudella oli optimaaliset olosuhteet ja runsaasti ruokaa.

Kolmannessa tutkimuksessa selvitettiin, onko sekä suolistomikrobiomin ja lisääntymismenestyksen että suolistomikrobiomin ja yksilön selviytymisen välillä yhteyttä. Tutkimuksessa käytettiin luonnonvaraisia sepelsieppoja (*Ficedula albicollis*), jotka pesivät Gotlannissa Ruotsissa. Tutkimuksessa hyödynnettiin pitkäaikaista seuranta-aineistoa ja jokaiselta lintuyksilöltä kerättiin tiedot koko eliniän aikaisesta pesintämenestyksestä (yksilön kelpoisuus) ja yhden lisääntymiskauden pesintämenestyksestä. Lisäksi selvitettiin yksilöiden selviytyminen. Tulokset osoittivat, että suolistomikrobiomin ja lisääntymismenestyksen välillä on yhteys, ja että tämä yhteys on merkitsevä erityisesti koirailla. On mahdollista, että suolistomikrobiomi on kytkeytynyt koiraiden käyttäytymiseen (aggressiivisuus) ja lisääntymiseen kytkeytyneisiin hormoneihin (testosteroni), ja että monipuolisempi suolistomikrobiomi vaikuttaa positiivisesti esimerkiksi paremman pesintäreviirin hankintaan. Suolistomikrobiomin ja yksilöiden selviytymisen välillä ei ollut yhteyttä.

Koko väitöskirjatyön ja sen artikkeleiden perusteella voidaan sanoa, että ympäristöllä on huomattava merkitys suolistomikrobiomin muodostumisessa ja muovautumisessa, ja että suolistomikrobiomi on merkittävä tekijä yksilöiden lisääntymismenestyksessä. Koska suolistomikrobiomi kytkeytyy yksilön lisääntymismenestykseen, on mahdollista, että suolistomikrobiomilla on merkitys yksilön fenotyyppisessä vaihtelussa ja mahdollisesti evoluutiossa.

REFERENCES

Abdel-Moneim A.-M.E., Elbaz A.M., Khidr R.E.-S. & Badri F.B. 2020. Effect of in Ovo Inoculation of Bifidobacterium spp. on Growth Performance, Thyroid Activity, Ileum Histomorphometry, and Microbial Enumeration of Broilers. *Probiotics Antimicro.* 12: 873–882.

Adriansjach J., Baum S.T., Lefkowitz E.J., Van Der Pol W.J., Buford T.W. & Colman R.J. 2020. Age-Related Differences in the Gut Microbiome of Rhesus Macaques. *J Gerontol. A* 75: 1293–1298.

Alabrudzińska J., Kaliński A., Słomczyński R., Wawrzyniak J., Zieliński P. & Bańbura J. 2003. Effects of Nest Characteristics on Breeding Success of Great Tits Parus major. *Acta Ornithol.* 38: 151–154.

Alberdi A., Aizpurua O., Bohmann K., Zepeda-Mendoza M.L. & Gilbert M.T.P. 2016. Do Vertebrate Gut Metagenomes Confer Rapid Ecological Adaptation? *Trends Ecol. Evol.* 31: 689–699.

Alerstam T. 2003. Bird Migration Speed. In: Berthold P., Gwinner E. & Sonnenschein E. (eds.), *Avian Migration*, Springer, Berlin, Heidelberg, pp. 253–267.

Alerstam T. 2011. Optimal bird migration revisited. *J. Ornithol.* 152: 5–23.

Ampoorter E., Barbaro L., Jactel H., Baeten L., Boberg J., Carnol M., Castagneyrol B., Charbonnier Y., Dawud S.M., Deconchat M., Smedt P.D., Wandeler H.D., Guyot V., Hättenschwiler S., Joly F., Koricheva J., Milligan H., Muys B., Nguyen D., Ratcliffe S., Raulund-Rasmussen K., Scherer-Lorenzen M., Van Der Plas F., Keer J.V., Verheyen K., Vesterdal L. & Allan E. 2020. Tree diversity is key for promoting the diversity and abundance of forest-associated taxa in Europe. *Oikos* 129: 133–146.

Anderson K.J. & Jetz W. 2005. The broad-scale ecology of energy expenditure of endotherms. *Ecol. Lett.* 8: 310–318.

Anderson M.J. & Walsh D.C.I. 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? *Ecol. Monogr.* 83: 557–574.

Antwis R.E., Lea J.M.D., Unwin B. & Shultz S. 2018. Gut microbiome composition is associated with spatial structuring and social interactions in semi-feral Welsh Mountain ponies. *Microbiome* 6: 207.

Antwis R.E., Edwards K.L., Unwin B., Walker S.L. & Shultz S. 2019. Rare gut microbiota associated with breeding success, hormone metabolites and ovarian cycle phase in the critically endangered eastern black rhino. *Microbiome* 7: 27.

Apprill A., McNally S., Parsons R. & Weber L. 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* 75: 129–137.

Askenmo C. 1977. Effects of Addition and Removal of Nestlings on Nestling Weight, Nestling Survival, and Female Weight Loss in the Pied Flycatcher Ficedula hypoleuca. *Ornis Scand.* 8: 1–8.

Avena C.V., Parfrey L.W., Leff J.W., Archer H.M., Frick W.F., Langwig K.E., Kilpatrick, A.M., Powers, K.E., Foster, J.T. & McKenzie V.J. 2016. Deconstructing the bat skin microbiome: influences of the host and the environment. *Front. Microbiol.* 7: 1753.

Azad, M.B., Bridgman, S.L., Becker, A.B. & Kozyrskyj, A.L. 2014. Infant antibiotic exposure and the development of childhood overweight and central adiposity. *International journal of obesity*. 38(10): 1290-1298.

Bäckhed F., Roswall J., Peng Y., Feng Q., Jia H., Kovatcheva-Datchary P., Li Y., Xia Y., Xie H., Zhong H., Khan M.T., Zhang J., Li J., Xiao L., Al-Aama J., Zhang D., Lee Y.S., Kotowska D., Colding C., Tremaroli V., Yin Y., Bergman S., Xu X., Madsen L., Kristiansen K., Dahlgren J. & Wang J. 2015. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe* 17: 690–703.

Baiz M.D., Benavides C. A., Miller E.T., Wood A.W. & Toews D.P.L. 2023. Gut microbiome composition better reflects host phylogeny than diet diversity in breeding wood-warblers. *Mol. Ecol.* 32: 518–536.

Baniel A., Amato K.R., Beehner J.C., Bergman T.J., Mercer A., Perlman R.F., Petrullo L., Reitsema L., Sams S., Lu A. & Snyder-Mackler N. 2021. Seasonal shifts in the gut microbiome indicate plastic responses to diet in wild geladas. *Microbiome* 9: 26.

Bates D., Maechler M., Bolker B., Walker S., Christensen R.H.B., Singmann H., Dai, B., Scheipl, F., Grothendieck, G., Green, P., Fox, J., Bauer, A., Krivitsky, P.N., Tanaka, E. & Jagan, M. B. 2015. Package 'lme4'. *Convergence*. 12(1): 2.

Beli E., Yan Y., Moldovan L., Vieira C.P., Gao R., Duan Y., Prasad R., Bhatwadekar A., White F.A., Townsend S.D., Chan L., Ryan C.N., Morton D., Moldovan E.G., Chu F.-I., Oudit G.Y., Derendorf H., Adorini L., Wang X.X., Evans-Molina C., Mirmira R.G., Boulton M.E., Yoder M.C., Li Q., Levi M., Busik J.V. & Grant M.B. 2018. Restructuring of the Gut Microbiome by Intermittent Fasting Prevents Retinopathy and Prolongs Survival in db/db Mice. *Diabetes* 67: 1867–1879.

Benskin C.McW.H., Wilson K., Jones K. & Hartley I.R. 2009. Bacterial pathogens in wild birds: a review of the frequency and effects of infection. *Biol. Rev.* 84: 349–373.

Ben-Yosef, M., Jurkevitch, E. & Yuval, B. 2008. Effect of bacteria on nutritional status and reproductive success of the Mediterranean fruit fly Ceratitis capitata. Physiol. Entomol. 33: 145–154. https://doi.org/10.1111/j.1365-3032.2008.00617.x

Berlow M., Phillips J.N. & Derryberry E.P. 2021. Effects of Urbanization and Landscape on Gut Microbiomes in White-Crowned Sparrows. *Microb. Ecol.* 81: 253–266.

Besten G. den, Eunen K. van, Groen A.K., Venema K., Reijngoud D.-J. & Bakker B.M. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* 54: 2325–2340.

Blekhman R., Goodrich J.K., Huang K., Sun Q., Bukowski R., Bell J.T., Spector T.D., Keinan A., Ley R.E., Gevers D. & Clark A.G. 2015. Host genetic

variation impacts microbiome composition across human body sites. *Genome Biol.* 16: 191.

Blomqvist D., Johansson O.C. & Götmark F. 1997. Parental quality and egg size affect chick survival in a precocial bird, the lapwing Vanellus vanellus. *Oecologia* 110: 18–24.

Bodawatta K.H., Freiberga I., Puzejova K., Sam K., Poulsen M. & Jønsson K.A. 2021. Flexibility and resilience of great tit (Parus major) gut microbiomes to changing diets. *Anim. Microbiome* 3: 20.

Bodawatta K.H., Hird S.M., Grond K., Poulsen M. & Jønsson K.A. 2022a. Avian gut microbiomes taking flight. *Trends Microbiol.* 30: 268–280.

Bodawatta K.H., Klečková I., Klečka J., Pužejová K., Koane B., Poulsen M., Jønsson K.A. & Sam K. 2022b. Specific gut bacterial responses to natural diets of tropical birds. *Sci. Rep.* 12: 713.

Bolyen E., Rideout J.R., Dillon M.R., Bokulich N.A., Abnet C., Al-Ghalith G.A., Alexander H., Alm E.J., Arumugam M., Asnicar F., Bai Y., Bisanz J.E., Bittinger K., Brejnrod A., Brislawn C.J., Brown C.T., Callahan B.J., Caraballo-Rodríguez A.M., Chase J., Cope E., Da Silva R., Dorrestein P.C., Douglas G.M., Durall D.M., Duvallet C., Edwardson C.F., Ernst M., Estaki M., Fouquier J., Gauglitz J.M., Gibson D.L., Gonzalez A., Gorlick K., Guo J., Hillmann B., Holmes S., Holste H., Huttenhower C., Huttley G., Janssen S., Jarmusch A.K., Jiang L., Kaehler B., Kang K.B., Keefe C.R., Keim P., Kelley S.T., Knights D., Koester I., Kosciolek T., Kreps J., Langille M.G., Lee J., Ley R., Liu Y.-X., Loftfield E., Lozupone C., Maher M., Marotz C., Martin B.D., McDonald D., McIver L.J., Melnik A.V., Metcalf J.L., Morgan S.C., Morton J., Naimey A.T., Navas-Molina J.A., Nothias L.F., Orchanian S.B., Pearson T., Peoples S.L., Petras D., Preuss M.L., Pruesse E., Rasmussen L.B., Rivers A., Robeson, Ii M.S., Rosenthal P., Segata N., Shaffer M., Shiffer A., Sinha R., Song S.J., Spear J.R., Swafford A.D., Thompson L.R., Torres P.J., Trinh P., Tripathi A., Turnbaugh P.J., Ul-Hasan S., Van Der Hooft J.J., Vargas F., Vázquez-Baeza Y., Vogtmann E., Von Hippel M., Walters W., et al. 2018. *QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science*. *PeerJ Prepr.*

Bordenstein S.R. & Theis K.R. 2015. Host Biology in Light of the Microbiome: Ten Principles of Holobionts and Hologenomes. *PLOS Biol.* 13, e1002226.

Bouwhuis S., Vedder O. & Becker P.H. 2015. Sex-specific pathways of parental age effects on offspring lifetime reproductive success in a long-lived seabird. *Evolution* 69: 1760–1771.

Bray J.R. & Curtis J.T. 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecol. monogr. 27*(4): 326-349.

Brown E.M., Clardy J. & Xavier R.J. 2023. Gut microbiome lipid metabolism and its impact on host physiology. *Cell Host Microbe* 31: 173–186.

Bull J.J. 1987. Evolution of Phenotypic Variance. *Evolution* 41: 303–315.

Burness G.P., McClelland G.B., Wardrop S.L. & Hochachka P.W. 2000. Effect of Brood Size Manipulation on Offspring Physiology: An Experiment with Passerine Birds. *J. Exp. Biol.* 203: 3513–3520.

Burnham C.M., McKenney E.A., Heugten K.A.- van, Minter L.J. & Trivedi S. 2023. Effects of age, seasonality, and reproductive status on the gut microbiome of Southern White Rhinoceros (Ceratotherium simum simum) at the North Carolina zoo. *Anim. Microbiome* 5: 27.

Callahan B.J., McMurdie P.J., Rosen M.J., Han A.W., Johnson A.J.A. & Holmes S.P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13: 581–583.

Cao, S., Guo, D., Yin, H., Ding, X., Bai, S., Zeng, Q., Liu, J., Zhang, K., Mao, X. & Wang, J. 2023. Improvement in ovarian function following fecal microbiota transplantation from high-laying rate breeders. *Poul. Sci.* 102(3): 102467.

Carmody R.N., Gerber G.K., Luevano J.M., Gatti D.M., Somes L., Svenson K.L. & Turnbaugh P.J. 2015. Diet Dominates Host Genotype in Shaping the Murine Gut Microbiota. *Cell Host Microbe* 17: 72–84.

Carthey, A.J., Gillings, M.R. & Blumstein, D.T. 2018. The extended genotype: microbially mediated olfactory communication. *Trends Ecol. Evol.* 33(11): 885-894.

Caviedes-Vidal E., McWhorter T.J., Lavin S.R., Chediack J.G., Tracy C.R. & Karasov W.H. 2007. The digestive adaptation of flying vertebrates: High intestinal paracellular absorption compensates for smaller guts. *PNAS* 104: 19132–19137.

Chao A. 2006. Species Estimation and Applications. In: Kotz S., Read C.B., Balakrishnan N., Vidakovic B. & Johnson N.L. (eds.), *Encyclopedia of Statistical Sciences*, John Wiley & Sons, Inc., Hoboken, NJ, USA, p. ess5051.

Chevalier C., Stojanović O., Colin D.J., Suarez-Zamorano N., Tarallo V., Veyrat-Durebex C., Rigo D., Fabbiano S., Stevanović A., Hagemann S., Montet X., Seimbille Y., Zamboni N., Hapfelmeier S. & Trajkovski M. 2015. Gut Microbiota Orchestrates Energy Homeostasis during Cold. *Cell* 163: 1360–1374.

Cho, I., Yamanishi, S., Cox, L., Methé, B.A., Zavadil, J., Li, K., Gao, Z., Mahana, D., Raju, K., Teitler, I., Li, H., Alekseyenko, A.V. & Blaser, M.J. 2012. Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature*. 488(7413): 621-626.

Clemente J.C., Ursell L.K., Parfrey L.W. & Knight R. 2012. The Impact of the Gut Microbiota on Human Health: An Integrative View. *Cell* 148: 1258–1270.

Cody M.L. 1981. Habitat Selection in Birds: The Roles of Vegetation Structure, Competitors, and Productivity. *BioScience* 31: 107–113.

Colldén H., Landin A., Wallenius V., Elebring E., Fändriks L., Nilsson M.E., Ryberg H., Poutanen M., Sjögren K., Vandenput L. & Ohlsson C. 2019. The gut microbiota is a major regulator of androgen metabolism in intestinal contents. *Am. J. Physiol.-Endoc. M.* 317: E1182–E1192.

Cossin-Sevrin N., Hsu B.-Y., Marciau C., Viblanc V.A., Ruuskanen S. & Stier A. 2022. Effect of prenatal glucocorticoids and thyroid hormones on developmental plasticity of mitochondrial aerobic metabolism, growth and survival: an experimental test in wild great tits. *J. Exp. Biol.* 225: jeb243414.

Cossin-Sevrin N., Stier A., Hukkanen M., Zahn S., Viblanc V.A., Anttila K. & Ruuskanen S. 2023. Early-life environmental effects on mitochondrial

aerobic metabolism: a brood size manipulation in wild great tits. *J. Exp. Biol.* 226: jeb245932.

Costanzo A., Ambrosini R., Caprioli M., Gatti E., Parolini M., Canova L., Rubolini D., Romano A., Gianfranceschi L. & Saino N. 2017. Lifetime reproductive success, selection on lifespan, and multiple sexual ornaments in male European barn swallows. *Evolution* 71: 2457–2468.

Cox L.M., Yamanishi S., Sohn J., Alekseyenko A.V., Leung J.M., Cho I., Kim S., Li H., Gao Z., Mahana D., Rodriguez J.G.Z., Rogers A.B., Robine N., Loke P. & Blaser M.J. 2014. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell* 158: 705–721.

Crooks K.R. & Van Vuren D. 1995. Resource utilization by two insular endemic mammalian carnivores, the island fox and island spotted skunk. *Oecologia* 104: 301–307.

Davenport E.R., Mizrahi-Man O., Michelini K., Barreiro L.B., Ober C. & Gilad Y. 2014. Seasonal Variation in Human Gut Microbiome Composition. *PLoS ONE* 9: e90731.

David L.A., Maurice C.F., Carmody R.N., Gootenberg D.B., Button J.E., Wolfe B.E., Ling A.V., Devlin A.S., Varma Y., Fischbach M.A., Biddinger S.B., Dutton R.J. & Turnbaugh P.J. 2014. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505: 559–563.

Davidson G.L., Somers S.E., Wiley N., Johnson C.N., Reichert M.S., Ross R.P., Stanton C. & Quinn J.L. 2021. A time-lagged association between the gut microbiome, nestling weight and nestling survival in wild great tits. *J Anim. Ecol.* 90: 989–1003.

Davidson G.L., Wiley N., Cooke A.C., Johnson C.N., Fouhy F., Reichert M.S., De La Hera I., Crane J.M.S., Kulahci I.G., Ross R.P., Stanton C. & Quinn J.L. 2020. Diet induces parallel changes to the gut microbiota and problem solving performance in a wild bird. *Sci. Rep.* 10: 20783.

Davies C.S., Worsley S.F., Maher K.H., Komdeur J., Burke T., Dugdale H.L. & Richardson D.S. 2022. Immunogenetic variation shapes the gut microbiome in a natural vertebrate population. *Microbiome* 10: 41.

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*. 6: 1-14.

Dawkins, R. 2016. The extended phenotype: The long reach of the gene. Oxford University Press.

Debray R., Herbert R.A., Jaffe A.L., Crits-Christoph A., Power M.E. & Koskella B. 2022. Priority effects in microbiome assembly. *Nat. Rev. Microbiol.* 20: 109–121.

Deeming D.C. & Mainwaring M.C. 2015. Functional properties of nests. *Nests, eggs and incubation: new ideas about avian reproduction*, 29-49. (Oxford, 2015; online edition, Oxford Academic, 17 Sept. 2015), https://doi.org/10.1093/acprof:oso/9780198718666.001.0001, accessed 16 Apr. 2024.

Dietz M., Matson K.D., Versteegh M., Van Der Velde M., Parmentier H., Arts J., Salles J.F. & Tieleman B.I. 2022. Gut microbiota of homing pigeons shows summer-winter variation under constant diet indicating a substantial effect of temperature. *Anim. Microbiome.* 4: 64.

Diez-Méndez D., Bodawatta K.H., Freiberga I., Klečková I., Jønsson K.A., Poulsen M. & Sam K. 2023. Indirect maternal effects via nest microbiome composition drive gut colonization in altricial chicks. *Mol. Ecol.* 32: 3657–3671.

Dong Z., Zhang S., Cheng Y., He X., Haase I., Liang Y., Jiang Y. & Wu Y. 2022. Comparative analysis of the intestinal tract microbiota and feeding habits of five sympatric flycatchers. *Avian Res.* 13: 100050.

Drobniak S.M., Cichoń M., Janas K., Barczyk J., Gustafsson L. & Zagalska-Neubauer M. 2022. Habitat shapes diversity of gut microbiomes in a wild population of blue tits *Cyanistes caeruleus*. *J. Avian Biol.* 2022, e02829.

Drovetski S.V., O'Mahoney M.J.V., Matterson K.O., Schmidt B.K. & Graves G.R. 2019. Distinct microbiotas of anatomical gut regions display idiosyncratic seasonal variation in an avian folivore. *Anim. Microbiome.* 1: 2.

Du, X., Xiang, Y., Lou, F., Tu, P., Zhang, X., Hu, X., Lyu, W. & Xiao, Y. 2020. Microbial community and short-chain fatty acid mapping in the intestinal tract of quail. *Animals*. 10(6): 1006.

Ellegren H., Smeds L., Burri R., Olason P.I., Backström N., Kawakami T., Künstner A., Mäkinen H., Nadachowska-Brzyska K., Qvarnström A., Uebbing S. & Wolf J.B.W. 2012. The genomic landscape of species divergence in Ficedula flycatchers. *Nature* 491: 756–760.

Engel P. & Moran N.A. 2013. The gut microbiota of insects – diversity in structure and function. *FEMS Microbiol. Rev.* 37: 699–735.

Escallón, C., Becker, M.H., Walke, J.B., Jensen, R.V., Cormier, G., Belden, L.K. & Moore, I.T. 2017. Testosterone levels are positively correlated with cloacal bacterial diversity and the relative abundance of Chlamydiae in breeding male rufous-collared sparrows. *Funct. Ecol*. 31(1): 192-203.

Escallón, C., Belden, L.K. & Moore, I.T. 2019. The cloacal microbiome changes with the breeding season in a wild bird. *Integr. Org. Biol.* 1(1): oby009.

Fernández L., Langa S., Martín V., Maldonado A., Jiménez E., Martín R. & Rodríguez J.M. 2013. The human milk microbiota: Origin and potential roles in health and disease. *Pharmacol. Res.* 69: 1–10.

Ferretti P., Pasolli E., Tett A., Asnicar F., Gorfer V., Fedi S., Armanini F., Truong D.T., Manara S., Zolfo M., Beghini F., Bertorelli R., De Sanctis V., Bariletti I., Canto R., Clementi R., Cologna M., Crifò T., Cusumano G., Gottardi S., Innamorati C., Masè C., Postai D., Savoi D., Duranti S., Lugli G.A., Mancabelli L., Turroni F., Ferrario C., Milani C., Mangifesta M., Anzalone R., Viappiani A., Yassour M., Vlamakis H., Xavier R., Collado C.M., Koren O., Tateo S., Soffiati M., Pedrotti A., Ventura M., Huttenhower C., Bork P. & Segata N. 2018. Mother-to-Infant Microbial Transmission from Different Body Sites Shapes the Developing Infant Gut Microbiome. *Cell Host Microbe* 24: 133-145.e5.

Flint H.J., Bayer E.A., Rincon M.T., Lamed R. & White B.A. 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat. Rev. Microbiol.* 6: 121–131.

Florkowski, M.R., Hamer, S.A. & Yorzinski, J.L. 2023. Brief exposure to captivity in a songbird is associated with reduced diversity and altered composition of the gut microbiome. *FEMS Microbiol. Ecol.* 99(9): fiad096.

Fontaine S.S., Novarro A.J. & Kohl K.D. 2018. Environmental temperature alters the digestive performance and gut microbiota of a terrestrial amphibian. *J. Exp. Biol.* 221: jeb187559.

Forsman A. 2014. Effects of genotypic and phenotypic variation on establishment are important for conservation, invasion, and infection biology. *PNAS* 111: 302–307.

Fox R.J., Donelson J.M., Schunter C., Ravasi T. & Gaitán-Espitia J.D. 2019. Beyond buying time: the role of plasticity in phenotypic adaptation to rapid environmental change. *Philos. T. R. Soc. B.* 374: 20180174.

Funkhouser, L.J. & Bordenstein, S.R. 2013. Mom knows best: the universality of maternal microbial transmission. *PLoS biol.* 11(8): e1001631.

Furman O., Shenhav L., Sasson G., Kokou F., Honig H., Jacoby S., Hertz T., Cordero O.X., Halperin E. & Mizrahi I. 2020. Stochasticity constrained by deterministic effects of diet and age drive rumen microbiome assembly dynamics. *Nat. Commun.* 11: 1904.

Gadau A., Crawford M.S., Mayek R., Giraudeau M., McGraw K.J., Whisner C.M., Kondrat-Smith C. & Sweazea K.L. 2019. A comparison of the nutritional physiology and gut microbiome of urban and rural house sparrows (Passer domesticus). *Comp. Biochem. Phys. B.* 237: 110332.

Geiler-Samerotte K., Bauer C., Li S., Ziv N., Gresham D. & Siegal M. 2013. The Details in the Distributions: Why and how to study phenotypic variability. *Curr. opin. biotech.* 24: 752–759.

Glendinning, L., Jia, X., Kebede, A., Oyola, S.O., Park, J.E., Park, W., Assiri, A., Bak Holm, J., Kristiansen, K., Han, J. & Hanotte, O. 2024. Altitude-dependent agro-ecologies impact the microbiome diversity of scavenging indigenous chicken in Ethiopia. *Microbiome*. 12(1): 1-21.

Gong L., Liu B., Wu H., Feng J. & Jiang T. 2021. Seasonal Dietary Shifts Alter the Gut Microbiota of Avivorous Bats: Implication for Adaptation to Energy Harvest and Nutritional Utilization. *mSphere* 6: 10.1128/msphere.00467-21.

Góngora E., Elliott K.H. & Whyte L. 2021. Gut microbiome is affected by inter-sexual and inter-seasonal variation in diet for thick-billed murres (Uria lomvia). *Sci. Rep.* 11: 1200.

González-Braojos S., Vela A.I., Ruiz-de-Castañeda R., Briones V. & Moreno J. 2012. Age-related changes in abundance of enterococci and Enterobacteriaceae in Pied Flycatcher (Ficedula hypoleuca) nestlings and their association with growth. *J. Ornithol.* 153: 181–188.

Goodenough A.E., Stallwood B., Dandy S., Nicholson T.E., Stubbs H. & Coker D.G. 2017. Like mother like nest: similarity in microbial communities of adult female Pied Flycatchers and their nests. *J. Ornithol.* 158: 233–244.

Goodson N.J., Stevens D.R. & Bailey J.A. 1991. Effects of Snow on Foraging Ecology and Nutrition of Bighorn Sheep. *J. Wildl. Manag.* 55: 214–222.

Goossens E., Boonyarittichaikij R., Dekeukeleire D., Hertzog L., Van Praet S., Pasmans F., Bonte D., Verheyen K., Lens L., Martel A. & Verbrugghe E. 2022. Tree Species Diversity and Forest Edge Density Jointly Shape the Gut Microbiota Composition in Juvenile Great Tits (Parus major). *Front. Microbiol.* 13.

Gosler, A. 1993. The Great Tit. – Hamlyn.

Gould A.L., Zhang V., Lamberti L., Jones E.W., Obadia B., Korasidis N., Gavryushkin A., Carlson J.M., Beerenwinkel N. & Ludington W.B. 2018. Microbiome interactions shape host fitness. *PNAS* 115, e11951–e11960.

Grant P.R. & Grant B.R. 2000. Non–random fitness variation in two populations of Darwin's finches. *P. Roy. Soc. Lond. B. Bio.* 267: 131–138.

Grear, D.A., Perkins, S.E. & Hudson, P.J. 2009. Does elevated testosterone result in increased exposure and transmission of parasites?. *Ecol. Lett.* 12(6): 528-537.

Grond K., Lanctot R.B., Jumpponen A. & Sandercock B.K. 2017. Recruitment and establishment of the gut microbiome in arctic shorebirds. *FEMS Microbiol. Ecol.* 93: fix142.

Grond K., Sandercock B.K., Jumpponen A. & Zeglin L.H. 2018. The avian gut microbiota: community, physiology and function in wild birds. *J. Avian Biol.* 49, e01788.

Grond K., Santo Domingo J.W., Lanctot R.B., Jumpponen A., Bentzen R.L., Boldenow M.L., Brown, S.C., Casler, B., Cunningham, J.A., Doll, A.C., Freeman, S., Hill, B.L., Kendall, S.J., Kwon, E., Liebezeit, J.R., Pirie-Dominix, L., Rausch, J. & Sandercock B.K. 2019. Composition and drivers of gut microbial communities in arctic-breeding shorebirds. *Front. microbiol.* 10, 2258.

Grond K., Louyakis A.S. & Hird S.M. 2023. Functional and Compositional Changes in the Fecal Microbiome of a Shorebird during Migratory Stopover Ishaq S.L. (ed.). *mSystems* 8, e01128-22.

Gustafsson L. 1985. Fitness factors in the collared flycatcher Ficedula Albicollis. Unpublished Ph. D. thesis, Department of Zoology, University of Uppsala, Sweden.

Gustafsson L., 1986. Lifetime Reproductive Success and Heritability: Empirical Support for Fisher's Fundamental Theorem. The American Naturalist 128, 761–764. https://doi.org/10.1086/284601

Halupka L., Borowiec M., Neubauer G. & Halupka K. 2021. Fitness consequences of longer breeding seasons of a migratory passerine under changing climatic conditions. *J Anim. Ecol.* 90: 1655–1665.

Hammer T.J., Janzen D.H., Hallwachs W., Jaffe S.P. & Fierer N. 2017. Caterpillars lack a resident gut microbiome. *PNAS* 114: 9641–9646.

Hammer T.J., Sanders J.G. & Fierer N. 2019. Not all animals need a microbiome. *FEMS Microbiol. Lett.* 366: fnz117.

Hanning I. & Diaz-Sanchez S. 2015. The functionality of the gastrointestinal microbiome in non-human animals. *Microbiome* 3: 51.

Hansen C.H.F., Nielsen D.S., Kverka M., Zakostelska Z., Klimesova K., Hudcovic T., Tlaskalova-Hogenova H. & Hansen A.K. 2012. Patterns of Early Gut Colonization Shape Future Immune Responses of the Host. *PLOS ONE* 7, e34043.

Hartig F. & Hartig M.F. 2017. Package 'dharma'. *R package*.

Hawn A.T., Radford A.N. & Du Plessis M.A. 2007. Delayed Breeding Affects Lifetime Reproductive Success Differently in Male and Female Green Woodhoopoes. *Curr. Biol.* 17: 844–849.

Hegner R.E. & Wingfield J.C. 1987. Effects of Brood-Size Manipulations on Parental Investment, Breeding Success, and Reproductive Endocrinology of House Sparrows. *The Auk* 104: 470–480.

Hemborg C. & Lundberg A. 1998. Costs of overlapping reproduction and moult in passerine birds: an experiment with the pied flycatcher. *Behav. Ecol. Sociobiol.* 43: 19–23.

Henry, L.P., Bruijning, M., Forsberg, S.K. & Ayroles, J.F. 2021. The microbiome extends host evolutionary potential. *Nat. Comms.* 12(1): 5141.

Hicks A.L., Lee K.J., Couto-Rodriguez M., Patel J., Sinha R., Guo C., Olson S.H., Seimon A., Seimon T.A., Ondzie A.U., Karesh W.B., Reed P., Cameron K.N., Lipkin W.I. & Williams B.L. 2018. Gut microbiomes of wild great apes fluctuate seasonally in response to diet. *Nat. Commun.* 9: 1786.

Hird S.M., Carstens B.C., Cardiff S.W., Dittmann D.L. & Brumfield R.T. 2014. Sampling locality is more detectable than taxonomy or ecology in the gut microbiota of the brood-parasitic Brown-headed Cowbird (Molothrus ater). *PeerJ* 2, e321.

Hird S.M., Sánchez C., Carstens B.C. & Brumfield R.T. 2015. Comparative Gut Microbiota of 59 Neotropical Bird Species. *Front. Microbiol.* 6.

Hird S.M. 2017. Evolutionary Biology Needs Wild Microbiomes. *Front. Microbiol.*8: 725.

Holmes E., Li J.V., Marchesi J.R. & Nicholson J.K. 2012. Gut microbiota composition and activity in relation to host metabolic phenotype and disease risk. *Cell Metab.* 16: 559–564.

Hooper L.V., Littman D.R. & Macpherson A.J. 2012. Interactions Between the Microbiota and the Immune System. *Science* 336: 1268–1273.

Hõrak P., Tegelmann L., Ots I. & Møller A.P. 1999. Immune function and survival of great tit nestlings in relation to growth conditions. *Oecologia* 121: 316–322.

Hõrak P. 2003. When to pay the cost of reproduction? A brood size manipulation experiment in great tits (Parus major). *Behav. Ecol. Sociobiol.* 54: 105–112.

Houwenhuyse S., Stoks R., Mukherjee S. & Decaestecker E. 2021. Locally adapted gut microbiomes mediate host stress tolerance. *ISME J.* 15: 2401–2414.

Hussain, T., Murtaza, G., Kalhoro, D.H., Kalhoro, M.S., Metwally, E., Chughtai, M.I., Mazhar, M.U. & Khan, S.A., 2021. Relationship between gut microbiota and host-metabolism: Emphasis on hormones related to reproductive function. *Anim. Nutr.* 7:1–10. https://doi.org/10.1016/j.aninu.2020.11.005

Ingala M.R., Albert L., Addesso A., Watkins M.J. & Knutie S.A. 2021. Differential effects of elevated nest temperature and parasitism on the gut microbiota of wild avian hosts. *Anim. Microbiome* 3: 67.

Jašarević E., Morrison K.E. & Bale T.L. 2016. Sex differences in the gut microbiome–brain axis across the lifespan. *Philos. T. R. Soc. B* 371: 20150122.

Jensen H., SÆther B.-E., Ringsby T.H., Tufto J., Griffith S.C. & Ellegren H. 2004. Lifetime reproductive success in relation to morphology in the house sparrow Passer domesticus. *J Anim. Ecol.* 73: 599–611.

Jia T., Zhao S., Knott K., Li X., Liu Y., Li Y., Chen Y., Yang M., Lu Y., Wu J. & Zhang C. 2018. The gastrointestinal tract microbiota of northern white-cheeked gibbons (Nomascus leucogenys) varies with age and captive condition. *Sci. Rep.* 8: 3214.

Jin, J., Li, Q., Zhou, Q., Li, X., Lan, F., Wen, C., Wu, G., Li, G., Yan, Y., Yang, N. & Sun, C. 2024. Calcium Deposition in Chicken Eggshells: Role of Host Genetics and Gut Microbiota. *Poul. Sci.* 104073.

Jones I., Marsh K., Handby T.M., Hopkins K., Slezacek J., Bearhop S. & Harrison X.A. 2023. The influence of diet on gut microbiome and body mass dynamics in a capital-breeding migratory bird. *PeerJ* 11. e16682.

Kamada N., Chen G.Y., Inohara N. & Núñez G. 2013a. Control of pathogens and pathobionts by the gut microbiota. *Nat. Immunol.* 14: 685–690.

Kamada N., Seo S.-U., Chen G.Y. & Núñez G. 2013b. Role of the gut microbiota in immunity and inflammatory disease. *Nat. Rev. Immunol.* 13: 321–335.

Karasov W.H., Pinshow B., Starck J.M. & Afik D. 2004. Anatomical and Histological Changes in the Alimentary Tract of Migrating Blackcaps (Sylvia atricapilla): A Comparison among Fed, Fasted, Food-Restricted, and Refed Birds. *Physiol. Biochem. Zool.* 77: 149–160.

Karr J.R. 1976. Seasonality, Resource Availability, and Community Diversity in Tropical Bird Communities. *Am. Nat.* 110: 973–994.

Kartzinel T.R., Hsing J.C., Musili P.M., Brown B.R.P. & Pringle R.M. 2019. Covariation of diet and gut microbiome in African megafauna. *PNAS* 116: 23588–23593.

Kau A.L., Ahern P.P., Griffin N.W., Goodman A.L. & Gordon J.I. 2011. Human nutrition, the gut microbiome and the immune system. *Nature* 474: 327–336.

Knutie, S.A., Wilkinson, C.L., Kohl, K.D. & Rohr, J.R. 2017. Early-life disruption of amphibian microbiota decreases later-life resistance to parasites. *Nat. Commun.* 8(1): 86.

Knutie S.A. & Gotanda K.M. 2018. A Non-invasive Method to Collect Fecal Samples from Wild Birds for Microbiome Studies. *Microb. Ecol.* 76: 851–855.

Knutie S.A., Chaves J.A. & Gotanda K.M. 2019. Human activity can influence the gut microbiota of Darwin's finches in the Galapagos Islands. *Mol. Ecol.* 28: 2441–2450.

Kohl K.D. 2012. Diversity and function of the avian gut microbiota. *J. Comp. Physiol. B.* 182: 591–602.

Kohl K.D. & Yahn J. 2016. Effects of environmental temperature on the gut microbial communities of tadpoles. *Environ. Microbiol.* 18: 1561–1565.

Kohl K.D., Brun A., Bordenstein S.R., Caviedes-Vidal E. & Karasov W.H. 2018. Gut microbes limit growth in house sparrow nestlings (*Passer domesticus*) but not through limitations in digestive capacity. *Integr. Zool.* 13: 139–151.

Koskella, B., Hall, L.J. & Metcalf, C.J.E. 2017. The microbiome beyond the horizon of ecological and evolutionary theory. *Nat. Ecol. Evol.* 1(11): 1606-1615.

Krebs J.R. 1971. Territory and Breeding Density in the Great Tit, Parus Major L. *Ecology* 52: 2–22.

Krebs J.R., Erichsen J.T., Webber M.I. & Charnov E.L. 1977. Optimal prey selection in the great tit (Parus major). *Anim. Behav.* 25: 30–38.

Kreisinger, J., Čížková, D., Kropáčková, L. and Albrecht, T., 2015. Cloacal microbiome structure in a long-distance migratory bird assessed using deep 16sRNA pyrosequencing. *PloS one*. 10(9): p.e0137401.

Kvist L., Ruokonen M., Lumme J. & Orell M. 1999. The colonization history and present-day population structure of the European great tit (Parus major major). *Heredity* 82: 495–502.

Lee C.Y., Peralta-Sánchez J.M., Martínez-Bueno M., Møller A.P., Rabelo-Ruiz M., Zamora-Muñoz C. & Soler J.J. 2020. The gut microbiota of brood parasite and host nestlings reared within the same environment: disentangling genetic and environmental effects. *ISME J.* 14: 2691–2702.

Leonard M.L. 2000. Brood size and begging intensity in nestling birds. *Behav. Ecol.* 11: 196–201.

Le Roy, C.I., Woodward, M.J., Ellis, R.J., La Ragione, R.M. & Claus, S.P. 2019. Antibiotic treatment triggers gut dysbiosis and modulates metabolism in a chicken model of gastro-intestinal infection. *BMC Vet. Res.* 15: 1-13.

Ley R.E., Hamady M., Lozupone C., Turnbaugh P.J., Ramey R.R., Bircher J.S., Schlegel M.L., Tucker T.A., Schrenzel M.D., Knight R. & Gordon J.I. 2008. Evolution of Mammals and Their Gut Microbes. *Science* 320: 1647–1651.

Li M., Wang B., Zhang M., Rantalainen M., Wang S., Zhou H., Zhang Y., Shen J., Pang X., Zhang M., Wei H., Chen Y., Lu H., Zuo J., Su M., Qiu Y., Jia W., Xiao C., Smith L.M., Yang S., Holmes E., Tang H., Zhao G., Nicholson J.K., Li L. & Zhao L. 2008. Symbiotic gut microbes modulate human metabolic phenotypes. *PNAS* 105: 2117–2122.

Liang, X., Zhang, Z., Wang, H., Lu, X., Li, W., Lu, H., Roy, A., Shen, X., Irwin, D.M. & Shen, Y. 2023. Early-life prophylactic antibiotic treatment disturbs the stability of the gut microbiota and increases susceptibility to H9N2 AIV in chicks. *Microbiome*. 11(1): 163.

Liukkonen, M., Hukkanen, M., Cossin-Sevrin, N., Stier, A., Vesterinen, E., Grond, K. & Ruuskanen, S. 2023. No evidence for associations between brood size, gut microbiome diversity and survival in great tit (Parus major) nestlings. *Anim. Microbiome*. 5(1): 19.

Liukkonen, M., Muriel, J., Martínez-Padilla, J., Nord, A., Pakanen, V.-M., Rosivall, B., Tilgar, V., van Oers, K., Grond, K. & Ruuskanen, S. 2024. Seasonal and environmental factors contribute to the variation in the gut microbiome: A large-scale study of a small bird. *J. Anim. Ecol.* 00: 1–18.

Loo W.T., García-Loor J., Dudaniec R.Y., Kleindorfer S. & Cavanaugh C.M. 2019. Host phylogeny, diet, and habitat differentiate the gut microbiomes of Darwin's finches on Santa Cruz Island. *Sci. Rep.* 9: 18781.

Love M., Anders S. & Huber W. 2014. Differential analysis of count data–the DESeq2 package. *Genome Biol. 15*(550), 10-1186.

Lozupone C. & Knight R. 2005. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. *Appl. Environ. Microb.* 71: 8228–8235.

Lozupone C.A., Stombaugh J.I., Gordon J.I., Jansson J.K. & Knight R. 2012. Diversity, stability and resilience of the human gut microbiota. *Nature* 489: 220–230.

Luo J., Cheng Y., Guo L., Wang A., Lu M. & Xu L. 2021. Variation of gut microbiota caused by an imbalance diet is detrimental to bugs' survival. *Sci. Total Environ.* 771: 144880.

Ma L., Hahn M.E., Karchner S.I., Nacci D., Clark B.W. & Apprill A. 2024. Environmental and Population influences on Mummichog (Fundulus heteroclitus). *Gut Microbes*. 2024.01.26.577513.

Mackie R.I., Sghir A. & Gaskins H.R. 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am. J. Clin. Nutr.* 69: 1035S-1045S.

Mainwaring M.C., Hartley I.R., Bearhop S., Brulez K., Feu C.R. du, Murphy G., Plummer K.E., Webber S.L., James Reynolds S. & Deeming D.C. 2012. Latitudinal variation in blue tit and great tit nest characteristics indicates environmental adjustment. *J. Biogeogr.* 39: 1669–1677.

Maki, J.J., Klima, C.L., Sylte, M.J. & Looft, T. 2019. The microbial pecking order: utilization of intestinal microbiota for poultry health. Microorganisms. 7(10): 376.

Mallott E.K., Borries C., Koenig A., Amato K.R. & Lu A. 2020. Reproductive hormones mediate changes in the gut microbiome during pregnancy and lactation in Phayre's leaf monkeys. *Sci. Rep.* 10: 9961.

Mallott E.K. & Amato K.R. 2021. Host specificity of the gut microbiome. *Nat. Rev. Microbiol.* 19: 639–653.

Maraci Ö., Antonatou-Papaioannou A., Jünemann S., Engel K., Castillo-Gutiérrez O., Busche T., Kalinowski J. & Caspers B.A. 2022a. Timing matters: age-dependent impacts of the social environment and host selection on the avian gut microbiota. *Microbiome* 10: 202.

Maraci Ö., Corsini M., Antonatou-Papaioannou A., Jünemann S., Sudyka J., Di Lecce I., Caspers B.A. & Szulkin M. 2022b. Changes to the gut microbiota of a wild juvenile passerine in a multidimensional urban mosaic. *Sci. Rep.* 12: 6872.

Maritan E., Quagliariello A., Frago E., Patarnello T. & Martino M.E. 2024. The role of animal hosts in shaping gut microbiome variation. *Philos. T. Roy. Soc. B.* 379: 20230071.

Markle J.G.M., Frank D.N., Mortin-Toth S., Robertson C.E., Feazel L.M., Rolle-Kampczyk U., Bergen M. von, McCoy K.D., Macpherson A.J. & Danska J.S. 2013. Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity. *Science* 339: 1084–1088.

Martínez I., Maldonado-Gomez M.X., Gomes-Neto J.C., Kittana H., Ding H., Schmaltz R., Joglekar P., Cardona R.J., Marsteller N.L., Kembel S.W., Benson A.K., Peterson D.A., Ramer-Tait A.E. & Walter J. 2018. Experimental evaluation of the importance of colonization history in early-life gut microbiota assembly Ley R.E. & Garrett W.S. (eds.). *eLife* 7, e36521.

Martínez-Mota R., Kohl K.D., Orr T.J. & Denise Dearing M. 2020. Natural diets promote retention of the native gut microbiota in captive rodents. *ISME J.* 14: 67–78.

Mathewson N.D., Jenq R., Mathew A.V., Koenigsknecht M., Hanash A., Toubai T., Oravecz-Wilson K., Wu S.-R., Sun Y., Rossi C., Fujiwara H., Byun J., Shono Y., Lindemans C., Calafiore M., Schmidt T.M., Honda K., Young V.B., Pennathur S., Brink M. van den & Reddy P. 2016. Gut microbiome–derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. *Nat. Immunol.* 17: 505–513.

Maurice C.F., CL Knowles S., Ladau J., Pollard K.S., Fenton A., Pedersen A.B. & Turnbaugh P.J. 2015. Marked seasonal variation in the wild mouse gut microbiota. *ISME J.* 9: 2423–2434.

McFall-Ngai M., Hadfield M.G., Bosch T.C.G., Carey H.V., Domazet-Lošo T., Douglas A.E., Dubilier N., Eberl G., Fukami T., Gilbert S.F., Hentschel U., King N., Kjelleberg S., Knoll A.H., Kremer N., Mazmanian S.K., Metcalf J.L., Nealson K., Pierce N.E., Rawls J.F., Reid A., Ruby E.G., Rumpho M., Sanders J.G., Tautz D. & Wernegreen J.J. 2013. Animals in a bacterial world, a new imperative for the life sciences. *PNAS* 110: 3229–3236.

McMurdie P.J. & Holmes S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE* 8, e61217.

McMurdie P.J. & Holmes S. 2014. Waste not, want not: why rarefying microbiome data is inadmissible. *PLOS comput. biol.* 10(4): e1003531.

McWilliams S.R. & Karasov W.H. 2001. Phenotypic flexibility in digestive system structure and function in migratory birds and its ecological significance. *Comp. Biochem. Phys. A.* 128: 577–591.

Miller E.T., Svanbäck R. & Bohannan B.J.M. 2018. Microbiomes as Metacommunities: Understanding Host-Associated Microbes through Metacommunity Ecology. *Trends Ecol. Evol.* 33: 926–935.

Mitchell-Olds T., Willis J.H. & Goldstein D.B. 2007. Which evolutionary processes influence natural genetic variation for phenotypic traits? *Nat. Rev. Genet.* 8: 845–856.

Moeller A.H., Foerster S., Wilson M.L., Pusey A.E., Hahn B.H. & Ochman H. 2016. Social behavior shapes the chimpanzee pan-microbiome. *Sci. Adv.* 2, e1500997.

Moeller A.H., Suzuki T.A., Phifer-Rixey M. & Nachman M.W. 2018. Transmission modes of the mammalian gut microbiota. *Science* 362: 453–457.

Moreno J., Briones V., Merino S., Ballesteros C., Sanz J.J. & Tomás G. 2003. Beneficial Effects of Cloacal Bacteria on Growth and Fledging Size in Nestling Pied Flycatchers (Ficedula Hypoleuca) in Spain. *The Auk* 120: 784–790.

Morimoto J., Simpson S.J. & Ponton F. 2017. Direct and trans-generational effects of male and female gut microbiota in Drosophila melanogaster. *Biol. Letters* 13: 20160966.

Moriya T., Satomi Y., Murata S., Sawada H. & Kobayashi H. 2017. Effect of gut microbiota on host whole metabolome. *Metabolomics* 13: 101.

Mountzouris K.C., Tsirtsikos P., Kalamara E., Nitsch S., Schatzmayr G. & Fegeros K. 2007. Evaluation of the Efficacy of a Probiotic Containing *Lactobacillus, Bifidobacterium, Enterococcus,* and *Pediococcus* Strains in Promoting Broiler Performance and Modulating Cecal Microflora Composition and Metabolic Activities. *Poultry Sci.* 86: 309–317.

Moya A. & Ferrer M. 2016. Functional redundancy-induced stability of gut microbiota subjected to disturbance. *Trends microbiol.* 24(5): 402-413.

Muegge B.D., Kuczynski J., Knights D., Clemente J.C., González A., Fontana L., Henrissat B., Knight R. & Gordon J.I. 2011. Diet Drives Convergence in Gut Microbiome Functions Across Mammalian Phylogeny and Within Humans. *Science* 332: 970–974.

Mueller, U.G., & Sachs, J.L. 2015. Engineering microbiomes to improve plant and animal health. *Trends microbiol.* 23(10): 606-617.

Murga-Garrido S.M., Hong Q., Cross T.-W.L., Hutchison E.R., Han J., Thomas S.P., Vivas E.I., Denu J., Ceschin D.G., Tang Z.-Z. & Rey F.E. 2021. Gut microbiome variation modulates the effects of dietary fiber on host metabolism. *Microbiome* 9: 117.

Murillo T., Schneider D., Fichtel C. & Daniel R. 2022. Dietary shifts and social interactions drive temporal fluctuations of the gut microbiome from wild redfronted lemurs. *ISME Commun.* 2: 3.

Murray B.G. Jr. 2000. Measuring Annual Reproductive Success in Birds. *Condor* 102: 470–473.

Murray M.H., Lankau E.W., Kidd A.D., Welch C.N., Ellison T., Adams H.C., Lipp E.K. & Hernandez S.M. 2020. Gut microbiome shifts with urbanization and potentially facilitates a zoonotic pathogen in a wading bird Staley C. (ed.). *PLOS ONE* 15, e0220926.

Naef-Daenzer B. & Keller L.F. 1999. 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. *J Anim. Ecol.* 68: 708–718.

Naef-Daenzer L., Naef-Daenzer B. & Nager R.G. 2000. Prey selection and foraging performance of breeding Great Tits *Parus major* in relation to food availability. *J. Avian Biol.* 31: 206–214.

Nagata N., Tohya M., Fukuda S., Suda W., Nishijima S., Takeuchi F., Ohsugi M., Tsujimoto T., Nakamura T., Shimomura A., Yanagisawa N., Hisada Y., Watanabe K., Imbe K., Akiyama J., Mizokami M., Miyoshi-Akiyama T., Uemura N. & Hattori M. 2019. Effects of bowel preparation on the human gut microbiome and metabolome. *Sci. Rep.* 9: 4042.

Nager R.G. & Noordwijk A.J. van. 1995. Proximate and Ultimate Aspects of Phenotypic Plasticity in Timing of Great Tit Breeding in a Heterogeneous Environment. *Am. Nat.* 146: 454–474.

Neuenschwander S. 2003. Brood size, sibling competition, and the cost of begging in great tits (Parus major). *Behav. Ecol.* 14: 457–462.

Neuman H., Debelius J.W., Knight R. & Koren O. 2015. Microbial endocrinology: the interplay between the microbiota and the endocrine system. *FEMS Microbiol. Rev.* 39: 509–521.

Nichols R.G. & Davenport E.R. 2021. The relationship between the gut microbiome and host gene expression: a review. *Hum. Genet.* 140: 747–760.

Nicolaus M., Michler S.P.M., Ubels R., Van Der Velde M., Komdeur J., Both C. & Tinbergen J.M. 2009. Sex-specific effects of altered competition on nestling growth and survival: an experimental manipulation of brood size and sex ratio. *J Anim. Ecol.* 78: 414–426.

Noguera J.C., Aira M., Pérez-Losada M., Domínguez J. & Velando A. 2018. Glucocorticoids modulate gastrointestinal microbiome in a wild bird. *Roy. Soc. Open Sci.* 5: 171743.

Nowak J., Pawłowski B., Borkowska B., Augustyniak D. & Drulis-Kawa Z. 2018. No evidence for the immunocompetence handicap hypothesis in male humans. *Sci. Rep.* 8: 7392.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H. B. A., … Weedon, J. 2013. Package 'vegan'. Community ecology package, version 2.6-6.1.

Oliveira, B.C., Murray, M., Tseng, F. & Widmer, G. 2020. The fecal microbiota of wild and captive raptors. *Anim. Microbiome*. 2: 1-9.

O'Mahony, S.M., Felice, V.D., Nally, K., Savignac, H.M., Claesson, M.J., Scully, P., Woznicki, J., Hyland, N.P., Shanahan, F., Quigley, E.M., Marchesi, J.R., O'Toole, P.W., Dinan, T.G. & Cryan, J.F. 2014. Disturbance of the gut microbiota in early-life selectively affects visceral pain in adulthood without impacting cognitive or anxiety-related behaviors in male rats. *Neuroscience*. 277: 885-901.

Orell, M. 1989. Population fluctuations and survival of Great tits Par us major dependent on food supplied by man in winter. *Ibis*, *131*(1), 112-127.

Orkin J.D., Campos F.A., Myers M.S., Cheves Hernandez S.E., Guadamuz A. & Melin A.D. 2019. Seasonality of the gut microbiota of free-ranging white-faced capuchins in a tropical dry forest. *ISME J.* 13: 183–196.

Palmer C., Bik E.M., DiGiulio D.B., Relman D.A. & Brown P.O. 2007. Development of the Human Infant Intestinal Microbiota. *PLOS Biol.* 5: e177.

Parada A.E., Needham D.M. & Fuhrman J.A. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* 18: 1403–1414.

Parejo D. & Danchin E. 2006. Brood size manipulation affects frequency of second clutches in the blue tit. *Behav. Ecol. Sociobiol.* 60: 184–194.

Pasciullo Boychuck S., Brenner L.J., Gagorik C.N., Schamel J.T., Baker S., Tran E., vonHoldt B.M., Koepfli K.-P., Maldonado J.E. & DeCandia A.L. 2024. The gut microbiomes of Channel Island foxes and island spotted skunks exhibit

fine-scale differentiation across host species and island populations. *Ecol. Evol.* 14, e11017.

Pascoe E.L., Hauffe H.C., Marchesi J.R. & Perkins S.E. 2017. Network analysis of gut microbiota literature: an overview of the research landscape in non-human animal studies. *ISME J.* 11: 2644–2651.

Pérez-Cobas A.E., Maiques E., Angelova A., Carrasco P., Moya A. & Latorre A. 2015. Diet shapes the gut microbiota of the omnivorous cockroach Blattella germanica. *FEMS Microbiol. Ecol.* 91: fiv022.

Perofsky A.C., Lewis R.J., Abondano L.A., Di Fiore A. & Meyers L.A. 2017. Hierarchical social networks shape gut microbial composition in wild Verreaux's sifaka. *P. T. Roy. Soc. B* 284: 20172274.

Perrins C.M. 1979. *British tits* (Vol. 62). HarperCollins.

Pettifor R.A., Perrins C.M. & McCleery R.H. 1988. Individual optimization of clutch size in great tits. *Nature* 336: 160–162.

Phillips J.N., Berlow M. & Derryberry E.P. 2018. The Effects of Landscape Urbanization on the Gut Microbiome: An Exploration Into the Gut of Urban and Rural White-Crowned Sparrows. *Front. Ecol. Evol.* 6: 148.

Pickard J.M., Zeng M.Y., Caruso R. & Núñez G. 2017. Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. *Immunol. Rev.* 279: 70–89.

Piersma T. 1998. Phenotypic Flexibility during Migration: Optimization of Organ Size Contingent on the Risks and Rewards of Fueling and Flight? *J. Avian Biol.* 29: 511–520.

Pigeault R., Cozzarolo C.-S., Glaizot O. & Christe P. 2020. Effect of age, haemosporidian infection and body condition on pair composition and reproductive success in Great Tits Parus major. *Ibis* 162: 613–626.

Potti J. 1999. Environmental factors and sexual differences in mass and condition of nestling pied flycatchers Ficedula hypoleuca. *Ecoscience* 6: 19–24.

Potti J., Moreno J., Yorio P., Briones V., García-Borboroglu P., Villar S. & Ballesteros C. 2002. Bacteria divert resources from growth for magellanic penguin chicks: Bacteria affect penguin chick growth. *Ecol. Lett.* 5: 709–714.

Prüter, H., Gillingham, M.A., Krietsch, J., Kuhn, S. and Kempenaers, B., 2023. Sexual transmission may drive pair similarity of the cloacal microbiome in a polyandrous species. *J. Anim. Ecol. 92*(8): 1639-1657.

Ran J., Wan Q.-H. & Fang S.-G. 2021. Gut microbiota of endangered crested ibis: Establishment, diversity, and association with reproductive output. *PLOS ONE* 16, e0250075.

Rantala M.J., Moore F.R., Skrinda I., Krama T., Kivleniece I., Kecko S. & Krams I. 2012. Evidence for the stress-linked immunocompetence handicap hypothesis in humans. *Nat. Comm.* 3: 694.

Rao, A.V., Bested, A.C., Beaulne, T.M., Katzman, M.A., Iorio, C., Berardi, J.M. & Logan, A.C. 2009. A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. *Gut pathog*. 1: 1-6.

Raouf, S.A., Parker, P.G., Ketterson, E.D., Nolan Jr, V. & Ziegenfus, C. 1997. Testosterone affects reproductive success by influencing extra–pair

fertilizations in male dark–eyed juncos (Aves: Junco hyemalis). *Proc. R. Roc. B*. 264(1388): 1599-1603.

Rastelli M., Cani P.D. & Knauf C. 2019. The Gut Microbiome Influences Host Endocrine Functions. *Endocr. Rev.* 40: 1271–1284.

Ren T., Boutin S., Humphries M.M., Dantzer B., Gorrell J.C., Coltman D.W., McAdam A.G. & Wu M. 2017. Seasonal, spatial, and maternal effects on gut microbiome in wild red squirrels. *Microbiome* 5: 163.

Risely A., Waite D., Ujvari B., Klaassen M. & Hoye B. 2017. Gut microbiota of a long-distance migrant demonstrates resistance against environmental microbe incursions. *Mol. Ecol.* 26: 5842–5854.

Roberts M.L., Buchanan K.L. & Evans M.R. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. *Anim. Behav.* 68: 227–239.

RodrÍguez J.M., Murphy K., Stanton C., Ross R.P., Kober O.I., Juge N., Avershina E., Rudi K., Narbad A., Jenmalm M.C., Marchesi J.R. & Collado M.C. 2015. The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb. Ecol. Health D.* 26: 26050.

Rosenberg E. & Zilber-Rosenberg I. 2018. The hologenome concept of evolution after 10 years. *Microbiome* 6: 78.

Rosivall B., Szöllősi E., Hasselquist D. & Török J. 2009. Effects of extrapair paternity and sex on nestling growth and condition in the collared flycatcher, *Ficedula albicollis*. *Anim. Behav.* 77: 611–617.

Rosshart S.P., Vassallo B.G., Angeletti D., Hutchinson D.S., Morgan A.P., Takeda K., Hickman H.D., McCulloch J.A., Badger J.H., Ajami N.J., Trinchieri G., Villena F.P.-M. de, Yewdell J.W. & Rehermann B. 2017. Wild Mouse Gut Microbiota Promotes Host Fitness and Improves Disease Resistance. *Cell* 171: 1015-1028.e13.

Roughgarden J., Gilbert S.F., Rosenberg E., Zilber-Rosenberg I. & Lloyd E.A. 2018. Holobionts as Units of Selection and a Model of Their Population Dynamics and Evolution. *Biol. Theory* 13: 44–65.

Round J.L. & Mazmanian S.K. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9: 313–323.

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

Rätti, O., Hovi, M., Lundberg, A., Tegelström, H. & Alatalo, R. V. 1995. Extra-pair paternity and male characteristics in the pied flycatcher. *Behav. Ecol. Sociobiol.* 37: 419-425.

Salgado-Flores, A., Tveit, A.T., Wright, A.D., Pope, P.B. & Sundset, M.A. 2019. Characterization of the cecum microbiome from wild and captive rock ptarmigans indigenous to Arctic Norway. *PLoS One*. 14(3): e0213503.

Sanz J.J. 1997. Clutch Size Manipulation in the Pied Flycatcher: Effects on Nestling Growth, Parental Care and Moult. *J. Avian Biol.* 28: 157–162.

Sanz J.J. 1999. Energy expenditure, nestling age, and brood size: an experimental study of parental behavior in the great tit Parus major. *Behav. Ecol.* 10: 598–606.

Sanz J.J. & Tinbergen J.M. 1999. Energy expenditure, nestling age, and brood size: an experimental study of parental behavior in the great tit Parus major. *Behav. Ecol.* 10: 598–606.

Sarkar A., Harty S., Johnson K.V.A., Moeller A.H., Archie E.A., Schell L.D., Carmody R.N., Clutton-Brock T.H., Dunbar R.I.M. & Burnet P.W.J. 2020. Microbial transmission in animal social networks and the social microbiome. *Nat. Ecol. Evol.* 4: 1020–1035.

Schloss P.D. 2024. Waste not, want not: revisiting the analysis that called into question the practice of rarefaction. *Msphere* 9, e00355-23.

Schmiedová L., Tomášek O., Pinkasová H., Albrecht T. & Kreisinger J. 2022. Variation in diet composition and its relation to gut microbiota in a passerine bird. *Sci. Rep.* 12: 3787.

Schroeder P.J. & Jenkins D.G. 2018. How robust are popular beta diversity indices to sampling error? *Ecosphere* 9, e02100.

Segura Munoz R.R., Mantz S., Martínez I., Li F., Schmaltz R.J., Pudlo N.A., Urs K., Martens E.C., Walter J. & Ramer-Tait A.E. 2022. Experimental evaluation of ecological principles to understand and modulate the outcome of bacterial strain competition in gut microbiomes. *ISME J.* 16: 1594–1604.

Sekirov I., Russell S.L., Antunes L.C.M. & Finlay B.B. 2010. Gut Microbiota in Health and Disease. *Physiol. Rev.* 90: 859–904.

Shapira, M. 2016. Gut microbiotas and host evolution: scaling up symbiosis. *Trends Ecol. Evol.* 31(7): 539-549.

Sharpton T.J. 2018. Role of the Gut Microbiome in Vertebrate Evolution. *mSystems* 3: 10.1128/msystems.00174-17.

Sheldon, B.C. & Ellegren, H. 1999. Sexual selection resulting from extrapair paternity in collared flycatchers. *Anim. Behav.* 57(2): 285-298.

Shin J.-H., Park Y.-H., Sim M., Kim S.-A., Joung H. & Shin D.-M. 2019. Serum level of sex steroid hormone is associated with diversity and profiles of human gut microbiome. *Res. Microbiol.* 170: 192–201.

Siikamäki P. 1998. Limitation of Reproductive Success by Food Availability and Breeding Time in Pied Flycatchers. *Ecology* 79: 1789–1796.

Sims T.T., El Alam M.B., Karpinets T.V., Dorta-Estremera S., Hegde V.L., Nookala S., Yoshida-Court K., Wu X., Biegert G.W.G., Delgado Medrano A.Y., Solley T., Ahmed-Kaddar M., Chapman B.V., Sastry K.J., Mezzari M.P., Petrosino J.F., Lin L.L., Ramondetta L., Jhingran A., Schmeler K.M., Ajami N.J., Wargo J., Colbert L.E. & Klopp A.H. 2021. Gut microbiome diversity is an independent predictor of survival in cervical cancer patients receiving chemoradiation. *Commun. Biol.* 4: 1–10.

Skeen H.R., Willard D.E., Jones A.W., Winger B.M., Gyllenhaal E.F., Tsuru B.R., Hackett S.J. & Novembre J. 2023. Intestinal microbiota of Nearctic-Neotropical migratory birds vary more over seasons and years than between host species. *Mol. Ecol.* 32: 3290–3307.

Smith H.G., Källander H. & Nilsson J.-Å. 1987. Effect of Experimentally Altered Brood Size on Frequency and Timing of Second Clutches in the Great Tit. *The Auk* 104: 700–706.

Somers S.E., Davidson G.L., Johnson C.N., Reichert M.S., Crane J.M.S., Ross R.P., Stanton C. & Quinn J.L. 2023. Individual variation in the avian gut microbiota: The influence of host state and environmental heterogeneity. *Mol. Ecol.* 32: 3322–3339.

Sommer F. & Bäckhed F. 2013. The gut microbiota — masters of host development and physiology. *Nat. Rev. Microb.* 11: 227–238.

Sommer F., Ståhlman M., Ilkayeva O., Arnemo J.M., Kindberg J., Josefsson J., Newgard C.B., Fröbert O. & Bäckhed F. 2016. The Gut Microbiota Modulates Energy Metabolism in the Hibernating Brown Bear *Ursus arctos*. *Cell Rep.* 14: 1655–1661.

Song S.J., Sanders J.G., Delsuc F., Metcalf J., Amato K., Taylor M.W., Mazel F., Lutz H.L., Winker K., Graves G.R., Humphrey G., Gilbert J.A., Hackett S.J., White K.P., Skeen H.R., Kurtis S.M., Withrow J., Braile T., Miller M., McCracken K.G., Maley J.M., Ezenwa V.O., Williams A., Blanton J.M., McKenzie V.J. & Knight R. 2020. Comparative Analyses of Vertebrate Gut Microbiomes Reveal Convergence between Birds and Bats. *mBio* 11: 10.1128/mbio.02901-19.

Sprockett D., Fukami T. & Relman D.A. 2018. Role of priority effects in the early-life assembly of the gut microbiota. *Nat. Rev. Gastro. Hepat.* 15: 197–205.

Stevens B.R., Roesch L., Thiago P., Russell J.T., Pepine C.J., Holbert R.C., Raizada M.K. & Triplett E.W. 2021. Depression phenotype identified by using single nucleotide exact amplicon sequence variants of the human gut microbiome. *Mol. Psychiatr.* 26: 4277–4287.

Sun F., Chen J. & Yang Y. 2022. The avian gut microbiota: Diversity, influencing factors, and future directions. *Front. Microbiol.* 13.

Szász E., Jablonszky M., Krenhardt K., Markó G., Hegyi G., Herényi M., Laczi M., Nagy G., Rosivall B., Szöllősi E., Török J. & Garamszegi L.Z. 2019. Male territorial aggression and fitness in collared flycatchers: a long-term study. *Sci. Nat.* 106: 11.

Szöllősi E., Rosivall B., Hasselquist D. & Török J. 2009. The effect of parental quality and malaria infection on nestling performance in the Collared Flycatcher (Ficedula albicollis). *J. Ornithol.* 150: 519–527.

Tang K., Tao L., Wang Y., Wang Q., Fu C., Chen B., Zhang Z. & Fu Y. 2023. Temporal Variations in the Gut Microbiota of the Globally Endangered Sichuan Partridge (Arborophila rufipectus): Implications for Adaptation to Seasonal Dietary Change and Conservation. *Appl. Environ. Microb.* 89, e00747-23.

Tap J., Mondot S., Levenez F., Pelletier E., Caron C., Furet J.-P., Ugarte E., Muñoz-Tamayo R., Paslier D.L.E., Nalin R., Dore J. & Leclerc M. 2009. Towards the human intestinal microbiota phylogenetic core. *Environ. Microbiol.* 11: 2574–2584.

Těšický M., Schmiedová L., Krajzingrová T., Samblas M.G., Bauerová P., Kreisinger J. & Vinkler M. 2024. Nearly (?) sterile avian egg in a passerine bird. *FEMS Microbiol. Ecol.* 100: fiad164.

Teullet S., Tilak M.-K., Magdeleine A., Schaub R., Weyer N.M., Panaino W., Fuller A., Loughry W.J., Avenant N.L., Thoisy B. de, Borrel G. & Delsuc F. 2023.

Metagenomics uncovers dietary adaptations for chitin digestion in the gut microbiota of convergent myrmecophagous mammals. *mSystems* 8, e00388-23.

Teyssier A., Lens L., Matthysen E. & White J. 2018a. Dynamics of Gut Microbiota Diversity During the Early Development of an Avian Host: Evidence From a Cross-Foster Experiment. *Front. Microbiol.* 9: 1524.

Teyssier A., Rouffaer L.O., Saleh Hudin N., Strubbe D., Matthysen E., Lens L. & White J. 2018b. Inside the guts of the city: Urban-induced alterations of the gut microbiota in a wild passerine. *Sci. Total Environ.* 612: 1276–1286.

Teyssier A., Matthysen E., Hudin N.S., De Neve L., White J. & Lens L. 2020. Diet contributes to urban-induced alterations in gut microbiota: experimental evidence from a wild passerine. *P. Roy. Soc. B-Biol. Sci.* 287: 20192182.

Theis K.R., Dheilly N.M., Klassen J.L., Brucker R.M., Baines J.F., Bosch T.C.G., Cryan J.F., Gilbert S.F., Goodnight C.J., Lloyd E.A., Sapp J., Vandenkoornhuyse P., Zilber-Rosenberg I., Rosenberg E. & Bordenstein S.R. 2016. Getting the Hologenome Concept Right: an Eco-Evolutionary Framework for Hosts and Their Microbiomes Gilbert J.A. (ed.). *mSystems* 1, e00028-16.

Thie N., Corl A., Turjeman S., Efrat R., Kamath P.L., Getz W.M., Bowie R.C.K. & Nathan R. 2022. Linking migration and microbiota at a major stopover site in a long-distance avian migrant. *Mov. Ecol.* 10: 46.

Thompson I.D., Wiebe P.A., Mallon E., Rodgers A.R., Fryxell J.M., Baker J.A. & Reid D. 2015. Factors influencing the seasonal diet selection by woodland caribou (Rangifer tarandus tarandus) in boreal forests in Ontario. *Can. J. Zool.* 93: 87–98.

Thursby E. & Juge N. 2017. Introduction to the human gut microbiota. *Biochem. J.* 474: 1823–1836.

Tian Y., Li G., Chen L., Bu X., Shen J., Tao Z., Zeng T., Du X. & Lu L. 2020. High-temperature exposure alters the community structure and functional features of the intestinal microbiota in Shaoxing ducks (Anas platyrhynchos). *Poult. Sci.* 99: 2662–2674.

Tilg H. & Kaser A. 2011. Gut microbiome, obesity, and metabolic dysfunction. *J. Clin. Invest.* 121: 2126–2132.

Tinbergen J.M. & Verhulst S. 2000. A fixed energetic ceiling to parental effort in the great tit? *J Anim. Ecol.* 69: 323–334.

Tinya F., Kovács B., Bidló A., Dima B., Király I., Kutszegi G., Lakatos F., Mag Z., Márialigeti S., Nascimbene J., Samu F., Siller I., Szél G. & Ódor P. 2021. Environmental drivers of forest biodiversity in temperate mixed forests – A multi-taxon approach. *Sci. Total Environ.* 795: 148720.

Trevelline, B.K., MacLeod, K.J., Knutie, S.A., Langkilde, T. & Kohl, K.D. 2018. In ovo microbial communities: a potential mechanism for the initial acquisition of gut microbiota among oviparous birds and lizards. *Biol. Lett.* 14(7): 20180225.

Trevelline, B.K., Sosa, J., Hartup, B.K. & Kohl, K.D. 2020. A bird's-eye view of phylosymbiosis: weak signatures of phylosymbiosis among all 15 species of cranes. *Proc. Soc. B*. 287(1923): 20192988.

Trevelline, B.K. & Kohl, K.D. 2022. The gut microbiome influences host diet selection behavior. *Proc. Nat. Acad. Sci.* 119(17): e2117537119.

Trujillo S.M., McKenney E.A., Hilderbrand G.V., Mangipane L.S., Rogers M.C., Joly K., Gustine D.D., Erlenbach J.A., Mangipane B.A. & Lafferty D.J.R. 2022. Intrinsic and extrinsic factors influence on an omnivore's gut microbiome. *PLOS ONE* 17, e0266698.

Tung J., Barreiro L.B., Burns M.B., Grenier J.-C., Lynch J., Grieneisen L.E., Altmann J., Alberts S.C., Blekhman R. & Archie E.A. 2015. Social networks predict gut microbiome composition in wild baboons Alm E. (ed.). *eLife* 4, e05224.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R. E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knirght, R. & Gordon, J.I. 2009. A core gut microbiome in obese and lean twins. *Nature*. 457(7228): 480-484.

Vel'ký M., Kaňuch P. & Krištín A. 2011. Food composition of wintering great tits (Parus major): habitat and seasonal aspects. *Folia Zool.* 60: 228–236.

Verboven N. & Visser M.E. 1998. Seasonal Variation in Local Recruitment of Great Tits: The Importance of Being Early. *Oikos* 81: 511–524.

Verhulst S., Balen J.H. van & Tinbergen J.M. 1995. Seasonal Decline in Reproductive Success of the Great Tit: Variation in Time or Quality? *Ecology* 76: 2392–2403.

Videvall E., Strandh M., Engelbrecht A., Cloete S. & Cornwallis C.K. 2018. Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. *Mol. Ecol. Resour*. 18: 424–434.

Videvall E., Song S.J., Bensch H.M., Strandh M., Engelbrecht A., Serfontein N., Hellgren O., Olivier A., Cloete S., Knight R. & Cornwallis C.K. 2019. Major shifts in gut microbiota during development and its relationship to growth in ostriches. *Mol. Ecol.* 28: 2653–2667.

Vitousek M.N., Taff C.C., Ardia D.R., Stedman J.M., Zimmer C., Salzman T.C. & Winkler D.W. 2018. The lingering impact of stress: brief acute glucocorticoid exposure has sustained, dose-dependent effects on reproduction. *P. Roy. Soc. B-Biol. Sci.* 285: 20180722.

Vrieze, A., Out, C., Fuentes, S., Jonker, L., Reuling, I., Kootte, R.S., van Nood, E., Holleman, F., Knaapen, M., Romjin, J.A., Soeters, M.R., Blaak, E.E., Dallinga-Thie, G.M., Reijnders, D., Ackermans, M.T., Serlie, M.J., Knop, F.K., Holst, J.J., van der Ley, C., Kema, I.P., Zoetendal, E.G., de Vos, W.M., Hoekstra, H.B.L., Stroes, E.S., Groen, A.K. & Nieuwdorp, M. 2014. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. *Journal of hepatology*. 60(4): 824-831.

Wagner G.P. & Altenberg L. 1996. Perspective: complex adaptations and the evolution of evolvability. *Evolution* 50: 967–976.

Waite D.W. & Taylor M.W. 2014. Characterizing the avian gut microbiota: membership, driving influences, and potential function. *Front. microbiol.* 5: 91622.

Wang, Feng J.H., Zhang M.H., Li X.M., Ma D.D. & Chang S.S. 2018. Effects of high ambient temperature on the community structure and composition of ileal microbiome of broilers. *Poult. Sci.* 97: 2153–2158.

Wang, J., Wan, C., Shuju, Z., Yang, Z., Celi, P., Ding, X., Bai, S., Zeng, Q., Mao, X., Xu, S., Zhang, K. & Li, M. 2021. Differential analysis of gut microbiota and the effect of dietary Enterococcus faecium supplementation in broiler breeders with high or low laying performance. *Poult. Sci*. 100(2): 1109-1119.

Ward T.L., Weber B.P., Mendoza K.M., Danzeisen J.L., Llop K., Lang K., Clayton J.B., Grace E., Brannon J., Radovic I., Beauclaire M., Heisel T.J., Knights D., Cardona C., Kogut M., Johnson C., Noll S.L., Arsenault R., Reed K.M. & Johnson T.J. 2019. Antibiotics and Host-Tailored Probiotics Similarly Modulate Effects on the Developing Avian Microbiome, Mycobiome, and Host Gene Expression Zhang Q. (ed.). *mBio* 10, e02171-19.

Wendeln H. & Becker P.H. 1999. Effects of parental quality and effort on the reproduction of common terns. *J Anim. Ecol.* 68: 205–214.

Whitham, T.G., Bailey, J.K., Schweitzer, J.A., Shuster, S.M., Bangert, R.K., LeRoy, C.J., Lonsdorf, E.V., Allan, G.J., DiFazio, S.P., Potts, B.M., Fischer, D.G., Gehring, C.A., Lindroth, R.L., Marks, J.C., Hart, S.C., Wimp, G.M & Wooley, S.C. 2006. A framework for community and ecosystem genetics: from genes to ecosystems. *Nat. Rev. Gen*. 7(7): 510-523.

Wienemann, T., Schmitt-Wagner, D., Meuser, K., Segelbacher, G., Schink, B., Brune, A., & Berthold, P. (2011). The bacterial microbiota in the ceca of Capercaillie (Tetrao urogallus) differs between wild and captive birds. *Syst. Appl. Microbiol*. 34(7): 542-551.

Wilkin T.A., King L.E. & Sheldon B.C. 2009. Habitat quality, nestling diet, and provisioning behaviour in great tits Parus major. *J. Avian Biol.* 40: 135–145.

Williams C.M., Henry H.A.L. & Sinclair B.J. 2015. Cold truths: how winter drives responses of terrestrial organisms to climate change. *Biol. Rev.* 90: 214–235.

Williams C.L., Garcia-Reyero N., Martyniuk C.J., Tubbs C.W. & Bisesi J.H. 2020. Regulation of endocrine systems by the microbiome: Perspectives from comparative animal models. *Gen. Comp. Endocr.* 292: 113437.

Willing B.P., Dicksved J., Halfvarson J., Andersson A.F., Lucio M., Zheng Z., Järnerot G., Tysk C., Jansson J.K. & Engstrand L. 2010. A Pyrosequencing Study in Twins Shows That Gastrointestinal Microbial Profiles Vary With Inflammatory Bowel Disease Phenotypes. *Gastroenterology* 139: 1844-1854.e1.

Willmore K.E., Young N.M. & Richtsmeier J.T. 2007. Phenotypic Variability: Its Components, Measurement and Underlying Developmental Processes. *Evol. Biol.* 34: 99–120.

Wingfield, J.C. 1984. Androgens and mating systems: testosterone-induced polygyny in normally monogamous birds. *The Auk* 101(4): 665-671.

Witte C.L., Hungerford L.L., Papendick R., Stalis I.H. & Rideout B.A. 2010. Investigation of factors predicting disease among zoo birds exposed to avian mycobacteriosis. *J. Am. Vet. Med. Assoc.* 236: 211–218.

Woodhams D.C., Bletz M.C., Becker C.G., Bender H.A., Buitrago-Rosas D., Diebboll H., Huynh R., Kearns P.J., Kueneman J., Kurosawa E., LaBumbard

B.C., Lyons C., McNally K., Schliep K., Shankar N., Tokash-Peters A.G., Vences M. & Whetstone R. 2020. Host-associated microbiomes are predicted by immune system complexity and climate. *Genom. Biol.* 21: 23.

Worsley S.F., Davies C.S., Mannarelli M.-E., Hutchings M.I., Komdeur J., Burke T., Dugdale H.L. & Richardson D.S. 2021. Gut microbiome composition, not alpha diversity, is associated with survival in a natural vertebrate population. *Anim. Microbiome* 3: 84.

Worsley S.F., Mazel F., Videvall E., Harrison X.A., Björk J.R. & Wanelik K.M. 2024. Probing the functional significance of wild animal microbiomes using omics data. *EcoEvoRxiv*. DOI: 10.32942/x28p68.

Wright, Both, Cotton, & Bryant. 1998. Quality vs. quantity: energetic and nutritional trade-offs in parental provisioning strategies. *J Anim. Ecol.* 67: 620–634.

Wysocki D., Jankowiak Ł., Cholewa M. & Zyskowski D. 2019. Natal conditions, lifespan and lifetime reproductive success of European blackbirds. *Behav. Ecol.* 30: 1707–1714.

Xiao G., Liu S., Xiao Y., Zhu Y., Zhao H., Li A., Li Z. & Feng J. 2019. Seasonal Changes in Gut Microbiota Diversity and Composition in the Greater Horseshoe Bat. *Front. Microbiol.* 10.

Xue B., Sartori P. & Leibler S. 2019. Environment-to-phenotype mapping and adaptation strategies in varying environments. *PNAS* 116: 13847–13855.

Yang Y., Gao H., Li X., Cao Z., Li M., Liu J., Qiao Y., Ma L., Zhao Z. & Pan H. 2021. Correlation analysis of muscle amino acid deposition and gut microbiota profile of broilers reared at different ambient temperatures. *Animal* 34: 93–101.

Yoon H., Shaw J.L., Haigis M.C. & Greka A. 2021. Lipid metabolism in sickness and in health: Emerging regulators of lipotoxicity. *Mol. Cell* 81: 3708–3730.

Youngblut N.D., Reischer G.H., Walters W., Schuster N., Walzer C., Stalder G., Ley R.E. & Farnleitner A.H. 2019. Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. *Nat. Commun.* 10: 2200.

Zaneveld J.R., McMinds R. & Vega Thurber R. 2017. Stress and stability: applying the Anna Karenina principle to animal microbiomes. *Nat. Microbiol.* 2: 1–8.

Zenner, C., Hitch, T.C., Riedel, T., Wortmann, E., Tiede, S., Buhl, E.M., Abt, B., Neuhaus, K., Velge, P., Overmann, J., Kaspers. B. & Clavel, T. 2021. Early-life immune system maturation in chickens using a synthetic community of cultured gut bacteria. *Msystems*. 6(3): 10-1128.

Zhang C., Derrien M., Levenez F., Brazeilles R., Ballal S.A., Kim J., Degivry M.-C., Quéré G., Garault P., Hylckama Vlieg J.E.T. van, Garrett W.S., Doré J. & Veiga P. 2016. Ecological robustness of the gut microbiota in response to ingestion of transient food-borne microbes. *ISME J.* 10: 2235–2245.

Zhang Z., Yang Z. & Zhu L. 2021. Gut microbiome of migratory shorebirds: Current status and future perspectives. *Ecol. Evol.* 11: 3737–3745.

Zhu L., Liao R., Wu N., Zhu G. & Yang C. 2019. Heat stress mediates changes in fecal microbiome and functional pathways of laying hens. *Appl. Microbiol. Biot.* 103: 461–472.

Zilber-Rosenberg I. & Rosenberg E. 2008. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol. Rev.* 32: 723–735.

ORIGINAL PAPERS

I

SEASONAL AND ENVIRONMENTAL FACTORS CONTRIBUTE TO THE VARIATION IN THE GUT MICROBIOME: A LARGE-SCALE STUDY OF A SMALL BIRD

by

Martta Liukkonen, Jaime Muriel, Jesús Martínez-Padilla, Andreas Nord, Veli-Matti Pakanen, Balázs Rosivall, Vallo Tilgar, Kees van Oers, Kirsten Grond & Suvi Ruuskanen 2024

Journal of Animal Ecology 00: 1-18

https://doi.org/10.1111/1365-2656.14153

Received: 14 November 2023 | Accepted: 25 June 2024

DOI: 10.1111/1365-2656.14153

Journal of Animal Ecology | BRITISH ECOLOGICAL SOCIETY

RESEARCH ARTICLE

Seasonal and environmental factors contribute to the variation in the gut microbiome: A large-scale study of a small bird

Martta Liukkonen[1] | Jaime Muriel[2] | Jesús Martínez-Padilla[3] | Andreas Nord[4] | Veli-Matti Pakanen[5] | Balázs Rosivall[6] | Vallo Tilgar[7] | Kees van Oers[8] | Kirsten Grond[9] | Suvi Ruuskanen[1,2]

[1]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland; [2]Department of Biology, University of Turku, Turku, Finland; [3]Department of Biodiversity Conservation and Ecosystem Restoration, Pyrenean Institute of Ecology (IPE–CSIC), Jaca, Spain; [4]Department of Biology, Lund University, Lund, Sweden; [5]Ecology and Genetics Research Unit, University of Oulu, Oulu, Finland; [6]Behavioural Ecology Group, Department of Systematic Zoology and Ecology, ELTE Eötvös Loránd University, Budapest, Hungary; [7]Department of Zoology, Tartu University, Tartu, Estonia; [8]Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands and [9]Department of Biological Sciences, University of Alaska Anchorage, Anchorage, Alaska, USA

Correspondence
Martta Liukkonen
Email: martta.a.liukkonen@jyu.fi

Funding information
Vetenskapsrådet, Grant/Award Number: 2020-04686; Emil Aaltonen Foundation

Handling Editor: Hannah Watson

Abstract

1. Environmental variation can shape the gut microbiome, but broad/large-scale data on among and within-population heterogeneity in the gut microbiome and the associated environmental factors of wild populations is lacking. Furthermore, previous studies have limited taxonomical coverage, and knowledge about wild avian gut microbiomes is still scarce.
2. We investigated large-scale environmental variation in the gut microbiome of wild adult great tits across the species' European distribution range. We collected fecal samples to represent the gut microbiome and used the 16S rRNA gene sequencing to characterize the bacterial gut microbiome.
3. Our results show that gut microbiome diversity is higher during winter and that there are compositional differences between winter and summer gut microbiomes. During winter, individuals inhabiting mixed forest habitat show higher gut microbiome diversity, whereas there was no similar association during summer. Also, temperature was found to be a small contributor to compositional differences in the gut microbiome. We did not find significant differences in the gut microbiome among populations, nor any association between latitude, rainfall and the gut microbiome.
4. The results suggest that there is a seasonal change in wild avian gut microbiomes, but that there are still many unknown factors that shape the gut microbiome of wild bird populations.

KEYWORDS
avian microbiome, ecological adaptation, environmental variation, gut microbiome, *Parus major*, seasonal adaptation, the 16S rRNA gene

1 | INTRODUCTION

The role of the gut microbiome on host traits has been of interest to many researchers, and it has been connected to issues such as host obesity (Tilg & Kaser, 2011), allergies (McKenzie et al., 2017) and mental health (Du Toit, 2019; Lucas, 2018). Additionally, the importance of gut microbiome in evolutionary biology, including its role in metabolism, pathogen susceptibility and adaptation has been discussed (Alberdi et al., 2016; Hird, 2017; Kopac & Klassen, 2016) and the biological mechanisms of host–microbiome interactions have been debated (Rosenberg & Zilber-Rosenberg, 2018; Zilber-Rosenberg & Rosenberg, 2008). However, many of the studies are focusing on captive-bred species such as birds (e.g., van Veelen et al., 2020; Xie et al., 2016; Zhu et al., 2021), mammals (e.g., Antwis et al., 2019; Beli et al., 2018; Grond et al., 2021) and invertebrates (e.g., Morimoto et al., 2017; Walters et al., 2020), or small-scale variation within or among closely located populations (birds e.g., Berlow et al., 2021; Gadau et al., 2019; Davidson et al., 2021; Drobniak et al., 2022; Phillips et al., 2018; Worsley et al., 2021; and mammals e.g., Baniel et al., 2021; Murillo et al., 2022; Ren et al., 2017; Roche et al., 2023). To our knowledge, there are no studies investigating the large-scale variation in the gut microbiome of wild sedentary birds. Understanding the role of the gut microbiome in eco-evolutionary research requires studying associations between host microbiome and environmental variation in natural environmental conditions, across large biogeographical scales among and within populations and across taxa.

Interestingly, previous studies have found that there is large-scale intraspecific variation in gut microbiome across populations (Rothschild et al., 2018; Sullam et al., 2012). Population-level differences in gut microbiome have been demonstrated in various taxa, including humans (Gilbert et al., 2018), wild red squirrels (Ren et al., 2017), brown frogs (Tong et al., 2020) and several insect (Sabree & Moran, 2014) and fish species (Liu et al., 2016; Sullam et al., 2012, 2015). However, the environmental drivers behind the population differences are not always well understood. Furthermore, whereas mammalian gut microbiomes are largely defined by phylogeny, many studies have highlighted that environmental variation is likely more important for explaining gut microbiome variation in other taxa, especially birds (Grond et al., 2019; Loo et al., 2019).

Historically, birds have been largely neglected in microbiome research and only the recent years have shown an increasing interest in gut microbiome studies with (wild) birds (Bodawatta, Hird, et al., 2022; Waite & Taylor, 2014). Birds are a good model species for gut microbiome studies because (1) they inhabit every continent on Earth and their varying ecology and species diversity enables us to study host life history and environmental effects simultaneously (Bibby, 1999; Pereira & Cooper, 2006; Pigot et al., 2020; Rahbek & Graves, 2001; Winkler et al., 2002). As a result of bird species' dispersal across the Earth and the biannual migration for some species, birds have developed ways to adapt to a wide range of environmental conditions (Gregory et al., 2005; Koskimies, 1989). This makes them an interesting taxon for studying different mechanisms, such as patterns in the gut microbiome, associated with environmental variation (Grond et al., 2018). (2) Within a species, populations are known to differ in phenotype (Charmantier et al., 2008; Husby et al., 2010), and the gut microbiome may contribute to this phenotypic variation among populations. (3) Due to life-history traits such as egg laying, powered flight and migration, the avian gut microbiome may be different from that of, for example, mammals (Grond et al., 2018). Distinct morphological characteristics and the ability to fly have resulted in a high-energy requirement and fast metabolism both of which are influenced by the gut microbiome (Kohl, 2012). Yet, surprisingly, large-scale studies focusing on among-population variation, and the environmental variables explaining variation in the gut microbiome of wild birds among and within populations are still poorly studied (Capunitan et al., 2020; Hird et al., 2015).

Population-level differences in avian gut microbiomes could be a result of a specific habitat (Drobniak et al., 2022; Loo et al., 2019; Wu et al., 2018), or a set of environmental factors such as diet (Singh et al., 2017), temperature (Sepulveda & Moeller, 2020) and humidity (Tajima et al., 2007). For example, there is a strong seasonal change in the gut microbiome composition of wild mice, which has been suspected to be a result of the transition from an insect to a seed-based diet (Maurice et al., 2015). In thick-billed murres, *Uria lomvia* variation in the gut microbiome across the breeding season was explained by prey specialization and differences in diet and sex during the breeding season (Góngora et al., 2021). Similar effect was found in barn swallows *Hirundo rustica*: The swallow diet varied across the breeding season and was correlated with gut microbiome (Schmiedová et al., 2022). In birds, the associations between habitat characteristics and gut microbiome have been studied to some extent. In blue tits, *Cyanistes caeruleus* a population living in dense deciduous forests had a higher gut microbiome diversity than a population inhabiting open areas and hay meadows. This may be explained by dense forests having higher overall species abundance and therefore, food item diversity (i.e., diet) and abundance (Drobniak et al., 2022). Diet is also shown to have a positive effect on eastern bluebirds' *Sialia sialis* nestling gut microbiome; food supplementation increased the relative abundance of *Clostridium* spp. and was positively correlated with antibody response and lower parasite abundance, thus increasing nestling survival (Knutie, 2020).

Among the abiotic environmental factors, the association between temperature and humidity and the gut microbiome have also been studied, but mostly in other taxa than birds. This study focuses on endothermic species (for ectothermic species see e.g., Bestion et al., 2017; Fontaine et al., 2018; Kohl & Yahn, 2016; Moeller et al., 2020), which maintain their body temperature by generating heat via metabolism (Chevalier et al., 2015; Rosenberg & Zilber-Rosenberg, 2016). Part of this temperature maintenance has been connected to the gut microbiome; the gut microbiome composition of cold-exposed laboratory-bred mice *Mus musculus* changed to so-called cold microbiota, potentially helping the host to tolerate periods of higher energy demand (Chevalier et al., 2015). In another study with laboratory-bred mice a change in temperature and humidity together with the exposure to wild environment led to different gut microbiome composition than their wild and laboratory-bred counterparts that resided in lower temperature and humidity (Bär et al., 2020). These changes in the gut

microbiome can mediate changes at molecular level and thus, enable adaptation to varying environmental conditions. Temperature has also been shown to have effects on poultry gut microbiomes. Higher temperature can lead to increased gut microbiome species richness and significantly different gut microbiome composition (Wang, Chen, et al., 2018), and lower temperatures correlate with changes in bacterial composition and muscle amino acid deposition (Yang et al., 2021). In domestic Shaoxing ducks, *Anas platyrhynchos* exposure to higher temperatures increased gut microbial abundance and changed the metabolic and transcription-related pathways, which suggests that gut microbiome may have enabled host adaptation to a new thermal environment (Tian et al., 2020). Recent work with wild birds has also shown associations between temperature, host gut microbiome and host health, although the results are not conclusive (Dietz et al., 2022; Ingala et al., 2021).

The overall aim of this study was to characterize variation in the gut microbiome of wild adult great tit *Parus major* populations across the species' distribution range in Europe. The great tit is a well-known study species in the fields of ecology and evolution (Krebs, 1971) and provides an attractive study system as this species inhabits vast geographical areas and lives in highly seasonal environments thus, offering the possibility to study the drivers that affect seasonal and population-level variation in the gut microbiome. Here, we investigated how (1) population and season contribute to the variation in the gut microbiome, and (2) how environmental factors associated with population and season, such as latitude, habitat, average rainfall, average temperature and supplementary feeding during winter shape the gut microbiome. We expected to see larger seasonal differences in populations living at higher latitudes because abiotic environmental conditions such as snow coverage, rainfall and temperature vary more towards the polar regions (Anderson & Jetz, 2005; Williams et al., 2015). We predicted that summer season would result in higher gut microbiome diversity, because food abundance, diversity and time for food foraging is generally higher during summer than winter (Cody, 1981; Karr, 1976). Moreover, we predicted that individual and population-specific factors such as habitat, average temperature and rainfall significantly contribute to variation in the gut microbiome (Lewis et al., 2017; Murray et al., 2020). For example, a more biodiverse habitat may offer more diverse and abundant prey items and warmer temperatures and moderate rainfall a higher insect abundance, which could lead to higher microbiome diversity and differences in composition (Cox et al., 2019). To our knowledge, this is the first study to characterize how environmental variation at a biogeographical scale shapes the variation of the gut microbiome in a wild bird.

2 | METHODS

2.1 | Study area

Faecal samples were collected from wild adult great tits across Europe during winter (January and February) and summer (May and June, breeding season) in 2021 from eight different locations (Figure 1; Data S1). The aim was to collect samples from ca. 20 to 25 individuals from winter and 20 to 25 from summer from each location. All winter samples were collected from a specific mist netting area located at the supplementary feeding (supplement: sunflower seeds or sunflower seeds + peanuts) site. Due to difficult winter conditions such as colder temperatures and deeper snow coverage, we failed in collecting winter samples from the Westerheide and La Hiruela populations. During summer, samples were collected from breeding adult great tits that were caught at the nest box during chick-rearing stage, that is, all the birds in our study had started reproduction. In total, we collected 285 samples, of which 124 samples from winter and 161 samples from summer.

2.2 | Faecal sample collection

To capture wild great tits, we used mist nets and feeding traps during winter, and nest box traps during summer. Sample collection followed a protocol by Knutie and Gotanda (2018): adult great tits were captured and put inside a paper bag until defecation, which usually took between 5 and 15 min. Faecal samples were then placed straight into 1.5 mL Eppendorf tubes and kept on ice until they were placed in long-term storage in a −80°C freezer. Each bird was also ringed for identification, sexed, weighed (~0.1 g) and their wing length was measured with a metal ruler (~1 mm). Habitat characteristics and latitude were recorded at each population (population level), and temperature (average ambient temperature) and rainfall (average mm per day) data from 2 weeks prior to sampling of each individual bird (individual level) were collected from the European Climate Assessment and Dataset (Winkler et al., 2002), using the nearest weather station to the sampling location. We chose this 2-week time window based on our own gut microbiome studies conducted in the laboratory environment where temperature caused changes in the gut microbiome after 2 weeks of exposure to a new temperature regime (Davies, Ruuskanen et al., in preparation). Moreover, Davidson et al. (2020) noticed similar diet-induced changes under a 2-week period. During winter, birds of this study very likely used supplementary winter feeding as the birds were caught near the feeding station (Data S1). Because the type of supplementary feeding (sunflower seeds or peanuts) could influence the birds' gut microbiome, all of our winter analyses included the type of supplementary feed (population level). We acknowledge that other types of supplementary feeding have likely occurred as it is common practice by the general public. We did not record summer diet because birds were caught straight from the nest boxes during summer and summer supplementary feeding is not that common. Permits for capturing birds and sample collection were acquired by collaborators at each population.

2.3 | DNA extraction and sequencing

We extracted DNA from the collected faecal samples using the Qiagen PowerFecal Pro Kit and followed the manufacturers

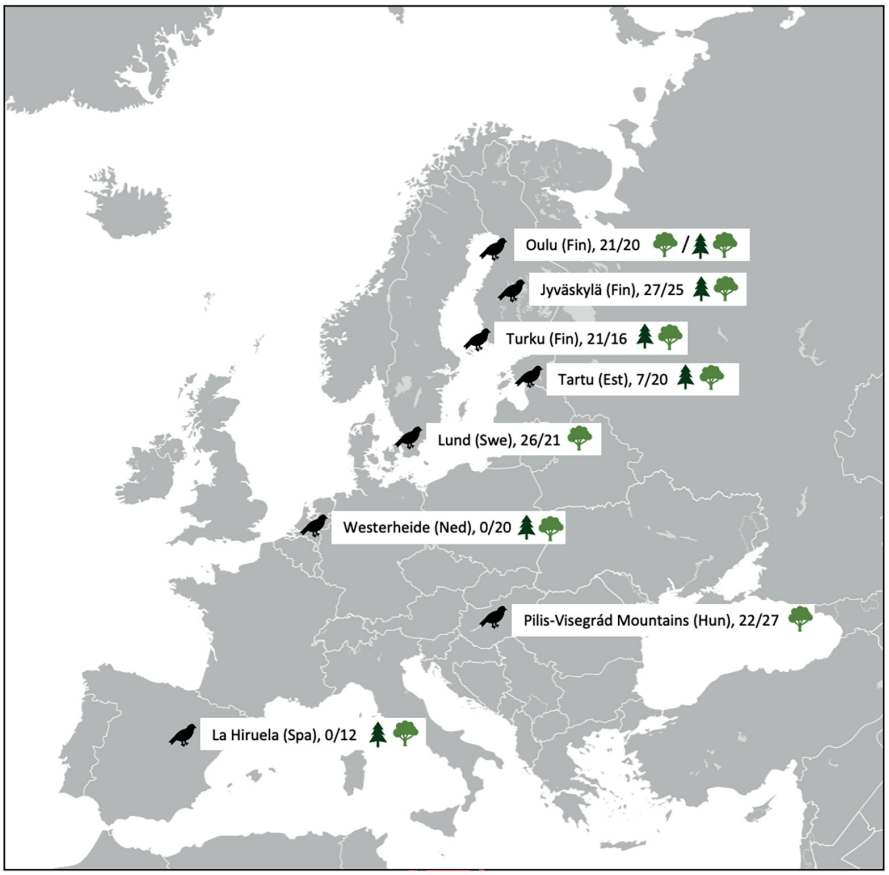

FIGURE 1 Locations, sample sizes (winter and summer) and habitat types of the eight different great tit populations across the species' distribution range.

protocol with minor adjustments: we added a 10-min incubation step at 65°C prior to lysis step and used a double elution (eluent was put through the filter twice) to improve DNA yield. To control for contamination and bias during DNA extraction, we included one negative control to each extraction batch and distributed samples from different populations to each extraction batch equally. After extraction, the V4 region of the 16S rRNA gene (approx. length 254 bp) was amplified using the following primers: 515F_Parada (5′-GTGYCAGCMGCCGCGGTAA-3′) (Parada et al., 2016) and 806R_Apprill (5′-GGACTACNVGGGTWTCTAAT-3′) (Apprill et al., 2015). A total volume of 12 μL was used in PCR reactions with MyTaq RedMix DNA polymerase (Meridian Bioscience, Cincinnati, OH, USA). We used the following PCR protocol: (1) an initial denaturation at 95°C for 3 min; (2) 30 cycles of 95°C for 45 s, 55°C for 60 s and 72°C for 90 s and (3) a 10-min extension at 72°C at the end. After the first round of PCR, a second round was conducted to apply barcodes for sample identification. For this, the protocol was (1) initial denaturation at 95°C for 3 min; (2) 18 cycles of 98°C for 20 s, 60°C for 15 s and 72°C for 30 s and (3) final extension at 72°C for 3 min. Each PCR plate also contained a negative control to control for contamination and a ZymoBIOMICS community standard (Zymo Research Corp., Irvine, CA, USA) to ensure successful amplification. PCR products' DNA concentration was measured with Quant-IT PicoGreen dsDNA Assay Kit (ThermoFischer Scientific, Waltham, MA, USA) and quality was checked with gel electrophoresis (1.5% TAE agarose gel). PCR products were then pooled equimolarly and purified using NucleoMag NGS Clean-up and Size Select beads (Macherey-Nagel, Düren, Germany). Finally, the pools were sequenced with Illumina Novaseq 6000 2 × 250 bp (San Diego, CA, USA) at the Finnish Functional Genomic Center at the University of Turku (Turku, Finland).

2.4 | Bioinformatics

The de-multiplexed sequence data was processed with QIIME2 version 2021.11 (Bolyen et al., 2018) following the 16S rRNA gene V4 region sequence processing protocol. Adapters were removed using the Cutadapt plugin version 4.4 (Martin, 2011) and quality scores were visually inspected. We used the DADA2 plugin version 2021.4.0 (Callahan et al., 2016) to truncate reads at 220 bp and to generate amplicon sequence variants (hereafter ASVs), which

stand for each individual bacterial sequence (Eren et al., 2013). We used the SILVA v132 database with the sk-learn classifier to assign taxonomy (Quast et al., 2013; Yilmaz et al., 2014). We used the phylogeny plugin to construct a rooted phylogenetic tree, and removed singletons, eukaryotes, mitochondria, archaea, chloroplasts and unassigned taxa in QIIME2 before further analysis. We then combined the resulting ASV table with metadata, taxonomy table and phylogenetic tree using the *phyloseq* package version 1.44.0 (McMurdie & Holmes, 2013) in R program version 4.3.0 (R Core Team). Contaminants ($N = 61$ ASVs) were removed using the *decontam* package version 1.20.0 (Davis et al., 2018). We also filtered samples that had less than 100 reads as they were likely a result of an error in amplification. The resulting data set had 15,288 ASVs in 284 samples (total number of reads in the whole data set 16,629,323, average number of reads per sample 57,740, median number of reads 17,189) (Data S2).

For downstream analyses of gut microbiome diversity (i.e., alpha diversity), the data set was rarified at 1000 reads based on the level at which the rarefaction curves plateaued. This was conducted to account for uneven sequencing depth between samples to normalize the data and to avoid the bias that rare taxa may have in the analyses (Cameron et al., 2020; Schloss, 2023; Weinroth et al., 2022). Seven samples were excluded from the data set in rarefying resulting in a total of 277 samples and 6883 ASVs, which divided into 121 winter samples and 156 summer samples. We tested both the rarefied and unrarefied data sets for consistency in gut microbiome diversity (Data S7). For analyses of gut microbiome composition (i.e., beta diversity), we used the unrarefied data set. For both gut microbiome diversity and composition analyses, we checked that the results were consistent between the unrarefied and rarefied data sets.

2.5 | Data analysis

2.5.1 | Gut microbiome diversity

We used Shannon Diversity Index and Chao1 Richness (Chao, 2006) as the gut microbiome diversity (i.e., alpha diversity) metrics using the *phyloseq* package version 1.44.0 (McMurdie & Holmes, 2013). In each model, we first ran the model with Shannon Diversity Index as the response variable and then with Chao1 Richness. We use these two metrics because Shannon Diversity Index considers both taxa abundance and evenness and Chao1 Richness measures the observed number of taxa. Chao1 Richness is more sensitive to rare taxa, whereas Shannon Diversity Index is more robust as it is not easily affected by the presence of rare taxa (Haegeman et al., 2013). For all gut microbiome diversity analyses, we use the rarefied dataset ($N = 277$). Additionally, we use both body condition (linear regression residual of weigh ~ wing) and weight as proxies for individual condition: We ran each model first with body condition and then with weight replacing body condition. Because some birds escaped prior to measurements, and in one population wing length was not recorded, we do not have a weight and wing measurement for every bird in this study (total of 46 birds from three different populations).

All statistical analyses were conducted in R program version 4.3.0 (R Core Team). Normality and homoscedasticity of the residuals were visually assessed. Variance inflation factors (VIFs) were assessed for each model with the package *DHARMa* version 0.4.6 (Hartig & Hartig, 2017). Linear mixed effects models were conducted using the packages *lme4* version 1.1-33 (Bates et al., 2014) and *car* version 3.1.2 (Fox et al., 2012).

First, we used a linear model to test if season (categories: winter and summer) and population (six categories) contribute to the gut microbiome diversity across all samples. We used gut microbiome diversity as the response variable and population and season as the predicting variables. VIF values suggest that there was no multicollinearity between factors (VIFs < 4). We also ran this same model with an interaction between season and population to test for population differences across seasons (Data S5). In these models, the Westerheide and La Hiruela populations were excluded as those populations were only measured during summer and including them may bias the results. Oulu was set as the population reference level because it was the northernmost of our study populations.

Second, we tested in more detail, which environmental factors across and within populations and seasons associated with the variation in gut microbiome diversity. We ran a linear mixed effects model with gut microbiome diversity as the response variable and the following fixed factors: latitude (continuous variable), habitat (categories: mixed and deciduous), rainfall (continuous variable) and temperature (continuous variable) using data across both seasons. Sex (category variable) and body condition/weight (continuous variable) were also included in the model as fixed factors to control for individual differences within population because physiological factors may contribute to variation in the gut microbiome (Amato et al., 2019; Corl et al., 2020; Góngora et al., 2021; Jašarević et al., 2016; Ley et al., 2008; Zhao et al., 2013). Population was included as a random effect as multiple individuals were sampled within each population. In these models, we excluded the Westerheide and La Hiruela populations as those populations were only recorded during summer. VIF values suggest that there was no multicollinearity between factors (VIFs < 4).

Third, because of the uneven sample sizes for winter and summer observations and because diet was only monitored during winter, we analysed gut microbiome diversity separately by season. For winter data ($N_{samples} = 121$), we ran a linear mixed effects model to analyse whether latitude, habitat, temperature, rainfall and supplementary feeding (categories: sunflower seeds and sunflower seeds + peanuts) contribute to gut microbiome diversity in populations during winter. Again, body condition/weight and sex were also included in the model and fixed factors and population as a random effect. The type of winter model supplementary feeding (categories: sunflower seeds and sunflower seeds + peanuts) as an explanatory variable as we sampled individuals at the supplementary winter-feeding site and the birds frequently visited the feeding site. VIF values suggest that there was no multicollinearity between factors (VIFs < 4).

For summer data ($N_{samples}=156$), we ran a similar model as we did for the winter data. We used gut microbiome diversity as the response variable and latitude, habitat, rainfall, temperature and body condition/weight and sex as explanatory variables. Population was included as a random effect in this model as well. VIF values suggest that there was no multicollinearity between factors (VIFs < 4). For each model, we tested the significance factors using *F*-test ratios in analysis of variance (ANOVA, Sattertwaithe's method for calculating degrees of freedom).

2.5.2 | Gut microbiome composition

For gut microbiome composition (i.e., beta diversity), we used the *microbiome* package version 1.22.0 (Lahti & Shetty, 2018). We visualized the gut microbiome compositions between populations and seasons with non-metric multidimensional scaling. For these visualizations, we used the Bray–Curtis dissimilarity metric that examines the dissimilarity of microbes among samples (Bray & Curtis, 1957). To analyse variation in gut microbiome communities among populations, we used permutational analysis of variance (PERMANOVA; *vegan* package, version 2.6-4, Oksanen et al., 2013) with the *adonis2* function and 9999 permutations. We constructed these PERMANOVA models the same way as we did the multiple linear regression models for the gut microbiome diversity measurements. First, we analysed whether season and population contribute to the variation in gut microbiome composition. Second, we analysed whether latitude, body condition/weight, habitat, rainfall and temperature and sex contribute to the variation in gut microbiome composition across both seasons. Third, we used the winter and summer data subsets to analyse associations between the gut microbiome composition and environmental variables within season. We tested the homogeneity of variance (beta dispersion), which showed similar dispersion for populations (BETADISPER^{9999}, $F_5=1.104$, $p=0.374$) and seasons (BETADISPER^{9999}, $F_1=1.417$, $p=0.235$), thus affirming that PERMANOVA is appropriate for comparing community compositions.

We also ran a differential abundance analysis (DESeq2) to see whether there are differences in bacterial taxa abundance within populations between winter and summer. For this analysis, we only used the populations that have both winter and summer data ($N=6$ populations) because the aim of this analysis was to compare within-population differences in taxa abundance between seasons. All taxa are identified at least to family level and some to genus level. Unfortunately, many of these observed taxa are less studied and their functions in the gastrointestinal tract, especially beyond humans, are not known. Furthermore, changes in functionality may not change gut microbiome diversity or composition (Moya & Ferrer, 2016). Here, we focus on the taxa that are more studied in gut microbiome research. For visualizing the DESeq2 results, we used order level to make the plot readable. We used the package *DESeq2* version 1.40.1 (Love et al., 2014) for the differential abundance analysis.

3 | RESULTS

3.1 | Gut microbiome diversity among populations and across both seasons

There were 27 bacterial phyla detected across all samples, and the most abundant phyla were Proteobacteria, Actinobacteria and Firmicutes. While there was variation in gut microbiome between populations, population did not significantly influence gut microbiome diversity (Figure 2a; Table 1; Data S4). Season significantly influenced gut microbiome diversity when Shannon Diversity Index was used as the response variable ($p=0.011$, Figure 2b; Table 1; Data S4): diversity was higher in winter than in summer. We found no

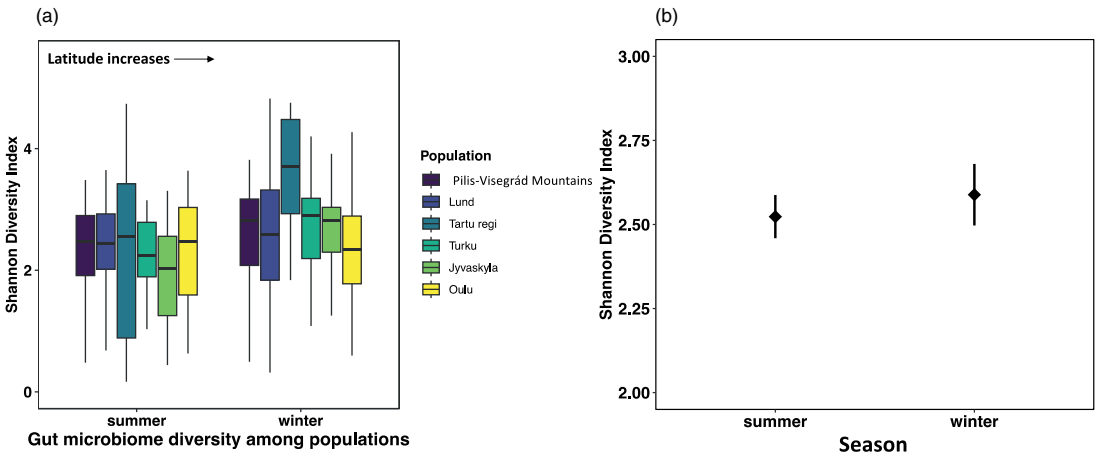

FIGURE 2 (a) Gut microbiome diversity (Shannon Diversity Index) among populations and between seasons ordered by latitude (south to north) and (b) season controlling for among-population gut microbiome diversity (mean and standard error).

TABLE 1 Association between gut microbiome diversity (Shannon and Chao1) and (A) population and season, (B) latitude, habitat, temperature, rainfall, body condition and sex between seasons, (C) latitude, habitat, temperature, rainfall, type of supplementary feeding, body condition and sex during winter and (D) latitude, habitat, temperature, rainfall, body condition and sex during summer.

Shannon	R^2/R adj.	df	F	p	Chao1	R^2/R adj.	df	F	p
(A) Both seasons	0.034/0.010	$N=239$				0.015/−0.010	$N=239$		
Population		5	0.541	0.745			5	0.491	0.783
Season		1	6.630	0.011*			1	1.618	0.205
	R^2/R adj.	df	χ^2	p	Chao1	R^2/R adj.	df	χ^2	p
(B) Both seasons		$N=225$					$N=225$		
Latitude		1	2.994	0.084			1	1.128	0.288
Habitat		1	2.304	0.129			1	0.674	0.412
Temperature		1	4.121	0.042*			1	2.030	0.154
Rainfall		1	0.291	0.590			1	0.151	0.698
Body condition		1	2.102	0.147			1	0.082	0.774
Sex		1	0.059	0.809			1	1.930	0.165
(C) Winter		$N=102$					$N=102$		
Latitude		1	0.069	0.792			1	1.342	0.247
Habitat		1	5.030	0.025*			1	0.594	0.441
Temperature		1	0.689	0.406			1	0.003	0.960
Rainfall		1	0.201	0.654			1	0.011	0.918
Suppl. feed. type		1	1.278	0.258			1	1.023	0.312
Body condition		1	2.627	0.105			1	1.179	0.278
Sex		1	0.258	0.612			1	1.023	0.312
(D) Summer		$N=129$					$N=129$		
Latitude		1	2.103	0.147			1	0.162	0.688
Habitat		1	0.428	0.513			1	1.960	0.162
Temperature		1	0.011	0.917			1	2.055	0.152
Rainfall		1	0.060	0.807			1	0.641	0.424
Body condition		1	1.819	0.177			1	0.137	0.712
Sex		1	2.007	0.157			1	0.003	0.959

Note: Linear model was used in (A) and linear mixed effects models in (B–D). The ANOVA output with Satterthwaite's method is reported in the table. Statistically significant values ($p < 0.05$) are indicated with a *.

significant interaction between season and population (p all >0.05, Data S5). Furthermore, latitude, habitat, average rainfall and body condition did not associate with gut microbiome diversity when the model included populations from both seasons (Table 1; Data S4). However, temperature negatively associated with gut microbiome diversity: lower temperatures correlated with higher Shannon diversity ($p=0.042$, Table 1; Data S4 and S8). When Chao1 Richness was used as the response variable, none of the explanatory factors significantly contributed to gut microbiome diversity (Table 1; Data S4). Population as a random effect did not contribute to gut microbiome diversity (var. < 0.000, SD < 0.000).

3.2 | Winter subset

In winter, gut microbiome diversity was higher in individuals inhabiting mixed forests than deciduous forests when measured with Shannon Diversity Index ($p=0.025$, Figure 3; Table 1; Data S4), but not when measured with Chao1 Richness (Table 1; Data S4). The result was the same for habitat when body condition was replaced with weight in the model (Shannon $p=0.033$, Data S9; Chao1 $p=0.218$, Data S4). Latitude, temperature, rainfall and supplementary feeding (p all >0.05) did not contribute to gut microbiome diversity in any of the models (Table 1, see Data S4) and neither did population as a random effect (var. < 0.000, SD < 0.000).

3.3 | Summer subset

Neither latitude, habitat, temperature and rainfall nor body condition/weight and sex contributed to gut microbiome diversity during summer (p all >0.05, Table 1; see Data S4). Population as a random effect did not contribute to gut microbiome diversity (var. < 0.000, SD < 0.000).

3.4 | Gut microbiome composition

As with gut microbiome diversity, visual observation of the gut microbiome composition showed that there was population-level variation in composition (Figure 4). However, PERMANOVA showed that population did not significantly contribute to differences in gut microbiome composition among populations ($R^2=0.021$, $p=0.397$, Figure 4; Data S6).

PERMANOVA showed that there were significant differences in composition between seasons, but season only explained 0.5% of these differences ($R^2=0.005$, $p=0.034$, Data S6). Of the environmental factors, temperature explained 0.6% of differences in gut microbiome composition in all data across both seasons ($R^2=0.006$, $p=0.012$, Figure 5; Data S6). When looking at the winter and summer subsets of data, none of the measured factors explained the differences in gut microbiome composition (p all >0.05, Data S6).

Seasonal differences in bacterial taxa abundance were detected in each population (Figure 6; Data S7), and some of these taxa were of interest to us due to their known beneficial or pathogenic effects. Of the well-known taxa, the order Bacillales were more abundant in Pilis-Visegrád Mountains, Turku and Jyväskylä during summer than winter, and more abundant in Oulu during winter than summer. The order Bifidobacteriales were more abundant in Turku during winter than summer. The order Chlamydiales were more abundant in Turku and Jyväskylä during summer than winter. The order Enterobacteriales were more abundant in Oulu during summer than winter. The order Lactobacillales were more abundant in Pilis-Visegrád Mountains during summer than winter and in Turku during winter than summer. The order Micrococcales were more abundant in Tartu, Lund, Pilis-Visegrád Mountains, Oulu and Turku during winter than summer.

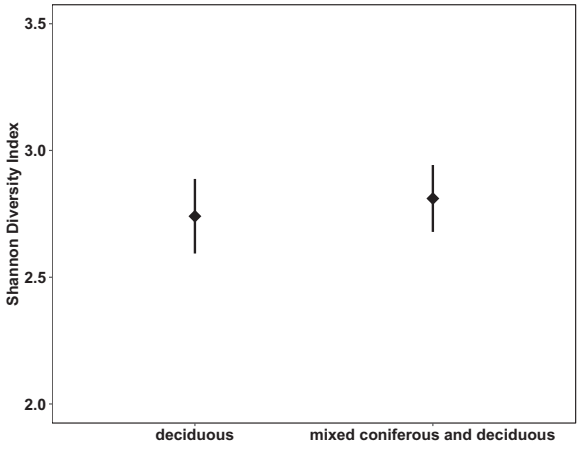

FIGURE 3 Gut microbiome diversity (mean and standard error) in two different habitats during winter. Populations inhabiting deciduous habitats are Oulu, Lund and Pilis-Visegrád Mountains and populations inhabiting mixed habitat are Jyväskylä, Turku and Tartu.

4 | DISCUSSION

The goal of this study was to characterize large-scale variation in wild adult great tit gut microbiomes and analyse whether environmental factors associated with population and season associate with the gut microbiome. Most of bacterial taxa in our samples belonged to the phyla Proteobacteria, Firmicutes and Actinobacteria, which was expected as they are the key phyla of

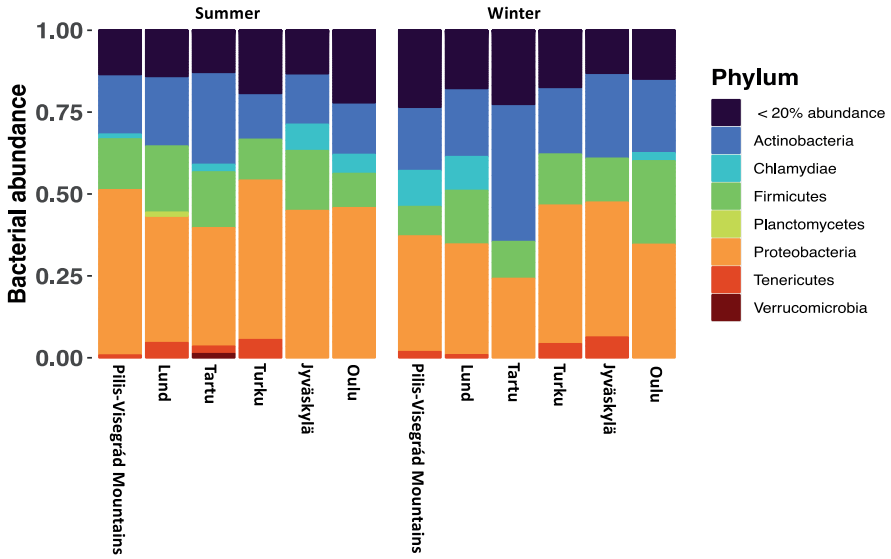

FIGURE 4 Among-population comparison of gut microbiome relative abundance on phylum level between seasons. Less abundant phyla are summed up as '<20% abundance' to improve plot readability.

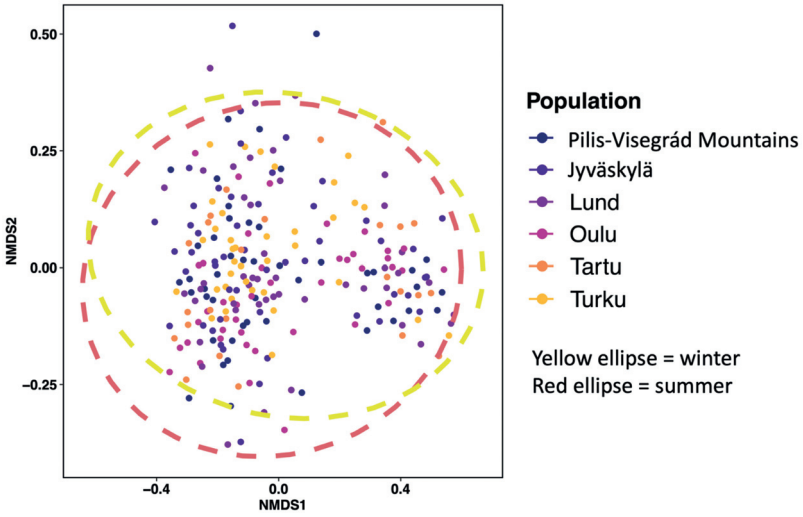

FIGURE 5 Non-metric multidimensional scaling (NMDS) measured with Bray–Curtis dissimilarity representing the microbial composition dissimilarity among populations between winter and summer. Points are coloured according to population. Ellipses represent 95% confidence intervals.

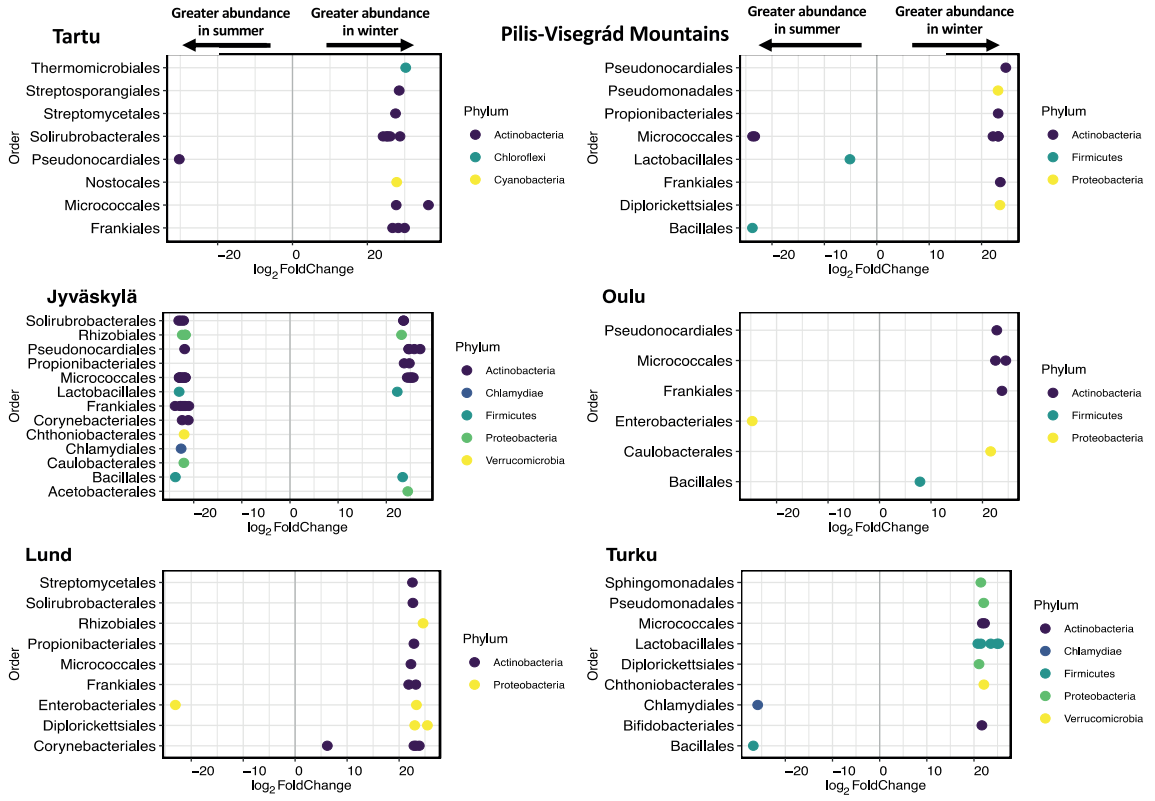

FIGURE 6 Visualization of the differential abundance analysis comparing six great tit populations between winter (positive log_2FoldChange) and summer (negative log_2FoldChange). Each dot represents one taxon within a bacterial order. All taxa are identified at least to Family level (Data S7), but for figure readability they are plotted on order level.

great tit gut microbiome (as described in Bodawatta, Freiberga, et al., 2021; Teyssier, Lens, et al., 2018). We did not find large-scale among population variation in the gut microbiome diversity or composition. Instead, we found the gut microbiome diversity (Shannon) and composition to be dependent on seasons and diversity (Shannon) to be dependent on habitats during the winter season. Variation in Chao1 Richness was not significantly explained by the predictors we investigated. This most likely means that there was no significant association between the number of bacterial taxa and our predictors. We found a slight

negative association between average temperature and Shannon diversity and average temperature was little associated with gut microbiome composition. We did not find evidence for the effects of latitude or average rainfall conditions, and much of the variation was left unexplained suggesting unknown sources of variation.

4.1 | No major differences among populations in microbiome diversity or composition

We found no evidence for population differences in the gut microbiome diversity or composition. This is surprising in light of apparent differences in environmental factors and given that great tits also show population-level differences in physiology (Saulnier et al., 2023) phenotypes (Dingemanse et al., 2012; Gamero et al., 2015) and minor genetic differentiation (Lemoine et al., 2016; Noordwijk et al., 2002), which have been shown to contribute to among-population variation in the gut microbiome of various taxa (Gadau et al., 2019; Meng et al., 2014; Spor et al., 2011; Wang, Chen, et al., 2018; Wen et al., 2021). We observed high within-population variation in gut microbiome diversity, which was expected because especially in smaller bird species individual variation in gut microbiome diversity has been found to be very high (as mentioned in Bodawatta, Koane, et al., 2021). In our study, all populations were in forested habitats with high plant species diversity, which could explain why large among-population differences in the gut microbiome were not observed [compared with e.g., the observed microbiome differences between urban and rural habitats (Phillips et al., 2018; Teyssier et al., 2020)].

4.2 | Gut microbiome diversity and composition differ across seasons

We found that gut microbiome diversity (Shannon) is higher in great tit populations during winter than summer and that gut microbiome composition varies between seasons. This is in line with many studies reporting seasonal variation in the gut microbiome (Baniel et al., 2021; Davenport et al., 2014; Góngora et al., 2021; Ren et al., 2017; Xiao et al., 2019). However, we expected that the gut microbiome diversity and composition would be lower during winter due to limited foraging times and the breadth of available dietary items for great tits (Grubb, 1978; McNamara et al., 1994; Vel'ký et al., 2011). During winter, great tits can use both insects (lepidopterans, coleopterans and dipterans), plant material (seeds and buds) and human provided food, compared to mostly insectivorous diet during summer (Vel'ký et al., 2011). The birds in our study populations were able to use supplementary feeding during winter. This supplementary feeding could reflect to their gut microbiome and possibly explain the higher gut microbiome diversity during winter: we did not provide any supplementary food during summer and therefore, part of the seasonal differences could be a result of the supplemented diet. The speculation would also follow some previous studies in which diet diversity has been connected to gut microbiome diversity (Jones et al., 2023; Knutie et al., 2019; Teyssier, Rouffaer, et al., 2018). Higher gut microbiome diversity during winter could also relate to bacterial functions in the gut. Cold exposure can increase gut microbiome diversity and enhance digestion (Fontaine et al., 2018). It can also increase energy intake and gut absorption and thus, improve the host's ability to tolerate cold (Chevalier et al., 2015; Zhang et al., 2018). Moreover, bacterial taxa such as Firmicutes that produce short-chain fatty acids and are responsible for carbohydrate and energy metabolic pathways could be more active during winter months when birds need to maintain their body temperature (Den Besten et al., 2013; Grond et al., 2018; Sun et al., 2016).

We observed within-population seasonal shifts in taxa abundances that could potentially associate with the variation between winter and summer diets. However, we can only speculate what the functions of these differentially abundant taxa are within the great tit gut microbiome, as there is a major lack of data regarding bacterial taxonomic functionality in wild animals (Worsley et al., 2024). Of the populations we sampled Oulu, Jyväskylä and Turku were the most northern and experienced the widest range of environmental changes between winter and summer and are therefore expected to show the largest changes. The rest of the populations were located more to the south and west of Europe, which can mean milder seasonal changes in environment and less snow cover (Baker, 1939). Of the six populations compared here, Jyväskylä showed the most differences in between-season taxa abundance and Oulu the least differences, which was opposite to what we expected. The order Enterobacteriales was more abundant in Lund, Pilis-Visegrád Mountains and Oulu during summer than winter. Many bacterial taxa belonging to the order Enterobacteriales such as *Salmonella enterica* and *Escherichia coli* are known pathogens in birds (Cheville & Arp, 1978; Tizard, 2004). The order Chlamydiales was more abundant in Jyväskylä and Turku during summer than winter. These pathogens are likely to be more abundant during summer, because individual birds can pass them on to other individuals during copulation (Escallón et al., 2019; Grond et al., 2018). The order Bacillales (not to be mixed with Lactobacillales), which contains several pathogenic genera such as *Staphylococcus*, *Bacillus* and *Listeria*, was also more abundant in Pilis-Visegrád Mountains, Jyväskylä and Turku during summer than winter and more abundant in Oulu during winter than summer.

Of the beneficial taxa, the order Lactobacillales abundance varied between populations: they were more abundant during winter than summer in Turku and more abundant during summer than winter in Pilis-Visegrád Mountains and Jyväskylä. Especially the genus *Lactobacillus* of the order Lactobacillales is known for its importance digestive health (Reid & Burton, 2002), and these beneficial health effects are also known from poultry (Al-Khalaifah, 2018). *Lactobacillus* species are found in the gut microbiome of many species, and they are known for their beneficial functions in the gut. Lactobacilli are involved in host metabolism via, for example, carbohydrate transport and metabolism, amino acid metabolism and protein synthesis and thus, influence the main metabolic pathways

of the host individual (De Angelis et al., 2016). Lactobacilli can protect the host against incoming potentially pathogenic microbes, and they influence host gene expression in, for example, immune and epithelial cells (Tappenden & Deutsch, 2007). Furthermore, it has been suggested that beneficial gut microbes such as Lactobacilli have coevolved with the host because of they improve host health (Backhed et al., 2005; Ley et al., 2006; Walter, 2008).

4.3 | Habitat associates with gut microbiome diversity, but not composition, during winter

Mixed forest associated with higher gut microbiome diversity than deciduous forest during winter, but not during summer. There were no differences in microbiome composition between habitats. Habitats with mixed tree and other plant species promote diversity in forest-associated taxa (Ampoorter et al., 2020; Tinya et al., 2021), resulting in a wider range of dietary items for the great tits. A more diverse diet has been found to associate with higher gut microbiome diversity (Bodawatta, Klečková, et al., 2022) and could also explain why great tits inhabiting mixed forest had more diverse gut microbiomes during winter. Higher gut microbiome diversity can potentially improve the stability of the gut microbiome and benefit the host. Generally, a more diverse gut microbiome is more stable because functionally similar taxa can potentially replace one another and therefore, the host is more tolerant to changes in the gut microbiome (Lozupone et al., 2012). Also, as the gut microbiome is involved in, for example, host metabolism and digestion by breaking down dietary items into compounds that can be used by the host, a diverse gut microbiome can influence host nutritional uptake and physiology (Grond et al., 2018).

Furthermore, breeding greatly influences physiology (Norte et al., 2010) and gut microbiome diversity (Escallón et al., 2019; Góngora et al., 2021; Zheng et al., 2020). Such physiological changes could overrun effects of the environment, such as the habitat, in the samples collected during the breeding season (but see Drobniak et al., 2022). It also leaves us questioning whether the differences in gut microbiome diversity between habitats would appear later during summer. As breeding comes with a great physiological cost (Norte et al., 2010), the gut microbiome may change prior, during and after the breeding season (Escallón et al., 2019).

4.4 | Weak associations between abiotic and intrinsic biotic factors on the gut microbiome variation

We found no association between latitude, rainfall, winter supplementary feeding and gut microbiome diversity or body condition/weight, sex and gut microbiome diversity. However, we did find that lower average temperature was associated with higher gut microbiome diversity (Shannon, but not Chao1) and that temperature was also weakly linked to gut microbiome composition.

The result regarding gut microbiome diversity was opposite to our prediction that lower temperature would lead to lower gut microbiome diversity. However, as expected temperature associated with microbiome composition, which follows previous studies with bird gut microbiomes (Dietz et al., 2022; Ingala et al., 2021; Tian et al., 2020; Wang, Chen, et al., 2018; Yang et al., 2021) and mammal studies (e.g., Worthmann et al., 2017; Zhang et al., 2018). Great tits are an endothermic species, which most likely means that ambient temperatures may not have major effects in the gut microbiome diversity/composition (Ingala et al., 2021) even though in mice studies effects between temperature and gut microbiome have been found (Chevalier et al., 2015). However, many of the previous studies were conducted with extreme temperatures and in captive conditions. For example, in egg laying hens spells of extreme hot temperatures lead to a decrease in Firmicutes abundance, a taxon that is known for its importance in short-chain fatty acid metabolism (Zhu et al., 2019). It is likely that the slight association between temperature and the gut microbiome is a result of the populations being in different parts of Europe and thus, they experience a varying range of temperatures throughout the year. Furthermore, this negative association between temperature and gut microbiome diversity was only significant in the analysis with both seasons included. It is likely that this result is connected to the result in which winter was associated with higher gut microbiome diversity.

Furthermore, both rainfall and snowfall can affect food item diversity and abundance and reflect on the gut microbiome diversity (Baniel et al., 2021; Schmiedová et al., 2023). For example, rainfall can influence insect abundances during summer, which are significant dietary items for great tits (Schöll et al., 2016). Severe weather can also limit foraging time leading to temporary depletion in food intake (Brittingham & Temple, 1988). This can result in increased physiological stress that has been shown to impact the gut microbiome diversity (Noguera et al., 2018). However, this limited foraging time may be more reflected on the nestlings (Radford et al., 2001) as the gut microbiome is established at the nestling stage (Davidson et al., 2021; Teyssier, Lens, et al., 2018).

We found no association between the type of supplementary food during winter and the gut microbiome. We provided sunflower seeds or peanuts or the mix of those two, which may not significantly change the gut microbiome, and most importantly, great tits will additionally use a wide variety of other food items within and across all populations. Finding associations between supplemented food quantity or quality/diet and the gut microbiome requires more fine-tuned experiments such as captive experiments in which dietary items and food intake are carefully monitored (such as Teyssier et al., 2020). Also, sampling the same individuals at multiple timepoints could be used to see possible longitudinal changes the gut microbiome (as suggested in Davidson et al., 2021).

Our results concerning body condition/weight are in line with recent studies that have not found a single conclusion between the gut microbiome diversity/composition and body condition. Here, gut microbiome diversity was not associated with individual body condition/weight. Generally, a higher body condition has been

connected to a higher gut microbiome diversity as it improves host gut stability (Lozupone et al., 2012) and this diversity is especially important in adult individuals as it can improve their overall fitness (Jones et al., 2023). Previous studies investigating the relationship between body condition and the gut microbiome with birds however show mixed results (Davidson et al., 2021; Kohl et al., 2018; Phillips et al., 2018; Teyssier, Lens, et al., 2018; Worsley et al., 2021). In nestling great tits, one study found that better body condition connected to higher gut microbiome diversity (Teyssier, Lens, et al., 2018), whereas in another study there was no association between the two factors (Liukkonen et al., 2023). In adult birds, there was no association between body condition and the gut microbiome diversity in Seychelles' warblers *Acrocephalus sechellensis* (Worsley et al., 2021) or in white-crowned sparrows *Zonotricia leucophrys* (Phillips et al., 2018). Yet, similarly to our results, in adult female steppe buzzards *Buteo buteo vulpinus* body condition associated with higher gut microbiome diversity, but no effect was found in male birds (Thie et al., 2022). It may be beneficial to sample birds at multiple timepoints throughout the year to detect possible longitudinal changes in gut microbiome and body condition. Furthermore, the association between sex and gut microbiome diversity has proven to be inconclusive in previous avian gut microbiome studies. In blue tits, sex did not associate with gut microbiome diversity or composition (Drobniak et al., 2022) and similar result was found in barn swallows (Kreisinger et al., 2015). During the breeding season, bird species that have multiple sexual partners pass cloacal microbiota during copulation, which could result in more similar gut microbiome samples between sexes (Grond et al., 2018). Also, sex-based differences in bird gut microbiomes may be difficult to detect with restricted sample sizes (Capunitan et al., 2020).

5 | CONCLUSIONS

This study is among the first to characterize the large-scale variation in the gut microbiome of wild adult great tits. It adds to the knowledge about the causes of variation in wild avian gut microbiomes. Our key finding is that season significantly associates with both gut microbiome diversity and composition and factors such as habitat and temperature, which are largely influenced by season, also associate with the gut microbiome. Our results indicate that changes in environmental conditions can alter the gut microbiome, thus highlighting the importance of studying the effects of environmental change on gut microbiomes. Future studies should try and incorporate omics methods to detect possible changes in gut microbiome functions between seasons and habitats. This would help us understand how variation in gut microbiome diversity and composition may influence host metabolism and, for example, reproduction. More work is needed to understand the origins of the observed within and among-population variation in great tit gut microbiomes and how this variation connects to population performance and the functionality of the gut microbiome in changing environmental conditions.

AUTHOR CONTRIBUTIONS

Martta Liukkonen planned the project, organized data collection processed the samples, prepared the sequence libraries, analysed the data and wrote the manuscript. Suvi Ruuskanen planned and funded the project, helped in sample processing, data analysis and manuscript writing. Kirsten Grond assisted in sequence library preparation protocols, bioinformatics and data analysis. Jaime Muriel, Jesús Martínez-Padilla, Andreas Nord, Veli-Matti Pakanen, Balázs Rosivall and Kees van Oers collected faecal samples and commented on the manuscript. All authors have approved the final manuscript.

ACKNOWLEDGEMENTS

For their help in the field, we would like to thank Cassandre Deparde, Juho Jolkkonen, Nelli Leskisenoja and Inka Ojanen for collecting samples in Jyväskylä. Jorma Nurmi (University of Turku) and Antoine Stier (IPHC Strasbourg, University of Turku) for collecting samples on Ruissalo island and Peter de Vries (NIOO-KNAW) for help in collecting samples in Westerheide. We would like to thank Emil Aaltonen Foundation (grant to Suvi Ruuskanen) for funding this research. Andreas Nord was funded by the Swedish Research Council (grant no. 2020-04686).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Sequencing data set and metadata are freely available from the NCBI Sequence Read Archive (BioProject: PRJNA1036439; Liukkonen et al., 2024). QIIME2 script and R codes are available at GitHub (https://github.com/marttal/GTvariation) and on request.

ORCID

Martta Liukkonen https://orcid.org/0000-0003-1765-9111
Jesús Martínez-Padilla https://orcid.org/0000-0003-2956-5163
Andreas Nord https://orcid.org/0000-0001-6170-689X
Veli-Matti Pakanen https://orcid.org/0000-0003-4838-9927
Vallo Tilgar https://orcid.org/0000-0003-2569-0143
Suvi Ruuskanen https://orcid.org/0000-0001-5582-9455

REFERENCES

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L., & Gilbert, M. T. P. (2016). Do vertebrate gut metagenomes confer rapid ecological adaptation? *Trends in Ecology & Evolution*, *31*, 689–699. https://doi.org/10.1016/j.tree.2016.06.008

Al-Khalaifah, H. S. (2018). Benefits of probiotics and/or prebiotics for antibiotic-reduced poultry. *Poultry Science*, *97*, 3807–3815. https://doi.org/10.3382/ps/pey160

Amato, K. R., Sanders, J. G., Song, S. J., Nute, M., Metcalf, J. L., Thompson, L. R., Morton, J. T., Amir, A., McKenzie, J. V., Humphrey, G., Gogul, G., Gaffney, J., Baden, A. L., Britton, G. A. O., Cuozzo, F. P., Di Fiore, A., Dominy, N., Goldberg, T. L., Gomez, A., … Leigh, S. R. (2019). Evolutionary trends in host physiology outweigh dietary niche in structuring primate gut microbiomes. *ISME Journal*, *13*, 576–587. https://doi.org/10.1038/s41396-018-0175-0

Ampoorter, E., Barbaro, L., Jactel, H., Baeten, L., Boberg, J., Carnol, M., Castagneyrol, B., Charbonnier, Y., Dawud, S. M., Deconchat, M., Smedt, P. D., Wandeler, H. D., Guyot, V., Hättenschwiler, S., Joly, F., Koricheva, J., Milligan, H., Muys, B., Nguyen, D., … Allan, E. (2020). Tree diversity is key for promoting the diversity and abundance of forest-associated taxa in Europe. *Oikos*, *129*, 133–146. https://doi.org/10.1111/oik.06290

Anderson, K. J., & Jetz, W. (2005). The broad-scale ecology of energy expenditure of endotherms. *Ecology Letters*, *8*, 310–318. https://doi.org/10.1111/j.1461-0248.2005.00723.x

Antwis, R. E., Edwards, K. L., Unwin, B., Walker, S. L., & Shultz, S. (2019). Rare gut microbiota associated with breeding success, hormone metabolites and ovarian cycle phase in the critically endangered eastern black rhino. *Microbiome*, *7*, 1–12. https://doi.org/10.1186/s40168-019-0639-0

Apprill, A., McNally, S., Parsons, R., & Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology*, *75*, 129–137. https://doi.org/10.3354/ame01753

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science*, *307*(5717), 1915–1920.

Baker, J. R. (1939). The relation between latitude and breeding seasons in birds. *Proceedings of the Zoological Society of London*, *A108*, 557–582. https://doi.org/10.1111/j.1096-3642.1939.tb00042.x

Baniel, A., Amato, K. R., Beehner, J. C., Bergman, T. J., Mercer, A., Perlman, R. F., Petrullo, L., Reitsema, L., Sams, S., Lu, A., & Snyder-Mackler, N. (2021). Seasonal shifts in the gut microbiome indicate plastic responses to diet in wild geladas. *Microbiome*, *9*, 26. https://doi.org/10.1186/s40168-020-00977-9

Bär, J., Leung, J. M., Hansen, C., Loke, P., Hall, A. R., Conour, L., & Graham, A. L. (2020). Strong effects of lab-to-field environmental transitions on the bacterial intestinal microbiota of *Mus musculus* are modulated by *Trichuris muris* infection. *FEMS Microbiology Ecology*, *96*, fiaa167. https://doi.org/10.1093/femsec/fiaa167

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. arXiv preprint arXiv:1406.5823. https://doi.org/10.48550/arXiv.1406.5823

Beli, E., Yan, Y., Moldovan, L., Vieira, C. P., Gao, R., Duan, Y., Prasad, R., Bhatwadekar, A., White, F. A., Townsend, S. D., Chan, L., Ryan, C. N., Morton, D., Moldovan, E. G., Chu, F.-I., Oudit, G. Y., Derendorf, H., Adorini, L., Wang, X. X., … Grant, M. B. (2018). Restructuring of the gut microbiome by intermittent fasting prevents retinopathy and prolongs survival in db/db mice. *Diabetes*, *67*(9), 1867–1879. https://doi.org/10.2337/db18-0158

Berlow, M., Phillips, J. N., & Derryberry, E. P. (2021). Effects of urbanization and landscape on gut microbiomes in white-crowned sparrows. *Microbial Ecology*, *81*(1), 253–266. https://doi.org/10.1007/s00248-020-01569-8

Bestion, E., Jacob, S., Zinger, L., Di Gesu, L., Richard, M., White, J., & Cote, J. (2017). Climate warming reduces gut microbiota diversity in a vertebrate ectotherm. *Nature Ecology & Evolution*, *1*, 1–3. https://doi.org/10.1038/s41559-017-0161

Bibby, C. J. (1999). Making the most of birds as environmental indicators. *Ostrich*, *70*(1), 81–88.

Bodawatta, K. H., Freiberga, I., Puzejova, K., Sam, K., Poulsen, M., & Jønsson, K. A. (2021). Flexibility and resilience of great tit (*Parus major*) gut microbiomes to changing diets. *BMC Animal Microbiome*, *3*, 20. https://doi.org/10.1186/s42523-021-00076-6

Bodawatta, K. H., Hird, S. M., Grond, K., Poulsen, M., & Jønsson, K. A. (2022). Avian gut microbiomes taking flight. *Trends in Microbiology*, *30*, 268–280. https://doi.org/10.1016/j.tim.2021.07.003

Bodawatta, K. H., Klečková, I., Klečka, J., Pužejová, K., Koane, B., Poulsen, M., Jønsson, K. A., & Sam, K. (2022). Specific gut bacterial responses to natural diets of tropical birds. *Science Reports*, *12*, 713. https://doi.org/10.1038/s41598-022-04808-9

Bodawatta, K. H., Koane, B., Maiah, G., Sam, K., Poulsen, M., & Jønsson, K. A. (2021). 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*, *288*, 20210446. https://doi.org/10.1098/rspb.2021.0446

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., … Caporaso, J. G. (2018). QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science (preprint). *PeerJ Preprints*. https://doi.org/10.7287/peerj.preprints.27295v2

Bray, J. R., & Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs*, *27*, 326–349. https://doi.org/10.2307/1942268

Brittingham, M. C., & Temple, S. A. (1988). Impacts of supplemental feeding on survival rates of black-capped chickadees. *Ecology*, *69*, 581–589. https://doi.org/10.2307/1941007

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*, 581–583. https://doi.org/10.1038/nmeth.3869

Cameron, E. S., Schmidt, P. J., Tremblay, B. J. M., Emelko, M. B., & Müller, K. M. (2020). To rarefy or not to rarefy: Enhancing diversity analysis of microbial communities through next-generation sequencing and rarefying repeatedly. *bioRxiv*. 2020-09.

Capunitan, D. C., Johnson, O., Terrill, R. S., & Hird, S. M. (2020). Evolutionary signal in the gut microbiomes of 74 bird species from Equatorial Guinea. *Molecular Ecology*, *29*, 829–847. https://doi.org/10.1111/mec.15354

Chao, A. (2006). Species estimation and applications. In S. Kotz, C. B. Read, N. Balakrishnan, B. Vidakovic, & N. L. Johnson (Eds.), *Encyclopedia of statistical sciences* (pp. 7907–7916). John Wiley & Sons, Inc. https://doi.org/10.1002/0471667196.ess5051

Charmantier, A., McCleery, R. H., Cole, L. R., Perrins, C., Kruuk, L. E. B., & Sheldon, B. C. (2008). Adaptive phenotypic plasticity in response to climate change in a wild bird population. *Science*, *320*, 800–803. https://doi.org/10.1126/science.1157174

Chevalier, C., Stojanović, O., Colin, D. J., Suarez-Zamorano, N., Tarallo, V., Veyrat-Durebex, C., Rigo, D., Fabbiano, S., Stevanović, A., Hagemann, S., Montet, X., Seimbille, Y., Zamboni, N., Hapfelmeier, S., & Trajkovski, M. (2015). Gut microbiota orchestrates energy homeostasis during cold. *Cell*, *163*, 1360–1374. https://doi.org/10.1016/j.cell.2015.11.004

Cheville, N. F., & Arp, L. H. (1978). Comparative pathologic findings of *Escherichia coli* infection in birds. *Journal of the American Veterinary Medicine Association*, *173*, 584–587.

Cody, M. L. (1981). Habitat selection in birds: The roles of vegetation structure, competitors, and productivity. *BioScience*, *31*, 107–113. https://doi.org/10.2307/1308252

Corl, A., Charter, M., Rozman, G., Toledo, S., Turjeman, S., Kamath, P. L., Getz, W. M., Nathan, R., & Bowie, R. C. K. (2020). Movement ecology and sex are linked to barn owl microbial community composition. *Molecular Ecology*, *29*, 1358–1371. https://doi.org/10.1111/mec.15398

Cox, A. R., Robertson, R. J., Lendvai, Á. Z., Everitt, K., & Bonier, F. (2019). Rainy springs linked to poor nestling growth in a declining avian aerial insectivore (*Tachycineta bicolor*). *Proceedings of the Royal Society B: Biological Sciences*, *286*, 20190018. https://doi.org/10.1098/rspb.2019.0018

Davenport, E. R., Mizrahi-Man, O., Michelini, K., Barreiro, L. B., Ober, C., & Gilad, Y. (2014). Seasonal variation in human gut microbiome composition. *PLoS One*, *9*, e90731. https://doi.org/10.1371/journal.pone.0090731

Davidson, G. L., Somers, S. E., Wiley, N., Johnson, C. N., Reichert, M. S., Ross, R. P., Stanton, C., & Quinn, J. L. (2021). A time-lagged

association between the gut microbiome, nestling weight and nestling survival in wild great tits. *Journal of Animal Ecology*, *90*, 989–1003. https://doi.org/10.1111/1365-2656.13428

Davidson, G. L., Wiley, N., Cooke, A. C., Johnson, C. N., Fouhy, F., Reichert, M. S., de la Hera, I., Crane, J. M. S., Kulahci, I. G., Ross, R. P., Stanton, C., & Quinn, J. L. (2020). Diet induces parallel changes to the gut microbiota and problem-solving performance in a wild bird. *Scientific Reports*, *10*(1), 20783.

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*, *6*, 226. https://doi.org/10.1186/s40168-018-0605-2

De Angelis, M., Calasso, M., Cavallo, N., Di Cagno, R., & Gobbetti, M. (2016). Functional proteomics within the genus *Lactobacillus*. *Proteomics*, *16*(6), 946–962.

Den Besten, G., Van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*, *54*(9), 2325–2340.

Dietz, M. W., Matson, K. D., Versteegh, M. A., van der Velde, M., Parmentier, H. K., Arts, J. A., Salles, J. F., & Tieleman, B. I. (2022). Gut microbiota of homing pigeons shows summer–winter variation under constant diet indicating a substantial effect of temperature. *Animal Microbiome*, *4*(1), 64.

Dingemanse, N. J., Bouwman, K. M., van de Pol, M., van Overveld, T., Patrick, S. C., Matthysen, E., & Quinn, J. L. (2012). Variation in personality and behavioural plasticity across four populations of the great tit *Parus major*. *Journal of Animal Ecology*, *81*, 116–126. https://doi.org/10.1111/j.1365-2656.2011.01877.x

Drobniak, S. M., Cichoń, M., Janas, K., Barczyk, J., Gustafsson, L., & Zagalska-Neubauer, M. (2022). Habitat shapes diversity of gut microbiomes in a wild population of blue tits *Cyanistes caeruleus*. *Journal of Avian Biology*, *2022*, e02829. https://doi.org/10.1111/jav.02829

Du Toit, A. (2019). The gut microbiome and mental health. *Nature Reviews Microbiology*, *17*, 196. https://doi.org/10.1038/s41579-019-0163-z

Eren, A. M., Maignien, L., Sul, W. J., Murphy, L. G., Grim, S. L., Morrison, H. G., Sogin, M. L., & Freckleton, R. (2013). Oligotyping: Differentiating between closely related microbial taxa using 16S rRNA gene data. *Methods in Ecology and Evolution*, *4*, 1111–1119. https://doi.org/10.1111/2041-210X.12114

Escallón, C., Belden, L. K., & Moore, I. T. (2019). The cloacal microbiome changes with the breeding season in a wild bird. *Integrative Organismal Biology*, *1*, oby009. https://doi.org/10.1093/iob/oby009

Fontaine, S. S., Novarro, A. J., & Kohl, K. D. (2018). Environmental temperature alters the digestive performance and gut microbiota of a terrestrial amphibian. *Journal of Experimental Biology*, *221*, 187559. https://doi.org/10.1242/jeb.187559

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S., Firth, D., Friendly, M., Gorjanc, G., Graves, S., & Heiberger, R. (2012). *Package 'car'*. R Foundation for Statistical Computing.

Gadau, A., Crawford, M. S., Mayek, R., Giraudeau, M., McGraw, K. J., Whisner, C. M., Kondrat-Smith, C., & Sweazea, K. L. (2019). A comparison of the nutritional physiology and gut microbiome of urban and rural house sparrows (*Passer domesticus*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *237*, 110332. https://doi.org/10.1016/j.cbpb.2019.110332

Gamero, A., Senar, J. C., Hohtola, E., Nilsson, J.-Å., & Broggi, J. (2015). Population differences in the structure and coloration of great tit contour feathers: Feather colour and microstructure. *Biological Journal of the Linnean Society London*, *114*, 82–91. https://doi.org/10.1111/bij.12409

Gilbert, J. A., Blaser, M. J., Caporaso, J. G., Jansson, J. K., Lynch, S. V., & Knight, R. (2018). Current understanding of the human microbiome. *Nature Medicine*, *24*, 392–400. https://doi.org/10.1038/nm.4517

Góngora, E., Elliott, K. H., & Whyte, L. (2021). Gut microbiome is affected by inter-sexual and inter-seasonal variation in diet for thick-billed murres (*Uria lomvia*). *Science Reports*, *11*, 1200. https://doi.org/10.1038/s41598-020-80557-x

Gregory, R. D., van Strien, A., Vorisek, P., Gmelig Meyling, A. W., Noble, D. G., Foppen, R. P. B., & Gibbons, D. W. (2005). Developing indicators for European birds. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, *360*, 269–288. https://doi.org/10.1098/rstb.2004.1602

Grond, K., Kurtz, C. C., Hatton, J., Sonsalla, M. M., & Duddleston, K. N. (2021). Gut microbiome is affected by gut region but robust to host physiological changes in captive active-season ground squirrels. *Animal Microbiome*, *3*, 1–11. https://doi.org/10.1186/s42523-021-00117-0

Grond, K., Sandercock, B. K., Jumpponen, A., & Zeglin, L. H. (2018). The avian gut microbiota: Community, physiology and function in wild birds. *Journal of Avian Biology*, *49*, e01788. https://doi.org/10.1111/jav.01788

Grond, K., Santo Domingo, J. W., Lanctot, R. B., Jumpponen, A., Bentzen, R. L., Boldenow, M. L., Brown, S. C., Casler, B., Cunningham, J. A., Doll, A. C., Freeman, S., Hill, B. L., Kendall, S. J., Kwon, E., Liebezeit, J. R., Pirie-Dominix, L., Rausch, J., & Sandercock, B. K. (2019). Composition and drivers of gut microbial communities in Arctic-breeding shorebirds. *Frontiers in Microbiology*, *10*, 2258.

Grubb, T. C., Jr. (1978). Weather-dependent foraging rates of wintering woodland birds. *The Auk*, *95*, 370–376. https://doi.org/10.1093/auk/95.2.370

Haegeman, B., Hamelin, J., Moriarty, J., Neal, P., Dushoff, J., & Weitz, J. S. (2013). Robust estimation of microbial diversity in theory and in practice. *ISME Journal*, *7*, 1092–1101. https://doi.org/10.1038/ismej.2013.10

Hartig, F., & Hartig, M. F. (2017). *Package 'DHARMa'*. R package.

Hird, S. M. (2017). Evolutionary biology needs wild microbiomes. *Fronters in Microbiology*, *8*, 725. https://doi.org/10.3389/fmicb.2017.00725

Hird, S. M., Sánchez, C., Carstens, B. C., & Brumfield, R. T. (2015). Comparative gut microbiota of 59 neotropical bird species. *Frontiers in Microbiology*, *6*, 1403.

Husby, A., Nussey, D. H., Visser, M. E., Wilson, A. J., Sheldon, B. C., & Kruuk, L. E. B. (2010). Contrasting patterns of phenotypic plasticity in reproductive traits in two great tit (*Parus major*) populations: Multivariate patterns of phenotypic plasticity. *Evolution*, *64*, 2221–2237. https://doi.org/10.1111/j.1558-5646.2010.00991.x

Ingala, M. R., Albert, L., Addesso, A., Watkins, M. J., & Knutie, S. A. (2021). Differential effects of elevated nest temperature and parasitism on the gut microbiota of wild avian hosts. *Animal Microbiome*, *3*, 67. https://doi.org/10.1186/s42523-021-00130-3

Jašarević, E., Morrison, K. E., & Bale, T. L. (2016). Sex differences in the gut microbiome–brain axis across the lifespan. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, *371*, 20150122. https://doi.org/10.1098/rstb.2015.0122

Jones, I., Marsh, K., Handby, T. M., Hopkins, K., Slezacek, J., Bearhop, S., & Harrison, X. A. (2023). The influence of diet on gut microbiome and body mass dynamics in a capital-breeding migratory bird. *PeerJ*, *11*, e16682.

Karr, J. R. (1976). Seasonality, resource Availability, and community diversity in tropical bird communities. *The American Naturalist*, *110*, 973–994. https://doi.org/10.1086/283121

Knutie, S. A. (2020). Food supplementation affects gut microbiota and immunological resistance to parasites in a wild bird species. *Journal of Applied Ecology*, *57*, 536–547. https://doi.org/10.1111/1365-2664.13567

Knutie, S. A., Chaves, J. A., & Gotanda, K. M. (2019). Human activity can influence the gut microbiota of Darwin's finches in the Galapagos Islands. *Molecular Ecology*, *28*, 2441–2450. https://doi.org/10.1111/mec.15088

Knutie, S. A., & Gotanda, K. M. (2018). A non-invasive method to collect fecal samples from wild birds for microbiome studies. *Microbial Ecology*, *76*, 851–855. https://doi.org/10.1007/s00248-018-1182-4

Kohl, K. D. (2012). Diversity and function of the avian gut microbiota. *Journal of Comparative Physiology B*, *182*, 591–602. https://doi.org/10.1007/s00360-012-0645-z

Kohl, K. D., Brun, A., Bordenstein, S. R., Caviedes-Vidal, E., & Karasov, W. H. (2018). Gut microbes limit growth in house sparrow nestlings (*Passer domesticus*) but not through limitations in digestive capacity. *Integrative Zoology*, *13*(2), 139–151.

Kohl, K. D., & Yahn, J. (2016). Effects of environmental temperature on the gut microbial communities of tadpoles. *Environmental Microbiology*, *18*, 1561–1565. https://doi.org/10.1111/1462-2920.13255

Kopac, S. M., & Klassen, J. L. (2016). Can they make it on their own? Hosts, microbes, and the holobiont niche. *Frontiers in Microbiology*, *7*, 1647.

Koskimies, P. (1989). Birds as a tool in environmental monitoring. *Annales Zoologici Fennici*, *26*, 153–166.

Krebs, J. R. (1971). Territory and breeding density in the great tit, *Parus major* L. *Ecology*, *52*, 2–22. https://doi.org/10.2307/1934734

Kreisinger, J., Čížková, D., Kropáčková, L., & Albrecht, T. (2015). Cloacal microbiome structure in a long-distance migratory bird assessed using deep 16sRNA pyrosequencing. *PLoS One*, *10*, e0137401. https://doi.org/10.1371/journal.pone.0137401

Lahti, L., & Shetty, S. (2018). *Introduction to the microbiome R package*. https://microbiome.github.io/tutorials

Lemoine, M., Lucek, K., Perrier, C., Saladin, V., Adriaensen, F., Barba, E., Belda, E. J., Charmantier, A., Cichoń, M., Eeva, T., Grégoire, A., Hinde, C. A., Johnsen, A., Komdeur, J., Mänd, R., Matthysen, E., Norte, A. C., Pitala, N., Sheldon, B. C., … Richner, H. (2016). Low but contrasting neutral genetic differentiation shaped by winter temperature in European great tits. *Biological Journal of the Linnean Society*, *118*, 668–685. https://doi.org/10.1111/bij.12745

Lewis, W. B., Moore, F. R., & Wang, S. (2017). Changes in gut microbiota of migratory passerines during stopover after crossing an ecological barrier. *The Auk*, *134*, 137–145. https://doi.org/10.1642/AUK-16-120.1

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., Schlegel, M. L., Tucker, T. A., Schrenzel, M. D., Knight, R., & Gordon, J. I. (2008). Evolution of mammals and their gut microbes. *Science*, *320*, 1647–1651. https://doi.org/10.1126/science.1155725

Ley, R. E., Peterson, D. A., & Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, *124*(4), 837–848.

Liu, H., Guo, X., Gooneratne, R., Lai, R., Zeng, C., Zhan, F., & Wang, W. (2016). The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. *Science Reports*, *6*, 24340. https://doi.org/10.1038/srep24340

Liukkonen, M., Hukkanen, M., Cossin-Sevrin, N., Stier, A., Vesterinen, E., Grond, K., & Ruuskanen, S. (2023). No evidence for associations between brood size, gut microbiome diversity and survival in great tit (*Parus major*) nestlings. *Animal Microbiome*, *5*, 19. https://doi.org/10.1186/s42523-023-00241-z

Liukkonen, M., Muriel, J., Martínez-Padilla, J., Nord, A., Pakanen, V.-M., Rosivall, B., Tilgar, V., van Oers, K., Grond, K., & Ruuskanen, S. (2024). Data from: *Seasonal and environmental factors contribute to the variation in the gut microbiome: A large-scale study of a small bird*. https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1036439

Loo, W. T., García-Loor, J., Dudaniec, R. Y., Kleindorfer, S., & Cavanaugh, C. M. (2019). Host phylogeny, diet, and habitat differentiate the gut microbiomes of Darwin's finches on Santa Cruz Island. *Science Reports*, *9*, 18781. https://doi.org/10.1038/s41598-019-54869-6

Love, M., Anders, S., & Huber, W. (2014). Differential analysis of count data–the DESeq2 package. *Genome Biology*, *15*(550), 10–1186.

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, *489*(7415), 220–230.

Lucas, G. (2018). Gut thinking: The gut microbiome and mental health beyond the head. *Microbial Ecology in Health and Disease*, *29*, 1548250. https://doi.org/10.1080/16512235.2018.1548250

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, *17*, 10–12. https://doi.org/10.14806/ej.17.1.200

Maurice, C. F., Knowles, C. L., Ladau, J., Pollard, K. S., Fenton, A., Pedersen, A. B., & Turnbaugh, P. J. (2015). Marked seasonal variation in the wild mouse gut microbiota. *ISME Journal*, *9*, 2423–2434. https://doi.org/10.1038/ismej.2015.53

McKenzie, C., Tan, J., Macia, L., & Mackay, C. R. (2017). The nutrition-gut microbiome-physiology axis and allergic diseases. *Immunological Reviews*, *278*, 277–295. https://doi.org/10.1111/imr.12556

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, *8*, e61217. https://doi.org/10.1371/journal.pone.0061217

McNamara, J. M., Houston, A. I., & Lima, S. L. (1994). Foraging routines of small birds in winter: A theoretical investigation. *Journal of Avian Biology*, *25*, 287–302. https://doi.org/10.2307/3677276

Meng, H., Zhang, Y., Zhao, L., Zhao, W., He, C., Honaker, C. F., Zhai, Z., Sun, Z., & Siegel, P. B. (2014). Body weight selection affects quantitative genetic correlated responses in gut microbiota. *PLoS One*, *9*, e89862. https://doi.org/10.1371/journal.pone.0089862

Moeller, A. H., Ivey, K., Cornwall, M. B., Herr, K., Rede, J., Taylor, E. N., & Gunderson, A. R. (2020). The Lizard gut microbiome changes with temperature and is associated with heat tolerance. *Applied and Environmental Microbiology*, *86*, e01181-20. https://doi.org/10.1128/AEM.01181-20

Morimoto, J., Simpson, S. J., & Ponton, F. (2017). Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biology Letters*, *13*(7), 20160966. https://doi.org/10.1098/rsbl.2016.0966

Moya, A., & Ferrer, M. (2016). Functional redundancy-induced stability of gut microbiota subjected to disturbance. *Trends in Microbiology*, *24*(5), 402–413. https://doi.org/10.1016/j.tim.2016.02.002

Murillo, T., Schneider, D., Fichtel, C., & Daniel, R. (2022). Dietary shifts and social interactions drive temporal fluctuations of the gut microbiome from wild redfronted lemurs. *ISME Communications*, *2*(1), 3. https://doi.org/10.1038/s43705-021-00086-0

Murray, M. H., Lankau, E. W., Kidd, A. D., Welch, C. N., Ellison, T., Adams, H. C., Lipp, E. K., & Hernandez, S. M. (2020). Gut microbiome shifts with urbanization and potentially facilitates a zoonotic pathogen in a wading bird. *PLoS One*, *15*, e0220926. https://doi.org/10.1371/journal.pone.0220926

Noguera, J. C., Aira, M., Pérez-Losada, M., Domínguez, J., & Velando, A. (2018). Glucocorticoids modulate gastrointestinal microbiome in a wild bird. *Royal Society Open Science*, *5*, 171743. https://doi.org/10.1098/rsos.171743

Noordwijk, A. J. V., Van Balen, J. H., & Scharloo, W. (2002). Heritability of ecologically important traits in the great tit. *Ardea*, *55*, 193–203. https://doi.org/10.5253/arde.v68.p193

Norte, A. C., Ramos, J. A., Sampaio, H. L., Sousa, J. P., & Sheldon, B. C. (2010). Physiological condition and breeding performance of the great tit. *The Condor*, *112*, 79–86. https://doi.org/10.1525/cond.2010.08007

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H. B. A., … Weedon, J. (2013). Package 'vegan'. Community ecology package, version 2.6-6.1.

Parada, A. E., Needham, D. M., & Fuhrman, J. A. (2016). Every base matters: Assessing small subunit rRNA primers for marine microbiomes

with mock communities, time series and global field samples. *Environmental Microbiology*, *18*, 1403–1414. https://doi.org/10.1111/1462-2920.13023

Pereira, H. M., & Cooper, H. D. (2006). Towards the global monitoring of biodiversity change. *Trends in Ecology & Evolution*, *21*, 123–129. https://doi.org/10.1016/j.tree.2005.10.015

Phillips, J. N., Berlow, M., & Derryberry, E. P. (2018). The effects of landscape urbanization on the gut microbiome: An exploration into the gut of urban and rural white-crowned sparrows. *Frontiers in Ecology and Evolution*, *6*, 148. https://doi.org/10.3389/fevo.2018.00148

Pigot, A. L., Sheard, C., Miller, E. T., Bregman, T. P., Freeman, B. G., Roll, U., Seddon, N., Trisos, C. H., Weeks, B. C., & Tobias, J. A. (2020). Macroevolutionary convergence connects morphological form to ecological function in birds. *Nature Ecology & Evolution*, *4*, 230–239. https://doi.org/10.1038/s41559-019-1070-4

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*, D590–D596. https://doi.org/10.1093/nar/gks1219

Radford, A. N., McCleery, R. H., Woodburn, R. J. W., & Morecroft, M. D. (2001). Activity patterns of parent great tits *Parus major* feeding their young during rainfall. *Bird Study*, *48*, 214–220. https://doi.org/10.1080/00063650109461220

Rahbek, C., & Graves, G. R. (2001). Multiscale assessment of patterns of avian species richness. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 4534–4539. https://doi.org/10.1073/pnas.071034898

Reid, G., & Burton, J. (2002). Use of *Lactobacillus* to prevent infection by pathogenic bacteria. *Microbes and Infection*, *4*, 319–324. https://doi.org/10.1016/S1286-4579(02)01544-7

Ren, T., Boutin, S., Humphries, M. M., Dantzer, B., Gorrell, J. C., Coltman, D. W., McAdam, A. G., & Wu, M. (2017). Seasonal, spatial, and maternal effects on gut microbiome in wild red squirrels. *Microbiome*, *5*, 163. https://doi.org/10.1186/s40168-017-0382-3

Roche, K. E., Bjork, J. R., Dasari, M. R., Grieneisen, L., Jansen, D., Gould, T. J., Gesquiere, L. R., Barreiro, L. B., Alberts, S. C., Blekhman, R., Gilbert, J. A., Tung, J., Mukherjee, S., & Archie, E. A. (2023). Universal gut microbial relationships in the gut microbiome of wild baboons. *eLife*, *12*, e83152. https://doi.org/10.7554/eLife.83152

Rosenberg, E., & Zilber-Rosenberg, I. (2016). Do microbiotas warm their hosts? *Gut Microbes*, *7*(4), 283–285.

Rosenberg, E., & Zilber-Rosenberg, I. (2018). The hologenome concept of evolution after 10 years. *Microbiome*, *6*, 78. https://doi.org/10.1186/s40168-018-0457-9

Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., Costea, P. I., Godneva, A., Kalka, I. N., Bar, N., Shilo, S., Lador, D., Vila, A. V., Zmora, N., Pevsner-Fischer, M., Israeli, D., Kosower, N., Malka, G., Wolf, B. C., … Segal, E. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature*, *555*, 210–215. https://doi.org/10.1038/nature25973

Sabree, Z. L., & Moran, N. A. (2014). Host-specific assemblages typify gut microbial communities of related insect species. *Springer Plus*, *3*, 138. https://doi.org/10.1186/2193-1801-3-138

Saulnier, A., Bleu, J., Boos, A., Millet, M., Zahn, S., Ronot, P., El Masoudi, I., Rojas, E. R., Uhlrich, P., Del Nero, M., & Massemin, S. (2023). Inter-annual variation of physiological traits between urban and forest great tits. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *279*, 111385. https://doi.org/10.1016/j.cbpa.2023.111385

Schloss, P. D. (2023). Waste not, want not: Revisiting the analysis that called into question the practice of rarefaction. *bioRxiv* 2023-06. https://doi.org/10.1101/2023.06.23.546312

Schmiedová, L., Kreisinger, J., Kubovčiak, J., Těšický, M., Martin, J.-F., Tomášek, O., Kauzálová, T., Sedláček, O., & Albrecht, T. (2023). Gut microbiota variation between climatic zones and due to migration strategy in passerine birds. *Frontiers in Microbiology*, *14*, 1080017.

Schmiedová, L., Tomášek, O., Pinkasová, H., Albrecht, T., & Kreisinger, J. (2022). Variation in diet composition and its relation to gut microbiota in a passerine bird. *Science Reports*, *12*, 3787. https://doi.org/10.1038/s41598-022-07672-9

Schöll, E. M., Ohm, J., Hoffmann, K. F., & Hille, S. M. (2016). Caterpillar biomass depends on temperature and precipitation, but does not affect bird reproduction. *Acta Oecologica*, *74*, 28–36. https://doi.org/10.1016/j.actao.2016.06.004

Sepulveda, J., & Moeller, A. H. (2020). The effects of temperature on animal gut microbiomes. *Frontiers in Microbiology*, *11*, 384.

Singh, R. K., Chang, H.-W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T. H., Bhutani, T., & Liao, W. (2017). Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine*, *15*, 73. https://doi.org/10.1186/s12967-017-1175-y

Spor, A., Koren, O., & Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. *Nature Reviews Microbiology*, *9*, 279–290. https://doi.org/10.1038/nrmicro2540

Sullam, K. E., Essinger, S. D., Lozupone, C. A., O'connor, M. P., Rosen, G. L., Knight, R., Kilham, S. S., & Russell, J. A. (2012). Environmental and ecological factors that shape the gut bacterial communities of fish: A meta-analysis. *Molecular Ecology*, *21*, 3363–3378. https://doi.org/10.1111/j.1365-294X.2012.05552.x

Sullam, K. E., Rubin, B. E., Dalton, C. M., Kilham, S. S., Flecker, A. S., & Russell, J. A. (2015). Divergence across diet, time and populations rules out parallel evolution in the gut microbiomes of Trinidadian guppies. *ISME Journal*, *9*, 1508–1522. https://doi.org/10.1038/ismej.2014.231

Sun, B., Wang, X., Bernstein, S., Huffman, M. A., Xia, D. P., Gu, Z., Chen, R., Sheeran, L. K., Wagner, R. S., & Li, J. (2016). Marked variation between winter and spring gut microbiota in free-ranging Tibetan Macaques (*Macaca thibetana*). *Scientific Reports*, *6*(1), 26035.

Tajima, K., Nonaka, I., Higuchi, K., Takusari, N., Kurihara, M., Takenaka, A., Mitsumori, M., Kajikawa, H., & Aminov, R. I. (2007). Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. *Anaerobe*, *13*, 57–64. https://doi.org/10.1016/j.anaerobe.2006.12.001

Tappenden, K. A., & Deutsch, A. S. (2007). The physiological relevance of the intestinal microbiota-contributions to human health. *Journal of the American College of Nutrition*, *26*(6), 679S–683S.

Teyssier, A., Lens, L., Matthysen, E., & White, J. (2018). Dynamics of gut microbiota diversity during the early development of an avian host: Evidence from a cross-foster experiment. *Frontiers in Microbiology*, *9*, 1524. https://doi.org/10.3389/fmicb.2018.01524

Teyssier, A., Matthysen, E., Hudin, N. S., De Neve, L., White, J., & Lens, L. (2020). Diet contributes to urban-induced alterations in gut microbiota: Experimental evidence from a wild passerine. *Proceedings of the Royal Society: Biology*, *287*, 20192182. https://doi.org/10.1098/rspb.2019.2182

Teyssier, A., Rouffaer, L. O., Saleh Hudin, N., Strubbe, D., Matthysen, E., Lens, L., & White, J. (2018). Inside the guts of the city: Urban-induced alterations of the gut microbiota in a wild passerine. *Science of the Total Environment*, *612*, 1276–1286. https://doi.org/10.1016/j.scitotenv.2017.09.035

Thie, N., Corl, A., Turjeman, S., Efrat, R., Kamath, P. L., Getz, W. M., Bowie, R. C. K., & Nathan, R. (2022). Linking migration and microbiota at a major stopover site in a long-distance avian migrant. *Movement Ecology*, *10*, 46. https://doi.org/10.1186/s40462-022-00347-0

Tian, Y., Li, G., Chen, L., Bu, X., Shen, J., Tao, Z., Zeng, T., Du, X., & Lu, L. (2020). High-temperature exposure alters the community structure and functional features of the intestinal microbiota in Shaoxing ducks (*Anas platyrhynchos*). *Poultry Science*, *99*, 2662–2674. https://doi.org/10.1016/j.psj.2019.12.046

Tilg, H., & Kaser, A. (2011). Gut microbiome, obesity, and metabolic dysfunction. *Journal of Clinical Investigation*, *121*, 2126–2132. https://doi.org/10.1172/JCI58109

Tinya, F., Kovács, B., Bidló, A., Dima, B., Király, I., Kutszegi, G., Lakatos, F., Mag, Z., Márialigeti, S., Nascimbene, J., Samu, F., Siller, I., Szél, G., & Ódor, P. (2021). Environmental drivers of forest biodiversity in temperate mixed forests – A multi-taxon approach. *Science of the Total Environment*, *795*, 148720. https://doi.org/10.1016/j.scitotenv.2021.148720

Tizard, I. (2004). Salmonellosis in wild birds. *Seminars in Avian and Exotic Pet Medicine*, *13*, 50–66. https://doi.org/10.1053/j.saep.2004.01.008

Tong, Q., Cui, L.-Y., Hu, Z.-F., Du, X.-P., Abid, H. M., & Wang, H.-B. (2020). Environmental and host factors shaping the gut microbiota diversity of brown frog *Rana dybowskii*. *Science of the Total Environment*, *741*, 140142. https://doi.org/10.1016/j.scitotenv.2020.140142

van Veelen, H. P. J., Falcão Salles, J., Matson, K. D., van der Velde, M., & Tieleman, B. I. (2020). Microbial environment shapes immune function and cloacal microbiota dynamics in zebra finches *Taeniopygia guttata*. *Animal Microbiome*, *2*, 1–17.

Vel'ký, M., Kaňuch, P., & Krištín, A. (2011). Food composition of wintering great tits (*Parus major*): Habitat and seasonal aspects. *Folia Zoologica*, *60*, 228–236. https://doi.org/10.25225/fozo.v60.i3.a7.2011

Waite, D. W., & Taylor, M. W. (2014). Characterizing the avian gut microbiota: Membership, driving influences, and potential function. *Frontiers in Microbiology*, *5*, 223.

Walter, J. (2008). Ecological role of lactobacilli in the gastrointestinal tract: Implications for fundamental and biomedical research. *Applied and Environmental Microbiology*, *74*(16), 4985–4996.

Walters, A. W., Hughes, R. C., Call, T. B., Walker, C. J., Wilcox, H., Petersen, S. C., Rudman, S. M., Newell, P. D., Douglas, A. E., Schmidt, P. S., & Chaston, J. M. (2020). The microbiota influences the *Drosophila melanogaster* life history strategy. *Molecular Ecology*, *29*(3), 639–653. https://doi.org/10.1111/mec.15344

Wang, S., Chen, L., He, M., Shen, J., Li, G., Tao, Z., Wu, R., & Lu, L. (2018). Different rearing conditions alter gut microbiota composition and host physiology in Shaoxing ducks. *Science Reports*, *8*, 7387. https://doi.org/10.1038/s41598-018-25760-7

Weinroth, M. D., Belk, A. D., Dean, C., Noyes, N., Dittoe, D. K., Rothrock, M. J., Jr., Ricke, S. C., Myer, P. R., Henniger, M. T., Ramírez, G. A., Oakley, B. B., Summers, K. L., Miles, A. M., Ault-Seay, T. B., Yu, Z., Metcalf, J. L., & Wells, J. E. (2022). Considerations and best practices in animal science 16S ribosomal RNA gene sequencing microbiome studies. *Journal of Animal Science*, *100*, skab346. https://doi.org/10.1093/jas/skab346

Wen, C., Yan, W., Mai, C., Duan, Z., Zheng, J., Sun, C., & Yang, N. (2021). Joint contributions of the gut microbiota and host genetics to feed efficiency in chickens. *Microbiome*, *9*, 126. https://doi.org/10.1186/s40168-021-01040-x

Williams, C. M., Henry, H. A. L., & Sinclair, B. J. (2015). Cold truths: How winter drives responses of terrestrial organisms to climate change. *Biological Reviews*, *90*, 214–235. https://doi.org/10.1111/brv.12105

Winkler, D. W., Dunn, P. O., & McCulloch, C. E. (2002). Predicting the effects of climate change on avian life-history traits. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(21), 13595–13599.

Worsley, S. F., Davies, C. S., Mannarelli, M.-E., Hutchings, M. I., Komdeur, J., Burke, T., Dugdale, H. L., & Richardson, D. S. (2021). Gut microbiome composition, not alpha diversity, is associated with survival in a natural vertebrate population. *Animal Microbiome*, *3*, 84. https://doi.org/10.1186/s42523-021-00149-6

Worsley, S. F., Mazel, F., Videvall, E., Harrison, X. A., Björk, J. R., & Wanelik, K. M. (2024). Probing the functional significance of wild animal microbiomes using omics data. *EcoEvoRxiv*. https://doi.org/10.32942/X28P68

Worthmann, A., John, C., Rühlemann, M. C., Baguhl, M., Heinsen, F.-A., Schaltenberg, N., Heine, M., Schlein, C., Evangelakos, I., Mineo, C., Fischer, M., Dandri, M., Kremoser, C., Scheja, L., Franke, A., Shaul, P. W., & Heeren, J. (2017). Cold-induced conversion of cholesterol to bile acids in mice shapes the gut microbiome and promotes adaptive thermogenesis. *Nature Medicine*, *23*, 839–849. https://doi.org/10.1038/nm.4357

Wu, Y., Yang, Y., Cao, L., Yin, H., Xu, M., Wang, Z., Liu, Y., Wang, X., & Deng, Y. (2018). Habitat environments impacted the gut microbiome of long-distance migratory swan geese but central species conserved. *Scientific Reports*, *8*(1), 13314.

Xiao, G., Liu, S., Xiao, Y., Zhu, Y., Zhao, H., Li, A., Li, Z., & Feng, J. (2019). Seasonal changes in gut microbiota diversity and composition in the greater horseshoe bat. *Frontiers in Microbiology*, *10*, 2247.

Xie, Y., Xia, P., Wang, H., Yu, H., Giesy, J. P., Zhang, Y., Mora, M. A., & Zhang, X. (2016). Effects of captivity and artificial breeding on microbiota in feces of the red-crowned crane (*Grus japonensis*). *Scientific Reports*, *6*(1), 33350.

Yang, Y., Gao, H., Li, X., Cao, Z., Li, M., Liu, J., Qiao, Y., Ma, L., Zhao, Z., & Pan, H. (2021). Correlation analysis of muscle amino acid deposition and gut microbiota profile of broilers reared at different ambient temperatures. *Animal Bioscience*, *34*, 93–101. https://doi.org/10.5713/ajas.20.0314

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., & Glöckner, F. O. (2014). The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Research*, *42*, D643–D648. https://doi.org/10.1093/nar/gkt1209

Zhang, X.-Y., Sukhchuluun, G., Bo, T.-B., Chi, Q.-S., Yang, J.-J., Chen, B., Zhang, L., & Wang, D.-H. (2018). Huddling remodels gut microbiota to reduce energy requirements in a small mammal species during cold exposure. *Microbiome*, *6*, 103. https://doi.org/10.1186/s40168-018-0473-9

Zhao, L., Wang, G., Siegel, P., He, C., Wang, H., Zhao, W., Zhai, Z., Tian, F., Zhao, J., Zhang, H., Sun, Z., Chen, W., Zhang, Y., & Meng, H. (2013). Quantitative genetic background of the host influences gut microbiomes in chickens. *Science Reports*, *3*, 1163. https://doi.org/10.1038/srep01163

Zheng, D., Liwinski, T., & Elinav, E. (2020). Interaction between microbiota and immunity in health and disease. *Cell Research*, *30*, 492–506. https://doi.org/10.1038/s41422-020-0332-7

Zhu, L., Liao, R., Wu, N., Zhu, G., & Yang, C. (2019). Heat stress mediates changes in fecal microbiome and functional pathways of laying hens. *Applied Microbiology and Biotechnology*, *103*, 461–472. https://doi.org/10.1007/s00253-018-9465-8

Zhu, Y., Li, Y., Yang, H., He, K., & Tang, K. (2021). Establishment of gut microbiome during early life and its relationship with growth in endangered crested ibis (*Nipponia nippon*). *Frontiers in Microbiology*, *12*, 723682.

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. *FEMS Microbiology Reviews*, *32*, 723–735. https://doi.org/10.1111/j.1574-6976.2008.00123.x

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1. Sample numbers by population and season with details about regional average rainfall and temperature per month.

Data S2. Sequence summaries for unrarefied and rarefied data and the number of contaminants removed.

Data S3. Gut microbiome relative abundance per sample.

Data S4. Linear mixed effects models measuring which population specific factors contribute to gut microbiome alpha diversity.

Data S5. A linear model with interaction to test whether there is a

significant interaction between season and population in relation to alpha diversity.

Data S6. Permutational analysis of variance to measure which factors contribute to the differences in gut microbiome beta diversity.

Data S7. Linear and linear mixed effects model summaries for the unrarefied dataset in gut microbiome diversity analyses.

Data S8. The negative association between average temperature and gut microbiome diversity (Shannon) when populations measured during winter and summer are included in the linear mixed effects model.

Data S9. deseq2_results.

How to cite this article: Liukkonen, M., Muriel, J., Martínez-Padilla, J., Nord, A., Pakanen, V.-M., Rosivall, B., Tilgar, V., van Oers, K., Grond, K., & Ruuskanen, S. (2024). Seasonal and environmental factors contribute to the variation in the gut microbiome: A large-scale study of a small bird. *Journal of Animal Ecology*, *00*, 1–18. https://doi.org/10.1111/1365-2656.14153

II

NO EVIDENCE FOR ASSOCIATIONS BETWEEN BROOD SIZE, GUT MICROBIOME AND SURVIVAL IN GREAT TIT (*PARUS MAJOR*) NESTLINGS

by

Martta Liukkonen, Mikaela Hukkanen, Nina Cossin-Sevrin, Antoine Stier, Eero Vesterinen, Kirsten Grond & Suvi Ruuskanen 2023

Animal Microbiome 5(1): 1-16

https://doi.org/10.1186/s42523-023-00241-z

Liukkonen *et al. Animal Microbiome* (2023) 5:19
https://doi.org/10.1186/s42523-023-00241-z

Animal Microbiome

RESEARCH Open Access

No evidence for associations between brood size, gut microbiome diversity and survival in great tit (*Parus major*) nestlings

Martta Liukkonen[1*], Mikaela Hukkanen[2,3], Nina Cossin-Sevrin[2], Antoine Stier[2,4,5], Eero Vesterinen[2], Kirsten Grond[6] and Suvi Ruuskanen[1,2]

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. As the gut microbiome is a vital part of organismal health, it is important to understand how it may connect to host performance. 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 significantly 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. We also demonstrated that early handling had no impact on nestling performance or gut microbiome. Furthermore, we found no significant association between gut microbiome diversity and short-term (survival to fledging) or mid-term (apparent juvenile) survival.

Conclusions We found no clear association between early-life environment, offspring condition and gut microbiome. This suggests that brood size is not a significantly contributing factor to great tit nestling condition, and that other environmental and genetic factors may be more strongly linked to offspring condition and gut microbiome. Future studies should expand into other early-life environmental factors e.g., diet composition and quality, and parental influences.

Keywords Avian microbiome, Brood size, Gut microbiome, *Parus major*, 16S rRNA gene

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 [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

*Correspondence:
Martta Liukkonen
martta.a.liukkonen@jyu.fi
Full list of author information is available at the end of the article

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, i.e., microbes that usually do not cause disease in healthy individuals, but may become harmful in individuals that are immunocompromised, 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 forms at a young age and remains somewhat stable in adulthood as found for example in laboratory bred mice [18–20]. Disruption in the gut microbiome that leads to a microbiome imbalance at a young age could result in both short-term and long-term changes in the gut microbiome [21, 22]. Of the environmental effects, diet [23], including e.g., macronutrient balance (carbohydrates, fats, amino acids) [3, 24] has been concluded to be major determinants of rat and mouse 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 most birds [8], have only started to attract attention [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]. In mammals, the offspring are exposed to bacterial colonization during vaginal birth [35] and lactation [36, 37], whereas bird hatchlings are exposed to bacteria first upon hatching [20, 38]. Few studies have investigated the possibility of bacterial colonization *in ovo*, but results are still lacking [39]. 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]. It has also been shown that environmental factors are major contributors in the formation of gut microbiome [48–51], one of these being the rearing environment in the nest [44].

As early-life environment is connected to the establishment of gut microbiome, brood size may affect gut microbiome [52]. Brood size is often associated with parents' performance and ability to feed their young [53], and the trade-off between offspring quality and quantity has been studied widely [54, 55]. Food quantity per nestling can decrease in enlarged broods, as parents may not be able to fully compensate for the additional amount of food an enlarged brood requires [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 [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.

Alterations in early-life gut microbiome could have long-term consequences on individual performance [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, a high abundance of bacteria in the order *Lactobacillales* of the phylum *Firmicutes* is related to higher individual fitness in Seychelles warblers [14] and great tits [61]. These bacteria are also known for the benefits for bird health in economically important species such as poultry, in which *Lactobacilli* are used as probiotics to boost immune functioning [62]. Besides *Lactobacillales,* gut bacteria belonging to other genera such as *Clostridium* and *Streptococcus* are important for the degradation of non-starch polysaccharides and for the synthesis of essential molecules such as the short-chain fatty acids [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 such key physiological processes that could influence for example nestling body mass, which is tightly linked to survival to fledging [58, 59]. Because the gut microbiome establishes at a young age and is less plastic later in life [18–20], gut microbiome and changes to its richness can have long-term effects on juvenile and adult survival [21, 22]. For example, antibiotic treatment at infancy can affect the expression of genes involved in immune system functioning and lead to long-term effects on host metabolism [20]. 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 manipulation influenced wild great tit nestlings' bacterial gut microbiome diversity on day 7 post-hatch. We also investigated whether brood size influenced nestling body mass on day 7 or on day 14 post-hatch, and if the 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 or enlarging the original brood size in order to analyze if this affected the gut microbiome. 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. This might impair nestling body mass and fitness prospects [13, 52]. We used a partial cross-fostering design that enabled us to disentangle the relative contributions of genetic background, early maternal effects, and rearing environment such as parents, nest and nestmates on gut microbiome. Furthermore, we used an unmanipulated control group in which no nestling was cross-fostered to control for the possible effects of moving the nestlings between nests. For example, early human handling such as marking and weighing at day 2 post-hatch could influence 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 on day 7 post-hatch would increase survival to fledging and potentially reflect apparent juvenile survival. Such knowledge could provide new information 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 a suitable model species. 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) on Ruissalo island (60°25′59.99″ N 22°09′60.00″ E). Ruissalo island habitat is a mostly temperate deciduous forest and meadows, and some areas have small patches of coniferous trees.

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. Increases in great tit brood size can lead to lowered weight in both the nestlings and adults [70–75], and our decision on the number (i.e., +2 or −2) of manipulated nestlings (i.e, +2 or −2) followed the cited studies. We had four treatment groups (see Fig. 1): in the 'enlarged group (henceforward called E)', we increased the brood size by two individuals that were taken from a 'reduced brood'. Correspondingly, in the 'reduced group (henceforward called R)', we decreased the brood size by two individuals, that were added to the enlarged broods. In the 'control group (henceforward called C)', we swapped nestlings between nests but did not change the brood size. And lastly, in the 'unmanipulated control group (henceforward called 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 for COU had both original and fostered nestlings. Control nests were used to control for potential cross-fostering effects unrelated to brood size. Additionally, in the unmanipulated control group nestlings were not moved or weighed on day 2 in order to control for 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 with a precision of 0.1 g and identified by clipping selected toenails. We aimed to add/remove nestlings that were of similar weight to avoid changing the sibling hierarchy in the brood. The moving procedure was performed as quickly as possible to minimize the risk of stress and the nestlings were kept in a warmed box during transportation. For each pair of nests in the brood size manipulation experiment, we selected nests that had a similar hatching date. In case of uneven number of nests hatching within a day, one or three nest(s) was/were allocated to the COU group. 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 brood size on average i.e., we did not want to only reduce the larger clutches and enlarge the smaller clutches. These is also a significant bias towards COU nests being later in the season on average (Table 1).

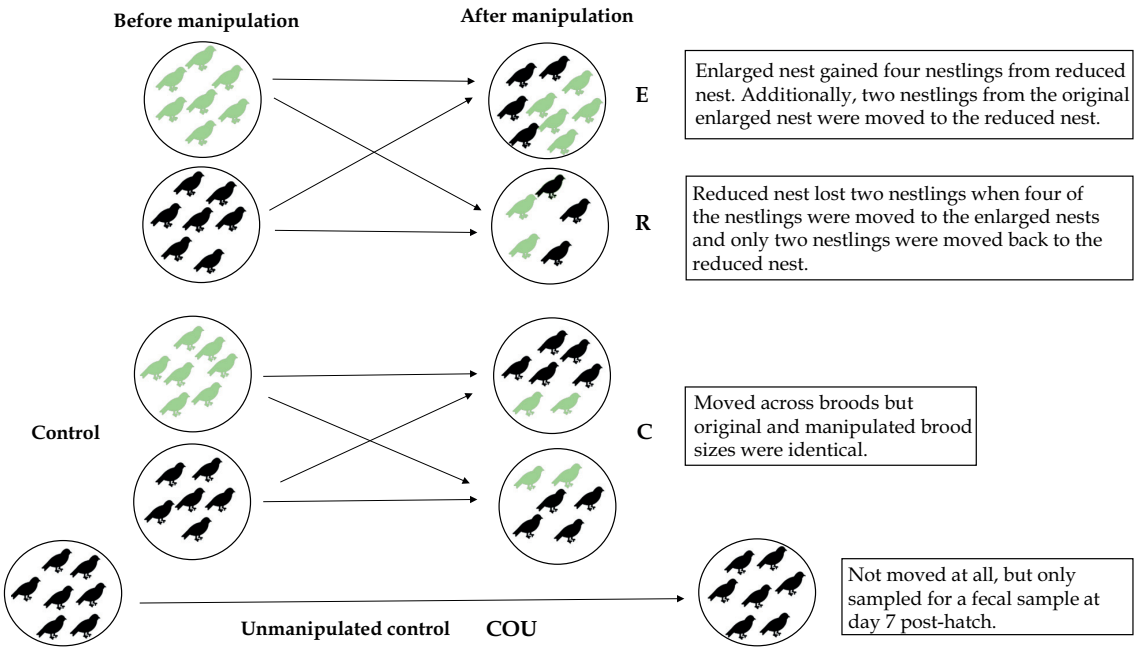

Fig. 1 Brood size manipulation experiment schematic diagram. 2-day-old nestlings were moved between nestboxes to enlarge or to reduce original brood size (an example with brood size of seven is given). Some nests were kept as control nests (nestlings were moved but brood size remained the same) and some 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

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 data from a larger experiment (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 two samples (one from original and one 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 (0.1 g), and the nestlings were ringed for individual identification using aluminum bands. The samples were stored in cool bags onsite and afterwards moved to a -80 °C freezer for storage until DNA extraction.

Apparent juvenile survival

We monitored all study nests until fledging to measure short-term 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. Additionally, we monitored our study 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 h 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 by dispersal. In a previous study in our population [76], none of the birds ringed as nestlings were recaptured outside the study area, suggesting that dispersal is likely limited.

Table 1 (A) Brood size before and after manipulation, (B) hatching date across treatments

(A)	Brood size	Before manipulation (mean ± SD)	After manipulation (mean ± SD)
	Enlarged broods (E)	7.700 ± 1.61	9.650 ± 1.309
	Reduced broods (R)	8.375 ± 1.637	6.375 ± 1.637
	Control broods (C)	7.565 ± 1.805	7.565 ± 1.805
	Unmanipulated broods	7.810 ± 2.112	na
	ANOVA	F3 = 0.987, $p = 0.403$	

(B)	Hatching date	Mean ± SD
	Enlarged broods (E)	58.60 ± 5.77
	Reduced broods (R)	59.83 ± 6.41
	Control broods (C)	58.74 ± 5.34
	Unmanipulated broods	63.81 ± 4.79

(B)	**Tukey's *post-hoc* for between-group comparisons Average hatching date**				
	ANOVA	**F3 = 3.964, $p = 0.011$***			
	Contrasts	**Estimate**	**SE**	**t.ratio**	***p***
	COU-C	5.070	1.70	2.983	0.019*
	COU-E	5.210	1.76	2.961	0.020*
	COU-R	3.976	1.68	2.363	0.092
	C-E	0.139	1.72	0.081	0.100
	C-R	− 1.094	1.64	− 0.666	0.910
	E-R	− 1.233	1.70	− 0.723	0.888

(E) enlarged brood size, (R) reduced brood size, (C) control brood size, (COU) unmanipulated control brood size. Brood size was successfully either reduced or enlarged by two chicks

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. 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 min followed by 30 cycles of 95 °C for 45 s, 55 °C for 60 s, and 72 °C for 90 s, and finished with a 10-min 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 min followed by 18 cycles of 98 °C for 20 s, 60 °C for 15 s, and 72 °C for 30 s, and finished with a 3-min 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 × 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

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 the single DNA sequences i.e., Amplicon Sequence Variants (henceforward ASV), and to construct a 'seqtab'. Seqtab is a matrix also known as otutable or readtable: ASVs in columns, samples in rows, number of reads in each cell, using default parameter settings. In total, our seqtab consisted of 6,929,537 high-quality reads. Reads were assigned to taxa against the SILVA v132 reference database [82] resulting in 8658 ASVs. 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 6566 ASVs (a total of 4,107,028 high-quality reads in all samples; mean per sample: 15,155.085; mean range per sample: 0–97,264). Singleton reads were removed from the dataset by the DADA2 pipeline. 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]).

The final dataset contained 92 samples from great tit nestlings resulting in a total of 3,161,696 reads (mean per sample: 34,366.261; mean range per sample 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 (see Additional file 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 (see Additional file 3) 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.

Statistical analyses

Nestling body mass

First, to analyze whether brood size manipulation affected nestling body mass in the C, E, and R treatment groups, we ran two linear mixed-effects models with the *lme4* package (v. 1.1-29; [91]). In these models we used either body mass on day 7 or 14 as the dependent variable and brood size manipulation treatment, hatching date, body mass on day 2 post-hatch and original brood size as predicting variables. Hatching date is used as a predicting variable because it is known to affect nestling body mass during the breeding season [92] and there were differences in hatching date between the COU and other treatment groups (see Table 1). We included the interaction between original brood size and brood size manipulation treatment in both models as the effect of manipulation may depend on the original brood size. For example, there could be 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 COU group in the analysis because we wanted to measure the effects of treatment on nestling body mass, and only enlarged, reduced or control broods' nestlings were moved between nests.

Second, to analyze whether the actual brood size affected nestling body mass, we ran two models where we used it as a continuous dependent variable to explain body mass either on day 7 or on day 14 post-hatch. Hatching date and body mass on day 2 post-hatch were used as predicting variables and nest of origin and nest of rearing as random intercepts to control for the non-independency of samples. We included the interaction between manipulated brood size and hatching date in the models because the effect of brood size may depend on the hatching date. For example, hatching date can reflect environmental conditions and large broods may perform poorly late in the season due to poorer food availability. The COU group was initially 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 origin explained more of the variation in the first model (see Additional file 4) and therefore, we used it in the full models with all treatment groups: C, COU, E and R. In these models, nestling body mass either on day 7 and or on day 14 post-hatch was used as a dependent variable and manipulated brood size as the explanatory variable. Hatching date and body mass on day 2 post-hatch were set as predicting variables. Nest of rearing was used as a random intercept to control for the non-independence of nestlings sharing the same foster nests. The significance of factors included in the models were tested using the F-test ratios in analysis of variance (Type III ANOVA).

Alpha diversity

For alpha diversity analyses, which measures within-sample species diversity, we ran two linear mixed-effects models with the *lme4* package (v. 1.1-29; [91]) to measure if either brood size manipulation or manipulated brood size as a continuous variable were associated with gut microbiome diversity. We used two alpha diversity metrics: the Shannon Diversity Index, which measures the number of bacterial ASVs and their abundance evenness within a sample, and Chao1 Richness, which is an estimation of the number of different bacterial ASVs in a sample. Both metrics were used to check if alpha diversity results were consistent across different metrics. Each diversity index was used as the dependent variable at a time and either brood size manipulation treatment or manipulated brood size as a predicting variable. In both models we included original brood size, weight on day 7 post-hatch and hatching date as covariates. We included interaction between brood size manipulation treatment and original brood size as there could be a stronger effect of enlargement in initially large broods. We also included interaction between manipulated brood size and weight on day 7 post-hatch because effect of brood size on microbiome may depend on nestling weight. 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. In this analysis we used weight on day 7 post-hatch as the dependent variable and alpha diversity (Shannon Diversity Index and Chao1 Richness), treatment and hatching date as predicting variables and nest of rearing as the random effect. In these sets of models, we first excluded the COU group to see which of the two random effects, nest of origin or nest of rearing, explained a larger proportion of variation in the treatment groups. Nest of rearing explained more of the variation in this model (see Additional file 4) 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 generalized linear models 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 predicting and there was no order between covariates, as they could not be ranked. Survival to fledging and recapture in Autumn 2020 were used as the binomial response variable (yes–no) in each model. Alpha diversity (Shannon Diversity Index and Chao1 Richness) was the main predicting variable, and weight on day 7 post-hatch (same time as sampling the fecal gut microbiome), hatching date and manipulated 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. 65 nestlings fledged successfully, while 8 nestlings were found dead in nest boxes. For 15 nestlings we 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 linear mixed-effects and generalized linear models.

Beta diversity

For visualizing beta diversity, i.e., the similarity or dissimilarity between the treatment group gut microbiomes, 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 tested with the R package vegan (*adonis2* function; v. 2.6-2; [97]) to investigate if any variables affected to the variation in gut microbiome composition. Nest of rearing was set as a blocking factor in the PERMANOVA to control for the non-desirable effects of the repeated sampling of foster siblings. The test for homogeneity of multivariate dispersions was used to measure the homogeneity of group dispersion values. We used the phyloseq package (v. 1.32.0; [84]) to run a differential abundance analysis with a significance cut-off $p < 0.01$ to test the differential abundance of ASVs between the treatment groups.

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: $F_{2,\ 25.832} = 0.441$, $p = 0.648$; see Additional file 5). Moreover, there was no significant interaction between brood size manipulation and original brood size (ANOVA: $F_{2,\ 24.610} = 0.678$, $p = 0.517$; see Additional file 5). On day 14 post-hatch, brood size manipulation did not significantly affect nestling body mass (ANOVA: $F_{2,\ 24.335} = 0.831$,

$p = 0.448$; see Additional file 5). However, body mass increased with increasing hatching date (ANOVA: $F_{1, 24.070} = 13.367$, $p = 0.001$; see Additional file 5). Next, we did not find any significant associations between manipulated brood size and nestling body mass (ANOVA for weight on day 7: $F_{1, 35.149} = 1.777$, $p = 0.191$; ANOVA for weight on day 14: $F_{1, 29.491} = 2.156$, $p = 0.153$; see Additional file 6). Nest of origin explained a larger proportion of the variation in weight than the nest of rearing on both day 7 (nest of origin 41.1% and nest of rearing 24.4%) and day 14 (nest of origin 65.5% and nest of rearing 21.9%) post-hatch, but this result was not statistically significant ($Pr > \chi 2 = 1$) (see Additional file 4).

Alpha diversity

As 7-day-old nestlings, most bacterial taxa belonged to the phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria* (Fig. 2).

Brood size manipulation did not significantly influence alpha diversity (Shannon Diversity Index) (ANOVA: $F_{3, 47.488} = 1.026$, $p = 0.390$, Fig. 3; see Additional file 7). Moreover, original brood size (ANOVA: $F_{1, 50.269} = 0.388$, $p = 0.536$; see Additional file 7), weight on day 7 post-hatch (ANOVA: $F_{1, 80.551} = 0.003$, $p = 0.959$; see Additional file 7), and hatching date (ANOVA: $F_{1, 50.276} = 1.073$, $p = 0.305$; see Additional file 7) did not significantly associate with alpha diversity. There was no significant interaction between brood size manipulation and original brood size (ANOVA: $F_{3, 48.053} = 0.126$, $p = 0.944$; see Additional file 7). Results for Chao1 Richness were quantitatively similar: brood size manipulation did not affect alpha diversity (ANOVA: $F_{3, 45.936} = 0.358$ $p = 0.784$, Fig. 3; see Additional file 7). Nest of rearing explained a larger proportion of the observed variance in alpha diversity (27.7%) than nest of origin (10.8%), but the result was not statistically significant ($Pr > \chi 2 = 1$) (see Additional file 4).

Next, we tested whether the manipulated brood size as a continuous variable was associated with alpha diversity (Shannon Diversity Index), but found no significant association (ANOVA: $F_{1, 63.001} < 0.001$, $p = 0.984$; see Additional file 8) in this analysis either. Weight on day 7 post-hatch (ANOVA: $F_{1, 82.840} = 0.015$, $p = 0.903$; see Additional file 8) and hatching date (ANOVA: $F_{1, 59.734} = 0.137$, $p = 0.713$; see Additional file 8) did not correlate with alpha diversity in this model either. There was no significant interaction between manipulated

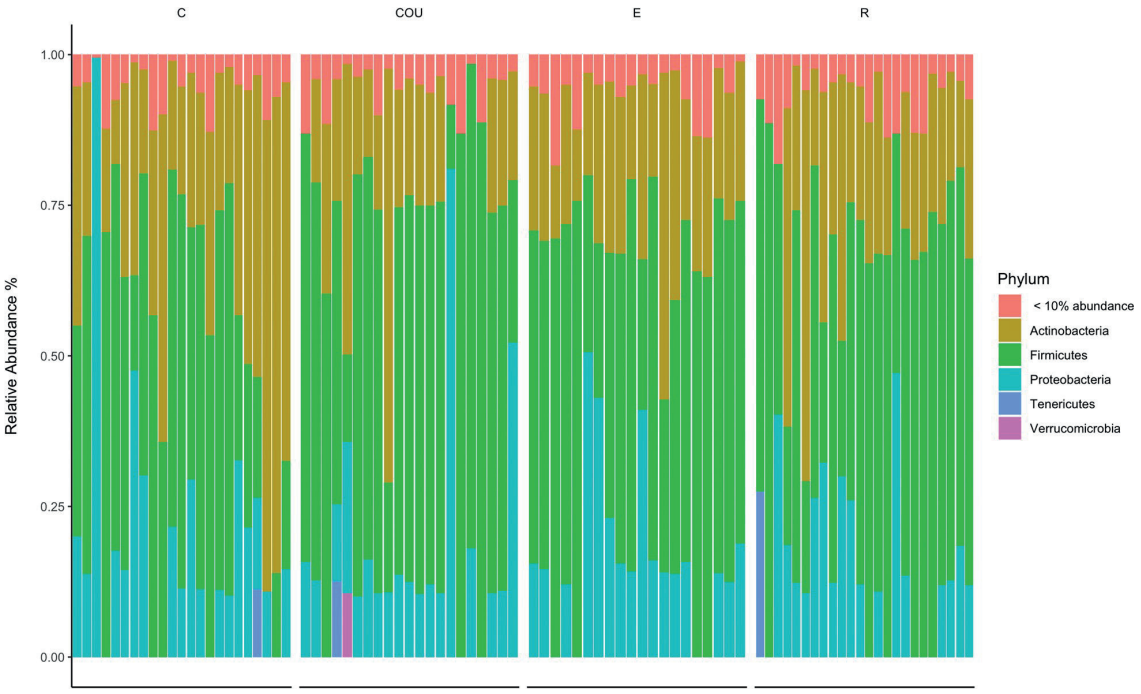

Fig. 2 Bacterial relative abundances on Phylum level across the four treatment groups. Each bar represents an individual sample. Treatment groups are control (C), unmanipulated control (COU), enlarged (E), and reduced (R). N = 88 samples divided into treatment groups as follows: C = 23, COU = 21, E =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

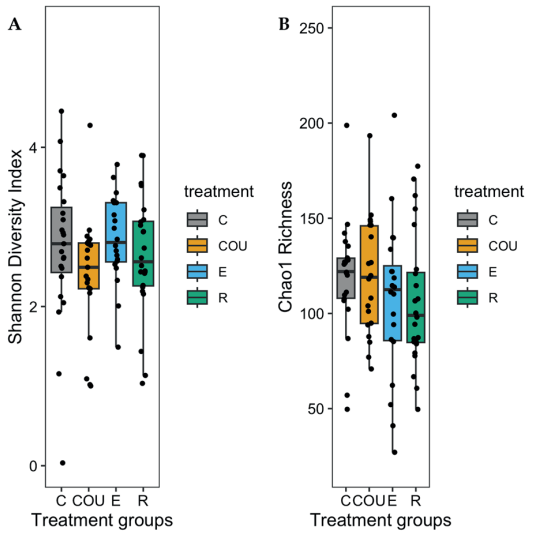

Fig. 3 The gut microbiome alpha diversity of 7-day-old great tit nestlings across the four treatment groups visualized with two diversity metrics: **A** Shannon Diversity Index and **B** Chao1 Richness. The black dots represent each observation within a treatment group. The whiskers represent 95% confidence 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

brood size and weight on day 7 post-hatch (ANOVA: $F_{1,\ 82.702} < 0.000$, $p = 0.998$; see Additional file 8). Results for Chao1 Richness were quantitatively similar (ANOVA: $F_{1,\ 65.064} = 0.246$, $p = 0.622$; see Additional file 8): manipulated brood size did not affect alpha diversity, and neither did weight on day 7 post-hatch (ANOVA: $F_{1\ 83.513} = 0.690$, $p = 0.409$; see Additional file 8) nor hatching date (ANOVA: $F_{1\ 57.110} = 1.133$, $p = 0.292$; see Additional file 8).

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 (Shannon Diversity Index: $\chi2 = 0.010$, df = 1, $p = 0.923$; see Additional files 9 and 10), manipulated brood size ($\chi2 = 0.090$, df = 1, $p = 0.764$; see Additional file 9), weight on day 7 post-hatch ($\chi2 = 0.388$, df = 1, $p = 0.533$; see Additional file 9) or hatching date ($\chi2 = 0.438$, df = 1, $p = 0.508$; see Additional file 9).

Apparent juvenile survival was not significantly associated with alpha diversity (Shannon Diversity Index: $\chi2 = 1.916$, df = 1, $p = 0.166$; see Additional file 9 and Additional file 10). Moreover, there was no significant interaction between alpha diversity and manipulated brood size ($\chi2 = 1.268$, df = 1, $p = 0.260$; see Additional file 9). However, apparent juvenile survival was negatively associated with hatching date ($\chi2 = 4.654$, df = 1, $p = 0.031$; see Additional file 9). Additional analyses to check for the consistency of results were tested the following way: survival to fledging with nestlings from the COU group removed and apparent juvenile survival without the nestlings with no recorded survival for fledging (see methods). These results were quantitatively similar as in the whole dataset for both Shannon Diversity Index (survival to fledging: $\chi2 = 2.285$, df = 1, $p = 0.131$; apparent juvenile survival: $\chi2 = 1.515$, df = 1, $p = 0.218$; see Additional file 11) and Chao1 Richness (survival to fledging: $\chi2 = 0.665$, df = 1, $p = 0.415$; apparent juvenile survival: $\chi2 = 2.654$, df = 1, $p = 0.103$; see Additional file 11).

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 (see Additional file 3). The test for homogeneity of multivariate dispersions supported the visual assessment of the NMDS ($Betadispersion_{9999\ permutations}$: $F_{3,\ 0.069} = 0.650$, $p < 0.001$; see Additional file 12). Pairwise PERMANOVA further indicated that the treatment (PERMANOVA: $R^2 = 0.061$, $F = 1.951$, $p = 0.278$; see Additional file 12), 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. Differential analysis of ASV abundance between the treatment groups showed that there is variation in taxa abundance. E group showed higher taxa abundance when compared to COU and C groups and was slightly higher than the R group. C and COU groups were generally lower in taxa abundance than R and E groups, and COU group showed lower abundance than the other groups in each comparison (see Additional file 13).

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 (Fig. 2), brood size did not significantly contribute to gut microbiome diversity. Neither did gut microbiome diversity explain

short-term (survival to nestling) nor mid-term (apparent juvenile) survival. Body mass was also not significantly affected by brood size manipulation. The COU group that functioned as a control for moving and handling effects, did not differ in this respect from the other groups. This suggests that human contact or handling nestlings 2 days post-hatch did not influence nestling gut microbiome or body mass. The partial cross-fostering design enabled us to disentangle the relative contributions of rearing environment (i.e., parents, nest and nestmates) from genetic, prenatal such as maternal allocation to egg, and early post-natal effects such as feeding up to day 2. Nest of rearing seemed to explain more of the variation in nestling gut microbiome diversity than the nest of origin (although not statistically significant), which follows previous studies. Contrastingly, nest of origin seemed to be a stronger contributor than the nest of rearing on nestling body mass on day 7 and day 14 post-hatch. This result was also not statistically significant.

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 reduction nor enlargement of the 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 [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 [52, 53, 57, 67, 99–104].

There are a few possible explanations why brood size manipulation did not affect nestling body mass. Firstly, 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. In that case the number of nestlings transferred between enlarged and reduced nests should probably have been larger to create differences in nestling body mass between the two treatments. Still, we think that the decision to transfer $+2/-2$ was reasonable since it was based on extensive evidence from previous studies [103]. Secondly, it could be that the enlarged brood size negatively influences some other physiological traits while body mass was retained at the expense of these other traits e.g., immune system functioning [105, 106]. Moreover, our analysis 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, changes in temperature conditions or in parental investment during the breeding season. As the season progresses, the abundance of insect taxa varies, and this can result in changes in nutrient rich food [103, 107]. For example, great tits can select certain lepidopteran larvae that vary in their abundance during the great tit breeding season [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. For example, parents were likely able to provide an equivalent amount of food, given that body mass was not significantly affected by the brood size manipulation. 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. Firstly, the time interval may not have been long enough to detect effects of the brood size manipulation. Secondly, 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]. 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 [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 [116, 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 [114–119]. 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, 107] this could affect the between-nestling and between-nest gut microbiome diversity. However, using wild bird populations in gut microbiome studies limits the ability to control the consumed dietary items because parents may use variable food resources and there can be variance in dietary between sexes and even individuals. Visual assessment of dietary items [116] and metabarcoding could be of use here as they enable the identification of food items on genus and even species level 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 indicated that the nest of rearing seemed to explain more of the gut microbiome variation than the nest of origin (although not significant), 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 [20].

Results from beta diversity analysis were similar to 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. The study suggested that individual homeostatic mechanisms linking to naturally occurring differences in individual gut microbiome could be why gut microbiome composition varied even with standardized housing conditions [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 behavior [115, 116].

Differential analysis of ASV abundance showed variation in differential abundance of taxa between the treatment groups. However, several ASVs were not taxonomically assigned beyond family level making it difficult to draw conclusions about the significance of these results. All treatment groups had taxa belonging to the order *Firmicutes, Proteobacteria* and *Actinobacteria*, which was to be expected because they are usually the most core phyla in passerine gut microbiomes [33]. Nestlings belonging to E, R or C group showed higher taxa abundance than the COU group in each comparison. This result could be a result of the COU nestlings generally hatching later in the season and potentially having a less diverse diet [103, 107]. Of the E, R and C groups, C group was less abundant than E and R groups. Both E and R group showed high taxa abundance, which is interesting because we hypothesized that nestlings belonging to the E group would potentially experience less parental investment per nestling and have lower gut microbiome diversity and therefore, be less abundant [56, 57, 67, 68]. We did not observe differential abundance in e.g., the order *Lactobacillales* which would have been of interest, because the order hosts taxa that are beneficial for gut microbiome health [14, 62]. The genus *Staphylococcus* was differentially abundant in the E group, but not in the other groups. *Staphylococcus* is a gram-positive genus of bacteria and known to cause infections in its host species [122]. Curiously, the COU group was differentially abundant in the genus *Dietzia*, which is a human pathogen [123].

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. 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 should be included in gut microbiome studies. Different bacterial taxa can have similar functions in the gut microbiome [5, 124] 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 [125]. Similarity in functions may also contribute to host's local adaptation e.g., to the changes in the host's early-life environment [124]: 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 contrasts with previous studies, but it should be noted that the majority of previous studies have been done with adult birds and not nestlings. Because nestling gut microbiome is still quite flexible compared to that of the adults [20], it is possible that our experiment did not result in a strong enough effect on the gut microbiome. In future studies, it would be important to study the parents as well as it could be more likely to find an association between adult microbiome and fitness than with nestling gut microbiome and survival. Also, 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, 126]. 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, 127]. A study with blackbirds (*Turdus merula*) showed that nestling body mass explained juvenile survival [128], and similar results have been shown with great tits and collared flycatchers (*Ficedula albicollis*; [31]). Contrastingly, Ringsby et al. [129] observed that in house sparrows (*Passer domesticus*) juvenile survival was independent of nestling mass and 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, 130, 131], yet we failed to demonstrate this in our study. Hatching date is also often positively correlated with fledging success [132] 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 between-individual 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) 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.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-023-00241-z.

Additional file 1: Brood size before and after manipulation: brood sizes between treatment groups were tested with a linear model to see if the differences were statistically significant.

Additional file 2: Rarefaction curves for the unrarefied dataset. Species (ASVs) plateaued at about 5000 reads which was used as the rarefying depth.

Additional file 3: Phylogenetic tree using the Newick-format. The tree describes the dissimilarity among the treatment groups. Each tip represents an individual sample, and each tip is colored and shaped based on treatment. Treatment groups are clustered using the UPGMA algorithm.

Additional file 4: (A) Linear mixed effects model for gut microbiome diversity (Shannon Diversity Index and Chao1 Richness) and brood size manipulation treatment. (B) Linear mixed effects model for GM diversity (Shannon Diversity Index and Chao1 Richness) and manipulated brood size.

Additional file 5: A linear mixed effects model investigating the effects of brood size manipulation on nestling body mass on day 7 and day 14 post-hatch.

Additional file 6: A linear mixed effects model investigating the effects of manipulated brood size on nestling body mass on day 7 and day 14 post-hatch.

Additional file 7: A linear mixed effects model investigating the associations between alpha diversity (Shannon Diversity Index and Chao1 Richness) and brood size manipulation.

Additional file 8: A linear mixed effects model investigating the association between alpha diversity (Shannon Diversity Index and Chao1 Richness) and manipulated brood size.

Additional file 9: 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.

Additional file 10: The gut microbiome alpha diversity (Shannon Diversity Index and Chao1 Richness) and short-term survival.

Additional file 11: Generalized linear model to measure the association between alpha diversity (Shannon Diversity Index and Chao1 Richness) survival to fledging and apparent juvenile survival.

Additional file 12: Ordination of the gut microbial communities.

Additional file 13: Differential analysis of abundance (DESeq2) to assess the ASV abundance between the treatment groups

Acknowledgements
We would like to thank Charli Davies, Petri Papponen, Sari Viinikainen and Elina Virtanen at the University of Jyväskylä for their help with bioinformatics (CD) laboratory optimizations (PP, SV and EV), 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.

Author contributions
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.

Funding
Open Access funding provided by University of Jyväskylä (JYU). This study was funded by the Emil Aaltonen Foundation. NCS was supported by EDUFI Fellowship (Opetushallitus). 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.

Availability of data and materials
Sequence files are available at NCBI database (BioProject PRJNA877058). Metadata and R scripts used to run the analyses are available in the Github Repository, https://github.com/marannli/bsm_analyses.

Declarations

Ethics approval and consent to participate
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.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Biological and Environmental Science, University of Jyväskylä, Jyvaskyla, Finland. [2]Department of Biology, University of Turku, Turku, Finland. [3]Institute for Molecular Medicine Finland, University of Helsinki, Helsinki, Finland. [4]Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA, 69622 Lyon, France. [5]Institut Pluridisciplinaire Hubert Curien, UMR7178, Université de Strasbourg, CNRS, Strasbourg, France. [6]Department of Biological Sciences, University of Alaska Anchorage, Anchorage, AK 99508, USA.

Received: 20 September 2022 Accepted: 13 March 2023
Published online: 22 March 2023

References

1. Kinross JM, Darzi AW, Nicholson JK. Gut microbiome-host interactions in health and disease. Genome Med. 2011;3(3):1–12.
2. van Dongen WF, White J, Brandl HB, Moodley Y, Merkling T, Leclaire S, Wagner RH. Age-related differences in the cloacal microbiota of a wild bird species. BMC Ecol. 2013;2013(13):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):559–63.
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. Mol Nutr Food Res. 2017;61(1):1600240.
5. Heintz-Buschart A, Wilmes P. Human gut microbiome: function matters. Trends Microbiol. 2018;26(7):563–74.
6. Egerton S, Culloty S, Whooley J, Stanton C, Ross RP. The gut microbiota of marine fish. Front Microbiol. 2018;9:873.
7. Pan D, Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. Gut microbes. 2014;5(1):108–19.
8. Hird SM. Evolutionary biology needs wild microbiomes. Front Microbiol. 2017;8:725.
9. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220–30.
10. Zaneveld JR, McMinds R, Vega TR. Stress and stability: applying the Anna Karenina principle to animal microbiomes. Nat Microbiol. 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. 2017;171(5):1015–28.
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. J Immunol. 2017;198:68–5.
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. Anim 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 Microbiol Rev. 2008;32:723–35.
16. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009;9:313–23.
17. Kopac SM, Klassen JL. Can they make it on their own? Hosts, microbiomes, and the holobiont niche. Front Microbiol. 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):705–21.
20. Grond K, Lanctot RB, Jumpponen A, Sandercock BK. Recruitment and establishment of the gut microbiome in arctic shorebirds. FEMS Microbiol Ecol. 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. Integr Comp Biol. 2017;57(4):732–42.
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 JM Jr, 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):1268–73.
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. Proc R Soc B. 1920;2020(287):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. 2021;3(1):1–14.
27. Góngora E, Elliott KH, Whyte L. Gut microbiome is affected by intersexual and inter-seasonal variation in diet for thick-billed murres (*Uria lomvia*). Sci Rep. 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. Sci Rep. 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. Int J Parasitol. 1998;28(8):1269–78.
30. Cotter SC, Simpson SJ, Raubenheimer D, Wilson K. Macronutrient balance mediates trade-offs between immune function and life history traits. Funct Ecol. 2011;25(1):186–98.
31. Linden M, Gustafsson L, Part T. Selection on fledging mass in the collared flycatcher and the great tit. Ecology. 1992;73(1):336–43.
32. Spor A, Koren O, Ley R. Unravelling the effects of the environment and host genotype on the gut microbiome. Nat Rev Microbiol. 2011;9(4):279–90.
33. Bodawatta KH, Hird SM, Grond K, Poulsen M, Jønsson KA. Avian gut microbiomes taking flight. Trends Microbiol. 2022;30:268–80.
34. Waite DW, Taylor M. Exploring the avian gut microbiota: current trends and future directions. Front Microbiol. 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. Front Immunol. 2021;12:1768.
38. Kohl KD. Diversity and function of the avian gut microbiota. J Comp Physiol 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. Biol Let. 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. Biol J Lin Soc. 2009;96(2):406–14.
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. Mol Ecol. 2010;19(24):5531–44.
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. ISME J. 2020;14(11):2691–702.
43. Lucas FS, Heeb P. Environmental factors shape cloacal bacterial assemblages in great tit *Parus major* and blue tit *P. caeruleus* nestlings. J Avian Biol. 2005;36:510–6.
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;2: e321.
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;92:123–30.
47. Ding J, Dai R, Yang L, He C, Xu K, Liu S, et al. Inheritance and establishment of gut microbiota in chickens. Front Microbiol. 2017;8:1967. https://doi.org/10.3389/fmicb.2017.01967.
48. Preest MR, Folk DG, Beuchat CA. Decomposition of nitrogenous compounds by intestinal bacteria in hummingbirds. Auk. 2003;120(4):1091–101.
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. Res Microbiol. 2012;163(3):211–20.
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 Vet Scand. 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. Front Microbiol. 2018;9:1524.
53. Pettifor RA, Perrins CM, McCleery RH. Individual optimization of clutch size in great tits. Nature. 1988;336(6195):160–2.
54. Wright J, Both C, Cotton PA, Bryant D. Quality vs. quantity: energetic and nutritional trade-offs in parental provisioning strategies. J Anim Ecol 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):2948–60.
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. J Anim Ecol. 1999;68:708–18.
57. de Kogel CH. Long-term effects of brood size manipulation on morphological development and sex-specific mortality of offspring. J Anim Ecol. 1997;66:167–78.
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*. J Anim Ecol. 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*. J Anim Ecol. 1989;58:383–401.
60. Simon K, Verwoolde MB, Zhang J, Smidt H, de Vries RG, Kemp B, Lammers A. Long-term effects of early life microbiota disturbance on adaptive immunity in laying hens. Poult Sci. 2016;95(7):1543–54.
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. J Anim Ecol. 2021;90(4):989–1003.
62. Kabir SM. The role of probiotics in the poultry industry. Int J Mol Sci. 2009;10(8):3531–46.
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. J Lipid Res. 2013;54(9):2325–40.
66. Harrison XA, Blount JD, Inger R, Norris DR, Bearhop S. Carry-over effects as drivers of fitness differences in animals. J Anim Ecol. 2011;80(1):4–18.
67. Leonard ML, Horn AG, Gozna A, Ramen S. Brood size and begging intensity in nestling birds. Behav Ecol. 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. J Anim Ecol. 2009;78(2):414–26.
69. Perrins CM. Population fluctuations and clutch size in the Great tit, *Parus major*. J Anim Ecol. 1965;34:601–47.

70. Smith HG, Källander H, Nilsson JÅ. Effect of experimentally altered brood size on frequency and timing of second clutches in the great tit. Auk. 1987;104(4):700–6.
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):316–22.
72. Sanz JJ, Tinbergen JM. Energy expenditure, nestling age, and brood size: an experimental study of parental behavior in the great tit Parus major. Behav Ecol. 1999;10(5):598–606.
73. Tinbergen JM, Verhulst S. A fixed energetic ceiling to parental effort in the great tit? J Anim Ecol. 2000;69(2):323–34.
74. Neuenschwander S, Brinkhof MW, Kölliker M, Richner H. Brood size, sibling competition, and the cost of begging in great tits (*Parus major*). Behav Ecol. 2003;14(4):457–62.
75. Tinbergen JM, Sanz JJ. Strong evidence for selection for larger brood size in a great tit population. Behav Ecol. 2004;15(4):525–33.
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. J Exp Biol. 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. ISME J. 2012;6(8):1621–4.
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. Environ Microbiol. 2016;18(5):1403–14.
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):10–2.
81. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581–3.
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 Res. 2012;41(D1):D590–6.
83. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018;2018(6):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. Data analysis. In *ggplot2* (pp. 189–201). 2016; 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):592–3.
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 Ecol Evol. 2017;8(1):28–36.
91. Bates D, Maechler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw. 2015;67(1):1–48. https://doi.org/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;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. p. 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. Ecol Monogr. 1957;27(4):326–49.
96. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol. 2005;71(12):8228–35.
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. J Anim Ecol. 1995;64:721–32.
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 Scand. 1977;8:1–8.
100. Nur N. The consequences of brood size for breeding blue tits II. Nestling weight, offspring survival and optimal brood size. J Anim Ecol. 1984;53:497–517.
101. Sanz JJ. Clutch size manipulation in the pied flycatcher: effects on nestling growth, parental care and moult. J Avian Biol. 1998;28:157–62.
102. Burness GP, McClelland GB, Wardrop SL, Hochachka PW. Effect of brood size manipulation on offspring physiology: an experiment with passerine birds. J Exp Biol. 2000;203(22):3513–20.
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. J Avian Biol. 2000;31(2):206–14.
104. Rytkönen S, Orell M. Great tits, Parus major, lay too many eggs:experimental evidence in mid-boreal habitats. Oikos. 2001;93:439–50.
105. van der Most PJ, de Jong B, Parmentier HK, Verhulst S. Trade-off between growth and immune function: a meta-analysis of selection experiments. Funct Ecol. 2011;25(1):74–80.
106. Rauw WM. Immune response from a resource allocation perspective. Front Genet. 2012;3:267.
107. 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. J Anim Ecol. 1999;6(84):708–18.
108. Barba E, Gil-Delgado JA. Seasonal variation in nestling diet of the great tit Parus major in orange groves in eastern Spain. Ornis Scand. 1990;21:296–8.
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. Funct Ecol. 1994;8:563–72.
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):148–54.
111. Kohl KD, Brun A, Caviedes-Vidal E, Karasov WH. Age-related changes in the gut microbiota of wild House Sparrow nestlings. Ibis. 2019;161(1):184–91.
112. Waite DW, Taylor MW. Characterizing the avian gut microbiota: membership, driving influences, and potential function. Front Microbiol. 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. Sci Rep. 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. Proc R Soc B. 2021;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. Sci Rep. 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 Ecol Evol. 2016;31(9):689–99.

118. Wilkin TA, King LE, Sheldon BC. Habitat quality, nestling diet, and provisioning behaviour in great tits Parus major. J Avian Biol. 2009;40(2):135–45.
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. Ecol Evol. 2019;9(1):631–9.
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*. J Avian Biol. 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. Ecol Evol. 2020;10(23):13345–54.
122. Kloos WE. Natural populations of the genus Staphylococcus. Annu Rev Microbiol. 1980;34(1):559–92.
123. Gharibzahedi SMT, Razavi SH, Mousavi SM. Characterization of bacteria of the genus Dietzia: an updated review. Ann Microbiol. 2014;64(1):1–11.
124. Hanning I, Diaz-Sanchez S. The functionality of the gastrointestinal microbiome in non-human animals. Microbiome. 2015;3:5. https://doi.org/10.1186/s40168-015-0113-6.
125. Doak DF, Bigger D, Harding EK, Marvier MA, O'malley RE, Thomson D. The statistical inevitability of stability-diversity relationships in community ecology. Am Nat. 1998;151(3):264–76.
126. Naef-Daenzer B, Widmer F, Nuber M. Differential post-fledging survival of great and coal tits in relation to their condition and fledging date. J Anim Ecol. 2001;70(5):730–8.
127. Nilsson JA, Svensson M. Sibling competition affects nestling growth strategies in marsh tits. J Anim Ecol. 1996;65:825–36.
128. Magrath RD. Nestling weight and juvenile survival in the blackbird, *Turdus merula*. J Anim Ecol. 1991;60:335–51.
129. Ringsby TH, Sæther BE, Solberg EJ. Factors affecting juvenile survival in house sparrow *Passer domesticus*. J Avian Biol. 1998;29:241–7.
130. Both C, Visser ME, Verboven N. Density–dependent recruitment rates in great tits: the importance of being heavier. Proc R Soc Lond Seri B Biol Sci. 1999;266(1418):465–9.
131. Cleasby IR, Nakagawa S, Gillespie DO, Burke T. The influence of sex and body size on nestling survival and recruitment in the house sparrow. Biol J Lin Soc. 2010;101(3):680–8.
132. Tinbergen JM, Boerlijst MC. Nestling weight and survival in individual great tits (*Parus major*). J Anim Ecol. 1990;59:1113–27.

Publisher's Note

III

GUT MICROBIOME DIVERSITY ASSOCIATES WITH ESTIMATED LIFETIME AND ANNUAL REPRODUCTIVE SUCCESS IN MALE BUT NOT FEMALE COLLARED FLYCATCHERS

by

Martta Liukkonen, Lars Gustafsson, Kirsten Grond & Suvi Ruuskanen 2024

Manuscript

Request a copy from the author.