Transmission ecology of the gut microbiota in wild mice

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Dedicated to the memory of Delilah the Mouse

Declaration

I hereby declare that the contents of this thesis are original and based on my own work and ideas. This includes designing the studies, collecting and analysisng data, interpreting the results and writing this thesis. Where I have used data collected by others or where others have significantly contributed in any other part of the research, this has been clearly stated in the Lists of contributions in the beginning of each chapter. The collection of the data has been conducted ethically in respect to the study subjects, in accordance with my personal license and the project license provided for this work by the Home Office.

This work, while partially published in Scientific Journals, has not been submitted for any other degree of qualification. The thesis contains less than 50 000 words excluding supplementary materials and references

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Abstract

Gut microbiota forms an influential part of its host animal's phenotype, but how variation in gut microbiota arises is poorly understood. Attempts to explain microbiota variation with traits of the host individual, such as physiology or genetics, have had limited success, and an emerging view is that microbiota is greatly shaped by processes operating between individuals, such as transmission of microbes among hosts. Parallel lines of research have shown that both transmission through social contacts and contacts with the natural environment can shape microbiota, but the relative importance of these transmission processes remains unclear. In this thesis, I use novel RFID-tracking technology and molecular microbiota profiling to study microbiota transmission in populations of wild wood mice. Combining methods from network theory and metacommunity theory I study how gut microbiota is transmitted through social contact networks among mice as well as through exposure to broader living environment and other sympatric species therein. I show that social contact networks strongly predict the microbiota composition independent of kinship and shared exposure to the same space and that transmission of microbes from the environment likewise shapes microbiota independently of social relationships, but far less so than social transmission. Further, these two transmission routes seem to transmit different sets of gut microbes, with environmental transmission being influential for aerotolerant bacteria while social networks functioning as a transmission pathway specifically for anaerobic microbes. Finally, I show that social contacts and space-sharing between individuals of different sympatric species can spread gut microbes across species boundaries within a mixed-species rodent community. The key findings are verified with alternative statistical frameworks and replicated in two separate study populations. These findings provide striking evidence for transmission processes in shaping the gut microbiota of wild mammals and emphasize the importance of considering contexts beyond individuals in explaining microbiota variation.

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Introduction

1.1 Motivation behind this thesis

All animals, including humans, harbour symbiotic microbes (their 'microbiotas' or 'microbiomes'). The most diverse of these microbial communities currently known to science resides in the mammalian gut, with thousands of bacterial species, and microbial genes, participating in physiological pathways outnumbering those of the host's own genome. As such, the gut microbiota is increasingly recognised as an influential part of its host's phenotype (Alberdi et al., 2016) with important effects on host digestion (Kohl et al., 2014; Turnbaugh et al., 2006), energy metabolism (Chevalier et al., 2015), immune function (Giannetti & Staiano, 2015; Round & Mazmanian, 2009) and even cognition and behaviour (Cryan & Dinan, 2012; Davidson et al., 2020; Montiel-Castro et al., 2013; Wu et al., 2021). Microbiota composition differs vastly between individuals and within individual over time, and this variation is thought to have important consequences for host health and fitness (Salosensaari et al., 2021; Suzuki, 2017). However, still rather little is known about how this natural variation arises. This is not due to a lack of effort, as a considerable body of biomedical literature has sought to explain variation in human or animal microbiotas with individual attributes (e.g. genetics, diet, physiology; Falony et al., 2016; Kurilshikov et al., 2021; Rothschild et al., 2018), but with limited success. For example, no study to date has been able to explain even half of the between-individual variation in human microbiota using individual-level host traits. An emerging perspective is that the inability to explain microbiota variation with host attributes stems from the fact that the microbiota is an *ecological community residing within a physiological system* (Costello et al., 2012a; Miller et al., 2018). As such, its composition is defined not only by its habitat (the host) but also by ecological processes happening within and between microbiotas. For example, recent research has emphasized the role of contact with natural soils (Lehtimäki et al., 2017; Liddicoat et al., 2020; Ottman et al., 2019; Ruokolainen et al., 2016) as well as social interactions (reviewed by Sarkar et al., 2020) in spreading gut microbes among individuals. Thus, it may be that, while the microbiota is an influential part of its host, as an ecological community it does not strictly belong to any one host, but rather to a network of hosts. To understand how variation in gut microbial profiles arises, we therefore need to consider contexts beyond the internal biology of one host, and pay attention to the landscape of contacts the host has with other hosts and the broader ecosystem.

Studying sources of microbiota variation beyond host individuals has been historically hindered by the limitations of studying model animals in a laboratory environment. In order to study contributions of both within- and between-host processes in structuring microbiota variation, studying animals in their natural environment is essential. While studying model animals in a laboratory environment has provided important insights on how microbiota works as part of host physiology, a major limitation of laboratory research on microbiota is that it cannot capture the vast microbiota variation seen in natural populations, nor many of the key processes, extending beyond a single host, that shape this variation. In nature, animals are exposed to a complex and dynamic microbial environment in which both the sources of microbial colonization (e.g. diet, soil, social interactions) as well as their consequences for the host (e.g. benefits of gut-microbially enhanced cold tolerance; Chevalier et al., 2015) or dietary flexibility (e.g. the ability to digest tannin-rich diets (Kohl et al., 2014)) vary greatly in space and time. Since these dynamics are missing from highly controlled laboratory settings, our understanding of the natural dynamics of microbiotas is still limited. Consequently, there is a need to study the causes and consequences of microbiota variation within more natural systems (Davidson et al., 2020; Greyson-Gaito et al., 2020; Hird, 2017), with a rigorous ecological framework that can accommodate the complexity of this trait as both an influential host phenotype and a dynamic ecological community nested inside a network of hosts.

In this introduction, I will briefly review current understanding of the causes and consequences of gut microbiota variation in mammals. I will also discuss the nature of this variability, how to best describe it and which analytical methods are most suitable for modelling it. Building on this, I will present an analytical framework to study microbiota variation arising from transmission of bacteria within a population of hosts, based on theories of metacommunity ecology and social network analysis. I will end by describing the main study questions, my study systems, and the outline of each chapter in this thesis.

1.2 Variation in the mammalian gut microbiota

Traditional culture-based microbiology provided early insights into the diversity and capabilities of mammalian gut microbiota (Berg, 1996; Cummings & Macfarlane, 1991; Moore & Holdeman, 1974), but this was prior to recent advances in molecular methods. The advent of bacterial phylogenetics and culture-independent profiling methods has transformed our understanding of microbial communities, including gut microbiotas. Over the last twenty years, the application of DNA-sequencing methods to characterize gut microbiota has revealed not only their high diversity but also how much their composition varies at all biological levels (Bäckhed et al., 2005). For example, although the Human Microbiome Project aimed to identify a 'core' gut microbiota shared by all humans, the emerging picture has been of variation at every level of organization: the microbiota is highly individualized (Human-Microbiome-Project-Consortium, 2012), varies across populations (Yatsunenko et al., 2012), and shows marked dynamics within individuals over time (Lloyd-Price et al., 2017). What's more, unlike macroecological communities, microbial communities can vary somewhat independently in "form and function"; because of widespread horizontal gene transfer between bacteria, different microbial taxa can have same functional properties (Louca et al., 2018). For the host, this means that the microbiota's functional potential is only partially determined by its taxonomic composition, and there can be a remarkable level of functional redundancy among taxonomically different microbial communities (Eng & Borenstein, 2018; Moya & Ferrer, 2016; Tian et al., 2020). For example, the human gut microbiota was found to retain marked functional similarity (the same microbial genes were active in similar proportions) despite temporal fluctuations in community composition (Moya & Ferrer, 2016).

1.2.1 Molecular methods for characterising microbiota variation

1.2.1.1 Identifying bacterial taxa

A microbiota's taxonomic composition can be revealed using a range of marker-gene based sequencing methods. The most common workflow to describe microbiota variation remains taxonomic community profiling with the 16S rRNA marker gene (hereafter "16S") (Langille et al., 2013), based on rRNA ribosomal gene phylogenetics developed by (Olsen & Woese, 1993). The 16S rRNA method relies on

nucleotide variation within a relatively short sequence (300-500 base pairs) of a highly conserved gene present in all bacteria and archaea (and consequently in all ancient bacterial endosymbionts of Eukaryota, such as mitochondria and plant plasmids). Sequence similarity of 97% in the 16S rRNA gene has been considered roughly equivalent to the definition of "species" in larger organisms, allowing researchers to profile a complex and diverse microbial community with a resolution similar to that of observational studies in the macro-world. However, due to the statistical nature of this microbial characterisation method and the fact there is no universally agreed species concept for bacteria, the lowest level units of microbial taxonomy are called "sequence variants" (ASVs) or "operational taxonomic units" (OTUs) rather than species. The power of the 16S method lies in the fact that genetic similarity in this gene is a robust measure of evolutionary distance between bacteria (Olsen & Woese, 1993; Woese & Fox, 1977), meaning that even bacterial taxa so far unknown to science can be recognised as separate sequence clusters for the use of higher-level community analysis, such as calculation of community diversity. This is especially useful in the study of wild animal microbiotas, where a lot of the microbial diversity remains uncultured and unclassified in the traditional microbiological sense. In fact, as discussed below, many microbiota research questions focus on properties of whole microbial communities such as alpha and beta diversity, and can be addressed without consideration of the specific bacterial taxa identified in each sample.

16S-method produces microbial community profiles with a resolution most suitable for study questions considering the whole microbiota, e.g. questions about community assembly or transmission among hosts. However, as 16S can only reliably differentiate between microbes on genus or ASV-level, describing microbiota variation with 16S profiles may hide cryptic variation present in lower taxonomic levels, such as strain-variation within bacterial ASVs. For this reason, other methods for finer-resolution taxonomic profiling of microbiota have been developed such as *strain-specific marker genes* and *metagenomics methods to identify microbial strains* within ASVs. Strain-level marker genes work much the same way as 16S, but profile the microbiota using genes that are evolving faster than 16S rRNA, providing higher taxonomic resolution (Ellegaard & Engel, 2016). For example, using a strain-level gyrase b marker gene method (Caro-Quintero & Ochman, 2015) to profile gut microbiota of hominin species, Moeller et al. revealed that some bacterial lineages had been exclusively maintained in their host

lineages for thousands of host generations and have co-speciated with their host species, leading to highly congruent phylogenetic patterns between host species and their microbes (Moeller, Caro-Quintero, et al., 2016) . A complication of such methods is that they are generally costlier than 16S and they have limited ability to strain-type low-abundance microbes. Faster evolving marker genes can differentiate between bacterial strains but they also vary a lot more across higher-level taxonomic groups. Consequently, it is hard to develop a universal genetic marker for strain-level variation across bacteria, as separate gene regions and primers need to be used for strain-typing different bacterial taxa (e.g. families).

1.2.1.2 Describing the functional properties of microbiota

While assessing taxonomic composition of microbiota will answer the question "who's there?", it won't give a comprehensive answer to the question "what are these microbes doing there?". At ASV-level of genetic resolution, bacterial ecology is much less reliant on specific taxonomies than the ecology of multicellular organisms, that lack horizontal gene transfer and fast evolutionary rates and thus are more "stuck" with their genomes. This makes microbial community profiling with methods such as 16S marker genes a crude method for describing any actual bacterial biology within a microbiota. Understanding the *functions* microbes exhibit requires understanding the microbial genes in action rather than the taxonomy present within a host. This is less important for the study of community-level properties such as microbiota similarity or diversity, where 16S marker gene data remains the standard approach. However, to ask more functional questions about microbiota, other methods exist, such as functional metagenomics, metatranscriptomics and metabolomics. *Functional metagenomics* can be used to characterise the microbiota's functional potential (in terms of what genes are present), while metatranscriptomics can be used to profile which genes are being actively transcribed, metaproteomics to analyse microbial proteins, and metabolomics to profile metabolites in a given sample (Zhang et al., 2019). For example, using a functional metagenomics approach, it was shown that compared to other carnivores, the gut microbiota of bamboo-feeding giant pandas was enriched in microbial genes participating in hemicellulose breakdown (Zhang et al., 2018). Lastly, perhaps the most comprehensive picture of both the taxonomy and the function of microbiota can be gained from *whole genome sequencing* (WGS) of all bacteria in a microbiota. Whole genomes of members of a microbiota are

typically not sequenced from isolated DNA libraries, but rather assembled together from shorter fragments within metagenomic datasets, such as those produced with *shotgun sequencing,* a method for probabilistic cataloguing of genes present in a sample by sequencing DNA randomly shredded into smaller pieces (Jovel et al., 2016). The assembly of genomes from shorter fragments happens with algorithms that stitch together sequences based on identical overlap. The resulting genome assemblages provide fine-resolution information on strain-level taxonomy as well as genes present within microbial taxa, but may miss rarer taxa with not enough sequences for a whole-genome assemblage. Other limitations of WGS methods are that they care costly to perform and produce amounts of sequencing data which are challenging for bioinformatic analysis (Jovel et al., 2016)

1.2.2 Data-analytical methods of describing microbiota variation

16S microbial sequence data is commonly presented in the form of a matrix containing proportional abundances of all microbial ASVs per sample or host. Variation in the microbiota is clearly multidimensional and compositional differences are not easily captured by a single number per host. Rather, they are commonly described through pairwise comparisons (e.g. microbiota similarity, "beta diversity") or multiple independent axes of variation based on ordination methods (e.g. NMDS, PCA, PCoA axes). Another consequence of high variability in microbiotas is that simple statistical questions such as "Does X affect the composition of microbiota?" very often yield positive answers but understanding the nature of these correlations requires exploring more quantitative questions, such as "how much variation is explained by X?". In fact, the majority of studies set out to find out whether or not one thing or another influences microbiota composition, have documented significant effects of their chosen predictor on microbiota. In other words, compared to simpler host traits with less variability, microbiota compositions harbor such multidimensional variation, that a multitude of weak but significant correlations exist between the overall composition and a range of predictors. These correlations may reflect the complex biological nature of microbiota (many things have an effect on one part or another of microbiota) or in some cases they may reflect underfitting the data, i.e. when a model is too simple for its predicted data and the predictive power assigned to the dimensions of the data exceed that of the model (Knights et al., 2011). As a host trait, microbiota is maybe more comparable to genotype as a

whole, rather than a single physiological parameter *per se,* and thus it is scarcely useful to ask asking whether simple variables are or aren't correlated with microbiota. Quantitative or comparative measures are needed to put these multitude of significant correlations in perspective. For example, studies that assess *how much* of total variation in microbiota is explained by some factor (e.g. (Sweeny et al., 2021) or whether one or another factor influences microbiota *more* (e.g. Rothschild et al., 2018) have been more useful than reports of mere significant correlations.

How, then, should such multidimensional variation be best measured, described and modelled? Borrowing methods designed to describe other complex systems (e.g. macroecological communities or genotypes), the most common measures of microbiota variation are i) pairwise measures of ecological community similarity ("beta diversity") and ii) emergent community properties, such as stability, alpha diversity or productivity.

1.2.2.1 Describing compositional variation in microbiota

First, compositional variation in microbiotas is possible to capture only by comparing two microbiotas to each other. This is commonly done using indices of ecological community similarity (Goodrich, di Rienzi, et al., 2014) ,such as the Jaccard index, describing the proportion of shared microbial taxa between two hosts, or Bray-Curtis dissimilarity, describing the level of differences in abundances of all taxa present between two hosts (Legendre & Legendre, 2012). Additionally, phylogenetically informed measures of community dissimilarity, such as weighted or unweighted UniFrac distances (Lozupone et al., 2011), describe how phylogenetically similar two microbial communities are. These dissimilarity/similarity indices present some challenges for traditional statistical approaches, because as pairwise metrics they are cannot be readily modeled as independent data points. However, even taking this autocorrelation into account, pairwise comparisons reveal manifold more variation in a system compared to measures of the independent units they compare (here, hosts), making them particularly powerful for explaining reasons behind and consequences of microbiota differences between hosts. Box 1 discusses the many statistical tools designed for modeling this type of pairwise data.

Box 1. Analytical tools for modeling pairwise data.

A central assumption of statistical modeling is that datapoints are independent. This means that the value of one observation is not influenced by the value of another observation. Failing to account for non-independence can result in pseudoreplication, which increases the rate of false positive results in statistical analyses (Hurlbert 1984). In natural systems, however, measurements are rarely truly independent and in fact a lot of the important variation is tied to the very connections and interactions between things. Consequently, a range of methods have been developed to account for non-independence of response values. A common example is the use of random factors used in multivariate models, to account for nested similarity structures in the data, such as repeated measurements from the same sites. Here, "dependence" between measurements means that we would expect them to be inherently more similar within sites than between sites, and by taking this into account, we can assess other important drivers of this variation. But if this site-similarity is due to geographic patterns in the values, then perhaps sites closer to each other might also be more similar to one another than sites further apart? Sometimes forcing values into categories (e.g. "site") is suboptimal compared to modeling their interdependence on a more continuous scale (e.g. "distance between sites"). Furthermore, not all dependencies present in biological data can be simplified by forcing their variation into separate categories in the first place. For example, genetic relationships, social associations or ecological community composition are difficult to represent as discrete groups, as their variation is inherently continuous, and can be fully revealed only by considering pairwise comparisons (e.g. genetic distance, social association strength, community similarity).

Pairwise ("dyadic") values are by definition not independent of each other. For instance, multiple values of distance end up at the same point, and many social relationships contain the same individual. This dependence structure does not involve datapoints simply falling into a nested a hierarchy of groups, as not only do individuals have many relationships, but all relationships belong to at least two individuals. This kind of dependence structure across pairwise values is best understood as a *network* where pairwise values are edges connecting a set of nodes (e.g. individuals) (Figure 1A) The power

of the network analogy is that it allows one to capture how even pairwise values that do not involve the same node still have indirect dependence on each other through other nodes and edges. In fact, the strength of autocorrelation between pairwise values degrades with increasing number of steps (nodes) between values. Using the words of network scientists, the dependence between edges degrades with distance in a fully connected network.

How do these correlational networks influence statistical power? Even though pairwise values are all dependent on each other and thus carry less information than the same number of independent values, they still carry more information than can be expressed by nodes alone. For instance, distances between five locations add up to 10 pairwise values (Figure 1A), which contain less information than would 10 independent values but still more than the information contained by the 5 independent nodes. Thus, modeling pairwise data is an important way of using all available information in a data set and maximizing statistical power. Consequently, many research fields such as genetics, social network analysis, evolutionary ecology and spatial and community ecology have developed statistical tools to effectively deal with pairwise correlation structures. Curiously, the development of these methods across research disciplines has happened somewhat independently, creating statistical subcultures within fields. For example, a popular method in quantitative genetics, the "animal model" specifies a genetic structure within a population as an inverse relatedness matrix within a multivariate model, to partition variation in a host trait of interest to that explained by relatedness compared to other (environmental) influences (Kruuk, 2004; see also Thomson et al., 2018 for other types of distance matrices). In spatial ecology, multiple linear modeling approaches have similarly been developed to explicitly account for spatial autocorrelation in the residuals of species distributions across locations (Dormann et al., 2007). In community ecology, pairwise measures of community similarity are commonly predicted with node-level attributes using permutational multivariate analysis of variance ("PERMANOVA"), a statistical test that partitions variation in a pairwise distance matrix across node-level variables and infers statistical significance by randomly permuting the distance matrix to create null models with no real correlations but a similar dependence structure (Anderson, 2017).

A lot of these tools have been developed in order to account for pairwise dependence structures in the data while statistically modeling values attached to the nodes. However, microbiota research has created a need for a new kind of pairwise model. This is because, as stated above, variation in microbiotas is best described by pairwise indices of distance or dissimilarity, which can be influenced both by node-level variables (e.g. host attributes) but also processes that happen between hosts, such as transmission of bacteria from one host to another, or exposure to more or less similar environments. Thus, drivers of microbiota variation are not optimally modelled by predicting a pairwise response with node-level predictors (as in PERMANOVA) nor a node-level variable predicted by pairwise matrices (as in the animal model). Microbiota research thus serves as a motivation to develop *fully dyadic models*, capable of modelling pairwise values (e.g. community distance metrics) with pairwise predictors. Various methods have been explored for predicting microbiota dissimilarity metrics, or similar measures of degree of microbe sharing. These include multiple regression quadratic assignment procedure or "MRQAP" (Amato et al., 2017; VanderWaal et al., 2014), generalized dissimilarity models or "GDMs" (Fountain-Jones et al., 2017), graphical network models (Fountain-Jones et al., 2019), Integrated nested Laplace approximation, or "INLA": (Gayawan et al., 2020) and regression with multi-membership random effects (Blyton et al., 2014; Springer et al., 2016). These approaches generally apply one of two alternative statistical strategies, with the network-like autocorrelation in the data accounted for by either 1) using null model permutations or 2) specifying a random dependence structure within the model. Here, I describe one of each method types, a pair of models that I find most promising and which are subsequently used in this thesis: Multiple regression quadratic assignment procedure ("MRQAP") and Bayesian regression using multi-membership random effects ("dyadic Bayesian regression").

1) **MRQAP**

MRQAP is a matrix correlation model, similar to a Mantel test, but with multiple predictor matrices predicting one response matrix (Dekker et al., 2007). MRQAP has been long used to correlate pairwise values among social network scientists (Dekker et al., 2007) and has become a commonly used method for linking contact networks with transmission of microbes (pathogenic: VanderWaal et al., 2014, or mutualistic:

Amato et al., 2017, sharing of information in real life social networks or social media (Cvetojevic & Hochmair, 2021; Holvoet et al., 2016) or sharing of resources, such as food among humans and other social animals (Carter et al., 2019; Koster, 2011). MRQAP deals with interdependence among datapoints by using null model permutations. The correlations among pairwise values across matrices are estimated as in a normal multivariate model, but to assess their significance, these coefficients are then compared to a set of coefficients derived from null models that use randomly shuffled versions of the response variable matrix. In other words, this procedure compares the observed covariation between pairwise values to covariation expected to arise from the network structure alone, i.e. correlations between pairwise values when any real signal is broken by shuffling, but the autocorrelation structure among datapoints is retained. Controlling the random shuffling process allows different types of inference. For instance, randomizing the residuals from regression on each predictor matrix instead of the response matrix, allows sequential testing of the effects (the effect of predictor 1 on response controlled against the effect of predictor 2; Dekker et al., 2007). The main limitation of MRQAP is its sensitivity to skewed response value distributions (e.g negative binomial distributions) and the difficulty of accounting for nested (hierarchical) autocorrelation across data (e.g. restricting randomisations to account for repeated samples from the same individuals complicates and reduces power of the permutation approach).

2) **Dyadic Bayesian regression**

Modeling pairwise values in a multivariate generalized linear model framework is made possible by specifying a non-nested, multi-membership random effect (Browne et al., 2001). This random effect resembles the common nested random factor, but in addition to hierarchical structure, each pairwise value is given membership of multiple (here exactly two) groups, which represent the independent nodes attached to each pairwise comparison (Figure 1B). For example, in the simplest case, the model formula would be:

$$y_{AB} \sim x_{AB} + mm(A + B),$$

where response variable y and predictor x are both pairwise comparisons between independent nodes A and B, and the last term specifies a random intercept across a multi-membership of nodes A and B.

This simple trick informs the model not only of the fact that some values have the common attribute that they, in network terms, connect to the same node, but moreover it lets the pairwise values not connecting to the same node depend on each other indirectly. After specifying the multi-membership random effect, pairwise values can be modeled as if they are independent measurements. This kind of dyadic regression was originally suggested by (Browne et al., 2001), utilized as a method to predict microbial sharing by (Blyton et al., 2014) and further developed by me in this thesis (Chapter 2, published as Raulo et. al 2021). Specifically, I extended this method to work in a Bayesian regression framework using the R package *brms*, allowing dyadic beta-regression of proportional data (such as microbiota data) while estimating the full posterior distribution of the multi-membership random structure alongside the predictor effects. Comparing results from this updated dyadic Bayesian regression model to MRQAP yielded very similar results (see Chapter 2, Supplementary Appendix S5), but unlike MRQAP, this method can also account for nested dependence structures and provides more flexibility over response distributions.

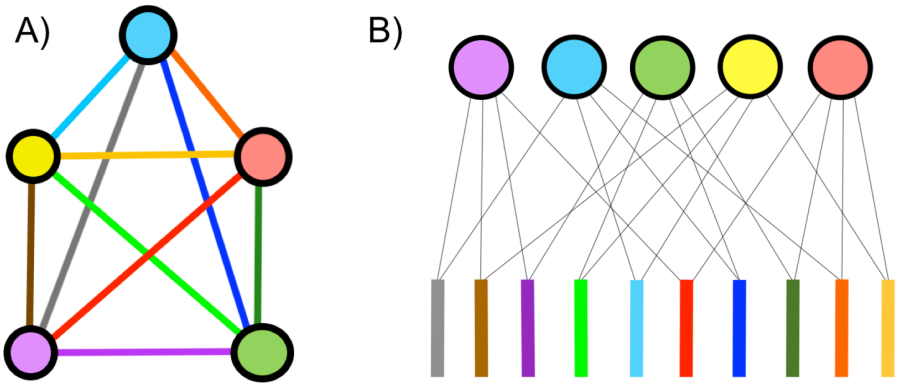

Figure 1. Multi-membership grouping of pairwise values with a network-like dependence structure. A) A dataset consisting of five independent units (nodes) and all pairwise comparisons between them (edges) in a network. Values tied to the edges (line colours) are not independent values, but reveal more variation of the full system than the independent values tied to the nodes (circle colour). B) One way to model values tied to the edges, is to give each edge membership of two groups, representing the nodes it connects. This kind of grouping can be specified within a regression model through multi-membership random effects.

As a final note, let it be said that these methods have been developed to account for the autocorrelation inherent for any type of pairwise comparison. However, just like many other metrics, different types of pairwise values have different features that come with varying autocorrelation structures. One simple example considers different types of distances: measures of Euclidean distances, such as real metric distance, are dependent on each other in a slightly different way than non-Euclidean distances. For instance, if you are standing on a two-dimensional space of Euclidean distance (e.g. football field), moving towards any point will affect your distance to all other points on the plain. But were you to stand on a field of non-Euclidean distances, e.g. a space describing similarity between ecological communities, you can become more similar to one other community while staying just as similar to another. Thus, in some ways, non-Euclidean distances are more independent of each other than Euclidean distances are. To date, I am not aware of any statistical methods differentiating between the autocorrelation structures of different types of distance values and future research is needed to assess how important these differences may be for dyadic modelling.

1.2.2.2 Describing emergent community properties of microbiota

Second, emergent community properties are of interest since many of them have been shown to be influential for host-microbiota relationships. While some specific microbial taxa have been shown to directly influence the host (e.g. the tannin-degrading microbes in Kohl et al., 2014), the emerging view seems to be that what matters most to the host seems not to be a fixed recipe of specific bacterial species, but rather that emergent properties of the microbiota as a whole, such as diversity, stability or productivity are important. As community-specific measures yielding one value per individual host, these are easier to model than compositional variation. Commonly explored community-level properties of interest are measures of alpha diversity, such as community richness describing the number of species (microbial taxa) in a given host, or Shannon diversity, measuring the taxonomic richness and evenness of a community (host). Other community properties of great interest are measures of community stability such as resilience and resistance, describing the rate of change and the ability to resist change respectively, in an ecological community (Gilbert & Lynch, 2019; Lozupone et al., 2012). Finally, functional metagenomics have also provided insights into microbiota productivity, a trait of great relevance for microbiota as part of metabolic pathways of the host. These emergent community properties all affect each other, and their specific relationships (e.g. the relationship between diversity and resilience of microbiota, see (Fassarella et al., 2021; McCann, 2000; Reese & Dunn, 2018), are subjects of rich ongoing debates in theoretical ecology as well as microbiota research, summarized in Box 2.

Box 2. Interplay between emergent community properties of microbiotas

In this thesis my focus is to study how variation in microbiota profiles between hosts arises, but to put these variation patterns in perspective it is useful to visit the ongoing debate over what different microbial community compositions may mean for their host's health and fitness. Variation in the microbiota most influential to the host is not necessarily tightly tied to the taxonomic composition of the microbial community, but rather the microbiota's properties as a whole. The microbiota is an ecological community in which the combined interactions among constituent microbes together define its emergent properties, such as *productivity, diversity or stability*. For example, community productivity is an emergent property of mouse gut microbiota which has been linked with host metabolic demands – wild mice living in colder climates were shown to host a microbiota with higher productivity, i.e. higher capacity to produce energy from the same quantity of food compared to mice living in warmer climates (Suzuki et al., 2020).

While emergent properties of the microbiota clearly matter to the host, the forces affecting productivity, diversity or stability of an ecological community is a complex matter even in macro-ecological systems. For this reason, the relationships between emergent properties of microbiota and their consequences for the host are still poorly understood and the subject of ongoing debate, with many assumptions based on hypotheses rather than robust evidence at present. An example of this concerns debate over the health implications of gut microbial alpha diversity. It is often assumed that a diverse gut microbiota is beneficial for the host because it is more stable and less prone to invasions (Belkaid & Hand, 2014; Lange et al., 2016; Tuddenham & Sears, 2015). This idea is based on some ground work in clinical antibiotic experiments as well as theoretical ecology. The former includes a line of experiments showing that a reduction of microbiota alpha diversity through antibiotic administration tends to destabilize microbiota and increase risk of pathogen invasions (Britton & Young, 2014). The latter includes theoretical work (based on simulations) suggesting that diverse ecological communities should be more stable (reviewed by McCann, 2000) for the same reasons as webs with more knots are more durable – breaking a few links won't collapse the whole system when

there are many links connecting different parts. In other words, higher diversity is expected to come with higher functional redundancy, whereby microbial species can take over each other's niches/functions if one disappears, stabilising the overall microbiota function in times of perturbations. Related to this, a lot of theoretical ecology studies have indeed implied that diverse ecosystems are less susceptible to invasions (reviewed by Levine & D'antonio, 1999). In line with these, clinical research has also shown that extremely low microbiota diversity in the human gut is indeed associated with more stochastic fluctuations and this microbiota state, called "dysbiosis" comes with a range of negative health consequences. Based on these insights, the status quo in biomedical research is that diverse gut microbiotas are more resilient and thus good for their host while low diversity microbiotas are less resilient and more unstable, impairing host health (Fassarella et al., 2021; Kriss et al., 2018; Lozupone et al., 2012).

Despite this widely accepted paradigm in biomedical field, significant debate remains about how microbiota diversity relates to stability (Reese & Dunn, 2018) and further to health. Apart from the extreme ends of the spectrum (e.g. very low diversity brought about through the use of broad-spectrum antibiotics), little evidence exists about how stability and diversity of the gut microbiota covary across a more natural range of diversity variation. Moreover, while extreme low diversity in the gut microbiota is associated with unstable dysbiotic community states, many diseases, which can destabilize microbiota, also come with higher-than-average gut microbial diversity. For example, gut microbiota diversity was negatively correlated with some forms of infection (macroparasites, nematodes), while positively associated with other forms of infection (viral infections) in wild mice (Weldon et al., 2015). In addition to inconsistent evidence, a lot of theoretical and experimental ecology work on microbial systems also provides alternative view points on the relationship between these emergent community properties: First, as explained above, diversity is assumed to be stabilising because diverse systems should have more ecological links providing more functional redundancy, but community complexity (number of links in the network) is not always readily captured by alpha diversity (number of nodes in a network), as shown by experimental work of free-living microbial systems (Shi et al., 2016). Second, the effect of diversity on stability can depend on multiple network properties, such as interaction type, link strength and connectivity. For example, increasing diversity may only increase functional redundancy in highly competitive ecological communities whereas

communities enriched in positive ecological interactions, whereby one microbial species heavily depends on another, in fact become more unstable the more diverse the get (Coyte et al., 2015). This is because in a network of species dependent on one another, disappearance of one species can create a cascade of collapses of others.

Evidence suggests that microbial communities are enriched in (stabilizing) competitive interactions rather than cooperative (Foster & Bell, 2012) and gut microbial communities may be also stabilized by host control over the emergent community state. However, even with optimal host control, the most beneficial microbiota state might not be completely stable. Especially for natural host populations living in seasonal habitats, gut microbiota should optimally not stay in a completely stable state but harbour a level of reactive plasticity in its function/composition (Davidson et al., 2020) just like in other so called "allostatic" traits, meaning physiological systems that fluctuate in an adaptive manner in response to fluctuating environmental demands (Sterling, 2004).

1.3 Consequences of microbiota variation for the host

The role of gut microbiota in digestion, especially in breaking down complex polysaccharides associated with plant-diet, has long been appreciated (Gilbert, 2020; Hungate et al., 1971; Kohl, et al., 2016; 2014; Troyer, 1984b), but the past decade has seen a surge of clinical work relating gut microbiota composition with the development and function of other physiological systems, from fine-tuned interactions with the immune system (Giannetti & Staiano, 2015; Round & Mazmanian, 2009; Zhang & He, 2015) to complex pathways between gut microbes and their metabolites and the central nervous system (Montiel-Castro et al., 2013; Wu et al., 2021) , consequently affecting host behaviour (Davidson et al., 2020). These effects have been primarily considered in the context of human health and disease within biomedical research, but more recently the gut microbiota has also been considered in relation to evolutionary fitness (Rowe et al., 2020; Suzuki, 2017) and successfully linked with overall mortality risk in humans (Salosensaari et al., 2021). Paralleling this, the microbiota is increasingly seen as providing adaptive phenotypic plasticity in wild animals (Alberdi et al., 2016), with downstream consequences for their ecology and evolution. Research on the consequences of microbiota on wild animal hosts has exciting potential to reveal evolutionarily influential homeostatic feedback loops, because in natural habitats, the same host traits that can be affected by the microbiota (e.g. digestive abilities, behaviour), can in turn determine which microbes can colonise the host from the environment (Davidson et al., 2020). Below, I briefly review evidence for the gut microbiota's role in three host traits relevant for ecology and evolution of wild animals: 1) metabolism, 2) immune function and 3) behaviour.

1.3.1 Gut microbiota effects on metabolism

Gut microbes are important metabolic agents in all vertebrates, breaking down complex molecules such as lipids and polysaccharides and producing short-chain fatty acids (SCFA) as a result. SCFAs have diverse downstream roles in host physiology, including as signalling molecules in the immune system (Tan et al., 2014) and in appetite control (Corfe et al., 2015). Gut microbial communities vary in their potential to harvest energy from the host's diet (Turnbaugh et al., 2006), and different specific microbes can enable the host to digest an otherwise indigestible diet. For example, population-level differences in

gut microbial composition and function were associated with the ability of woodrats to feed on a plant rich in toxic secondary compounds (Kohl et al., 2014). A follow-up study showed that enriching the gut microbiota of laboratory rats with a tannin-degrading microbiota transferred from wild woodrats enhanced their digestive capacity by allowing them to digest a tannin-rich plant diet that otherwise damaged their liver (Kohl, Stengel, et al., 2016). Mirroring these effects, host species with a narrow dietary niche maintain a strongly specialized microbiota, with remarkable convergence across distantly related species with similar specialist diets (Baldo et al., 2017; Delsuc et al., 2014). For example, a range of species across animal kingdom who feed exclusively on blood (e.g. lice, ticks, leeches, vampire bats) host a remarkably convergent microbiota dominated by bacteria producing vitamin B, which is lacking from their diet (Duron & Gottlieb, 2020). An extreme case of microbe-dependent digestion among vertebrates is rumination. The ruminant gut harbours the most diverse known symbiotic microbiota, nurtured by the host and specialized in metabolizing complex polysaccharides from grass. A major part of the grass-based diet cannot be metabolized with the host's own enzymes and thus the niche of ruminant herbivores relies largely on their gut microbiota for mobilizing nutrients in their diet. Consequently, gut microbial diversity is repeatedly suggested as an important source of variation in digestive plasticity between species (Milani et al., 2020), populations (Kohl et al., 2014) and seasonally shifting diets in wild animals (Amato et al., 2014; Gomez et al., 2015; Maurice et al., 2015). The symbiosis between the vertebrate host and its gut microbiota has been suggested as one of the key forces enabling the evolution of herbivory (reviewed by Gilbert, 2020).

Gut microbes are not only important for digestion but are also crucial for the gut's development. For example, a calf feeding on its mother's milk has a stomach system bypassing the rumen, which only activates, and grows to its full size, if and when the cow starts feeding on grass and subsequently hosting butyrate-producing fermentative bacteria in its gut (reviewed by Gilbert, 2020). The formation of digestive symbioses is laid in early postnatal development in other, non-ruminant vertebrates as well. For instance, development of the mouse gut is dependent on the presence of specific gut bacteria (Hooper, 2004) and young green iguanas (*Iguana iguana*) will not start foraging their normal plant diet before acquiring a specific gut microbiota through eating soil and adult faeces (Troyer, 1984a). Even feeding on animal tissue can require bacterial metabolism. For example, in young mammals including

human babies, digesting milk requires *Bifidobacterium* bacteria for optimal breakdown of its constituent oligosaccharides. These bacteria are maternally transmitted to the baby in birth (Funkhouser & Bordenstein, 2013), and to promote bifidobacterial abundance in the infant gut, human milk is enriched in oligosaccharides that are specifically aimed at nourishing these bacteria, rather than the baby itself (Hinde & German, 2012).

The mammalian gut microbiota's role in metabolism has been mainly studied in terms of digestion and nutrition. However, recent research has highlighted that another aspect of energy homeostasis influenced by the gut microbiota is thermal tolerance (Chevalier et al., 2015; Fontaine et al., 2021; Moeller et al., 2020). For instance, cold exposure resulted in a compositional change in the gut microbiota of mice, and transplanting this cold exposure microbiota to germ free mice enhanced their cold-resistance traits, by increasing insulin sensitivity and energy uptake from the gut (Chevalier et al., 2015). Evidence from wild mouse populations has further emphasized the gut microbiota's potential role in climatic adaptation. Mice from northern latitudes (and colder climates) host a microbiota with higher capacity for energy uptake from their diet (Suzuki et al., 2020), paralleling a similar finding in humans, whereby humans living in colder climates harbour a microbiota otherwise linked with obesity and fat accumulation (Suzuki & Worobey, 2014).

1.3.2 Gut microbiota effects on immunity

The gut microbiota plays such an important role in the immune system of animals, that the bacterial community in the gut has justifiably been termed "an extended immune phenotype" (Koch & Schmid-Hempel 2011). Firstly, many species rely on gut bacteria for the normal development of their gut immune system (Bates et al., 2006) as well as broader systemic immunity (Sjögren et al., 2009). In mammals, after birth the gut microbiota and the immune system develop in parallel and in close cross-talk with one another. The developing mammalian embryo is thought to be microbially sterile, and has limited immune function. Even though both innate and adaptive immune cells are present in the mammalian fetus, the pro-inflammatory pathways are suppressed and become activated only at birth in response to microbial stimulus as well as hormones of the HPA-axis that trigger cytokine production (Cho & Norman, 2013;

Gollwitzer & Marsland, 2015; McGovern et al., 2017; Yektaei-Karin et al., 2007). The activated innate immunity then forms the selective platform on which the first gut microbiota assembles, and this microbiota then in turn modifies the responsiveness of innate immunity (Muraille, 2015; Wang et al., 2013), programs acquired immunity (Buffie & Pamer, 2013; Muraille, 2015) and further affects immune function through effects on HPA-axis responsiveness (Sudo et al., 2004).

Secondly, the microbiota continues to play a key role modulating immune function throughout the host's lifetime. Or rather, the immune system has adapted, both developmentally and evolutionarily, to use the gut microbiota as part of its systemic processes. The mechanisms by which the microbiota interacts with the immune system constitute an area of intense research, and include the production of immunomodulatory SCFAs, activation of neutrophils, regulation of antimicrobial peptide expression by intestinal epithelial cells, and stimulating the development of multiple immune cells (e.g. antigen presenting cells). Importantly, in addition to taking regulatory part in systemic immune function, the microbiota is thought to protect the host against invasive pathogens through their own ecological community dynamics, a phenomenon termed 'colonisation resistance' (Britton & Young, 2014; Buffie & Pamer, 2013; Stecher & Hardt, 2011; N. Zhang & He, 2015). This can happen through for instance competitive exclusion of pathogenic taxa, that, in a healthy host, arrive in a saturated "full" ecosystem with locally well-adapted competitors and no free niches, but many other mechanisms of microbiota-induced colonization resistance of pathogens also exist (Kamada et al., 2012; Stecher & Hardt, 2011). This is an important theory in the discussion of the role of microbiota diversity in its resilience towards invasions (See Box 2), and an example of how the protective value of a microbiota may not rely on specific bacterial taxa but rather the emergent community properties of the microbial community as a whole.

1.3.3 Gut microbiota effects on host behaviour

Perhaps the most surprising documented effects of the gut microbiota on host biology, are those involving neuroendocrine pathways with downstream impacts on host behaviour. The evidence for gut microbiota modulation of host behaviour started with observations concerning altered behaviour in

laboratory mice that completely lacked a microbiota. A seminal study by Sudo et al., (2004) showed that germ-free mice have an exaggerated stress response due to developmental mistuning of the HPA-axis in the absence of gut microbes. Accumulating research built on this finding has demonstrated that normal neurobehavioral development in mice depends on gut microbes acquired at birth (Heijtza et al., 2011) and that experimental introduction of gut microbes such as *Bifidobacterium* and *Lactobacillus* can rectify behavioural abnormalities of germ-free mice, as well as reduce stress behaviours in mice with an intact microbiota (Bravo et al., 2011; Sudo et al., 2004). Two classes of behavioural phenotype that are emerging as being influenced by the gut microbiota are locomotor activity and social behaviour. Compared to specific-pathogen-free (SPF) mice, germ-free mice have been shown to exhibit increased locomotor activity (Heijtza et al., 2011) and microbial colonisation of GF-mice has been shown to reduce locomotor activity (Nishino et al., 2013). The gut microbiota also has nuanced and context-specific effects on social behaviour of mice, via mechanisms involving the vagus nerve and HPA-axis (Wu et al., 2021). These results serve as an important context for studying microbiota-behaviour links in natural systems, since both activity and social behaviour can influence a host's microbial exposures, while gut microbes can then in turn influence host behaviour, generating the potential for feedback loops to exist between the microbiota and host behaviour in the wild. A comprehensive outlook on microbiota-mediated behaviour in vertebrates and how we might study such links in wild animal systems is presented as part of our opinion piece "Identifying Microbiome-mediated behaviour in wild vertebrates", included in Supplementary Appendix.1.

1.4 Drivers of microbiota variation

Research in humans, model animals and wild populations has provided a catalogue of potential influences on the composition of the mammalian gut microbiota. These include host genetics (Campbell et al., 2012; Goodrich, Waters, et al., 2014), diet (David et al., 2014; Murphy et al., 2010), sex (Gomez et al., 2012; Markle et al., 2013), age (Leclaire et al., 2014; Reveles et al., 2019; Sandrini et al., 2015; Yatsunenko et al., 2012), hormonal status (Neuman et al., 2015), social relationships (Neuman et al., 2015) as well as contacts with the natural ecosystem (Lehtimäki et al., 2017; Ottman et al., 2019). However, ascertaining the relative importance of these different factors in natural populations remains a work in progress, and one that will require detailed population studies of identifiable individuals. For instance, a number of studies attempting to assess the heritability of gut microbiota have yielded inconsistent estimates of how much variation in human and other primate gut microbiotas can be explained by host genetics alone (Goodrich, Waters, et al., 2014; Rothschild et al., 2018; Turnbaugh et al., 2009; Turpin et al., 2016; Yatsunenko et al., 2012). A recent survey of these effects in baboons showed that a failure to consider within-individual temporal fluctuations in the microbiota (with no longitudinal sampling of the same individuals) could overestimate the heritability of specific taxa but under-estimate heritability of microbiota composition as a whole (Grienesen et al., 2021). Comparative studies on wild animal microbiota have also tried to estimate the relative importance of phylogenetic and environmental effects on gut microbiota composition. Host species are known have somewhat geographically constrained microbiotas (Moeller et al., 2017), with sympatric species hosting more overlapping gut microbial communities than allopatric species (Baxter et al., 2015; Gogarten et al., 2018; Moeller et al., 2013, 2017) . However, in primates a subset of the gut microbiota has also been shown to co-diversify with their hosts, generating patterns of co-phylogeny between host and gut microbes. Alltogether many processes operating in and outside the host have been shown to shape microbiota but the relative importance of these influences remains poorly understood.

1.4.1 A metacommunity framework for understanding microbiota assembly

Making sense of the evidence concerning microbiota community assembly has been greatly enhanced by bringing an ecological perspective into this originally more physiology-oriented research field. An important step in conceptualising the relationships between various forces driving microbiota composition was harnessing the theory of metacommunity ecology to explain how this variation arises (Miller et al., 2018; Miller & Bohannan, 2019). Metacommunity ecology was originally developed by combining the principles of traditional community ecology (Morin, 2009) with metapopulation ecology (Hanski & Gilpin, 1997) with historical roots island biogeography, (MacArthur & Wilson, 1967) to explain the dynamics of community assembly in multi-species communities living in spatially constrained patchy landscapes, such as archipelagos or tidepools (Leibold & Chase, 2018). Metacommunity ecology theory posits that in a spatially constrained but interconnected ecological community, the composition of sub-communities is determined by 1) ecological interactions between species within each patch (intra-community interactions), 2) selective filtering of species due to varying abiotic patch characteristics (selective processes), 3) movement of species between patches (dispersal) and 4) chance effects (drift). Host-associated microbiotas can be conceptualised as constituting a metacommunity, where each host is a patch with its own microbial sub-community, shaped by ecological interactions among bacteria, selective forces imposed by the host and processes of microbial transmission between the hosts (Miller et al., 2018). In this view, microbiotas in a population of hosts have been compared with island ecosystems, where the host population comprises an "archipelago" of habitable patches between which bacteria transmit across an inhospitable habitat matrix (Sarkar et al., 2020). However, some attributes of host gut microbiotas are not well-captured by the island analogy. For example, some gut microbes can live and even prosper in the matrix between host "islands", and the host 'islands' are not passive, with host-microbe interactions thought to have important feedback effects on community structure (Miller et al., 2018). Consequently, tools from traditional metacommunity theory need re-tailoring for the microbiota context (Miller & Bohannan, 2019).

The power of the metacommunity framework is that it allows us to consider microbiota variation as something fundamentally shaped by both host-level processes and between-host processes. Historically,

a common aim of metacommunity ecology has been partitioning variation in ecological community composition across dispersal effects and within-patch processes (Leibold et al., 2004). This differentiation between within and between-community processes shaping composition can be harnessed for robust comparison of forces shaping microbiotas. Instead of asking questions such as whether microbiota composition is more affected by different host-level attributes such as genetic background or environment, we can ask how much of the variation in microbiota composition is accounted for by transmission of bacteria from outside the host (dispersal), compared with similarity (e.g. genetic relatedness) between hosts (selective process). Microbiota researchers have begun to apply a metacommunity perspective to address pertinent research questions, for example using it to show that the gut microbiota can shift from transmission-driven to host-control driven composition during zebrafish development (Burns et al., 2016), and that gut microbiota is influenced by between-individual transmission processes and within-host selective forces differentially in western and non-western human societies (Martínez et al., 2015), and can be shaped by different ecological processes depending on the health status of the host. For example, neutral model of microbial transmission explained almost all variation in healthy human lung microbiomes, while cystic fibrosis patients had a lung microbiome more structured by local selective effects, implying that the disease state imposed selective pressures on some bacteria over others (Venkataraman et al., 2015).

Here, I will briefly summarise the main processes shaping variation in the mammalian gut microbiota, following a metacommunity framework. My aim is to describe how a microbial community assembles guided by three ecological processes which can be conceptually nested inside each other (Figure 2): 1) microbial *intracommunity interactions* (ICI) define how interactions among bacterial species, ranging from negative (competitive) to positive (cooperative), shape community dynamics within the gut. These ecological interactions happen in a selective landscape formed by 2) *host-microbiota interactions* (HMI), defining the environmental parameters favouring some microbes over others. The set of possible community members within a microbiota is ultimately defined by 3) *transmission* of microbes from outside to inside the host.

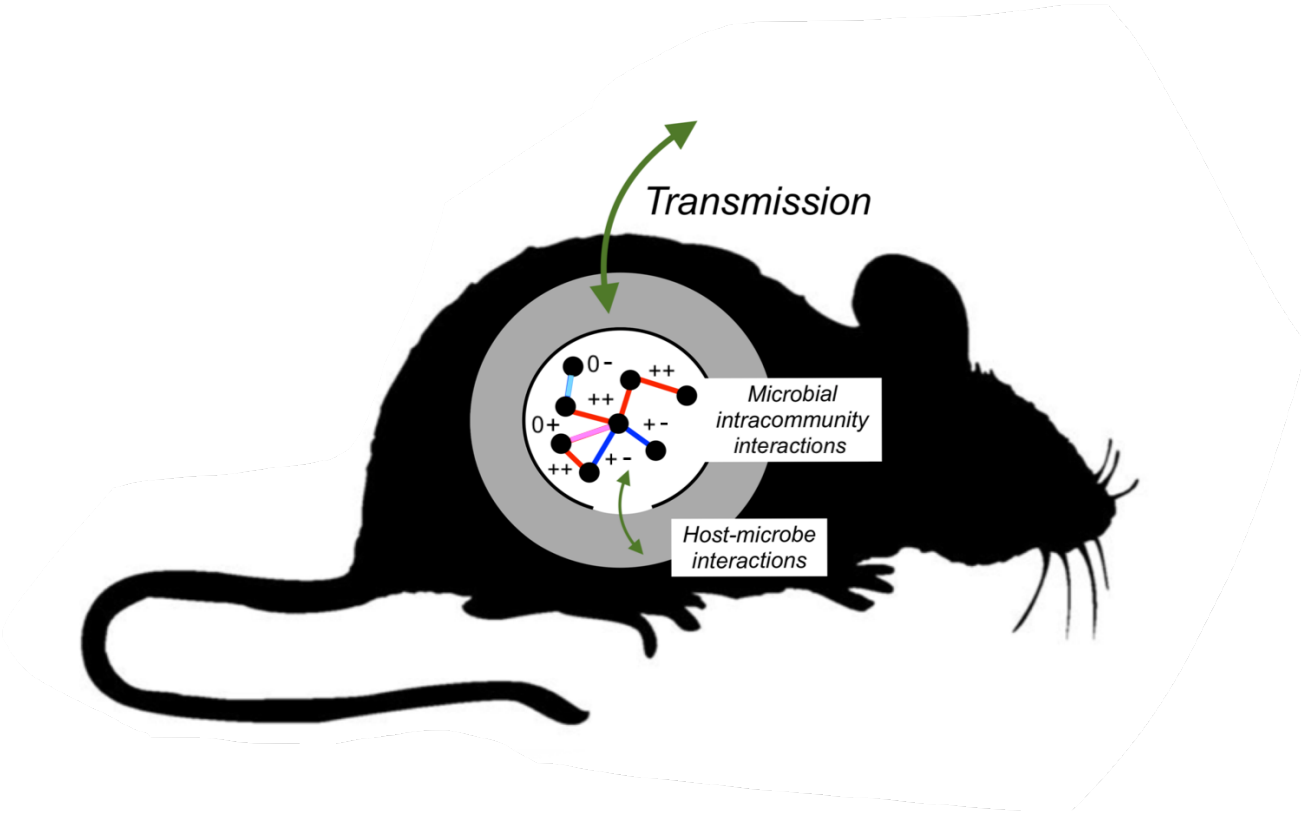

Figure 2. Metacommunity assembly defines the composition of a microbiota. Three nested processes of metacommunity assembly defining the composition of a host-associated microbiota: *Transmission* of microbes defines the taxa that get into a host, *host-microbe interactions* then set the selective environment in which *microbial intra-community interactions* take place, ultimately defining which microbes establish populations and persist in the gut.

1.4.1.1 Intra-community interactions

Microbes of the gut comprise an ecological community with ecological interactions comparable to those present in the macro-world. The interactions of microbial taxa can be either competitive, as evident from the competitive exclusion of similar-niche taxa from the same system (Wiles et al., 2016) or facilitating/cooperative, as is the case with cross-feeding between members of the genus *Bacteroides* (Rakoff-Nahoum et al., 2016). Recognising these ecological interactions among microbes led to a theory of microbiotas dividing into specific ecological community types, called "enterotypes", whereby certain community compositions arise driven by the dependencies between and inabilities of certain microbial

taxa to co-exist in the same microbiota (Cheng & Ning, 2019). Some evidence from low-diversity systems, such as insect guts, indeed showed that gut microbial communities may divide into specific compositional types or clusters driven by intra-community interactions alone (Martinson et al., 2017), but whether community variation in vertebrate gut microbiotas falls into real, discrete ecological clusters or arises from inappropriate statistical analysis has been debated (Gorvitovskaia et al., 2016). Paralleling processes in macro-ecosystems, microbiotas go through ecological shifts, such as succession guided by founder effects (Costello et al., 2012b; Karkman et al., 2017), shifts between alternative stable equilibrium states (Costello et al., 2012a; Gonze et al., 2018) or invasions, whereby one taxon has disproportionate growth and effects on the remaining community (Costello et al., 2012a). In other cases, one microbial taxon can be suppressed by many others. For example, growth of the pathogen *Clostridium difficile* is actively suppressed by a number of other bacteria in the genus *Clostridium* (Britton & Young, 2014; Buffie & Pamer, 2013).

It is important to understand that populations of gut microbes are regulated by ecological interactions, including likely frequent and sometimes intense competition from other bacterial species, as this means no microbial species interacts with the host in isolation. For example, some authors have suggested that certain microbial taxa could have evolved to manipulate host behaviour in ways that benefit their own transmission (Stilling, 2015; Wong et al., 2015; Yuval, 2017), but this is unlikely in a diverse ecological community; any microbe investing in a host manipulation strategy to enhance their own transmission would bear the costs of this adaptation alone, but share the benefits with millions of other free-riders, and would quickly be outcompeted (Johnson & Foster, 2018). In a system as diverse as the mammalian gut microbiota, microbes will rather primarily interact with each other while the host will interact with the microbiota as a whole (Foster et al., 2017). Indeed, the adaptations of single microbial species to interact with their host may be heavily constrained by other microbes of the community. This is in accordance with evidence from simulations and free-living microbial ecosystems which suggest microbial species in higher diversity communities face more constraints when evolutionarily adapting to their local environment (de Mazancourt et al., 2008; Lawrence et al., 2012).

Within the microbiota, microbe-microbe relationships range from competitive ("-/-", defined as two species growing better without each other than with), to neutral ("0/0" defined as two species having no effect on each other's growth) and to cooperative ("+/+", defined as two species growing better with each other than without), with all possible intermediate relationship types based on how two bacteria relate to each other (e.g. "0/+", "0/-", or "0/0"). The ratio of competitive vs. cooperative links in an ecological network is predicted to influence many aspects of the emergent composition and dynamics of the microbiota. For example, (Boyle et al., 2012) showed that cooperation between microbial species increases community productivity, because cross-feeding (a relationship between bacterial species where one feeds on another's metabolic waste product), is an effective way of using resources. Further, a simulation study by (Coyte et al., 2015) implied that competitive interactions are expected to enhance community stability, because when one community member dies, competition enables fast niche overtake instead of leading to cascading negative effects as is the case with highly dependent cooperative networks.

While basic rules of macro-ecology apply to some aspects of gut microbial communities, there are a few aspects in which microbiotas differ from larger scale ecological communities: Firstly, the remarkable spatial and temporal heterogeneity of their habitat creates a very unique environment for the coexistence of diverse taxa. Just as microbes may be transmitted among host "islands" (as described below), even within a host they grow in a patchy environment and exist in a highly dynamic habitat with a constant directional flow of nutrients and other microbes, like a river inside an island. This structural complexity allows chance effects to have a lot of power, even to the extent of allowing co-existence of species with near identical niches, enabling higher overall diversity. For example, spatial heterogeneity in microbial communities exist along the length of the gut (Donaldson et al., 2015), because environmental attributes (such as pH) and nutrients vary depending on upstream processes of local microbial and host metabolism. Further, host immune cells regularly clear excess growth of bacteria in the mucosal layers of gut (Wallace et al., 2018), creating temporal heterogeneity that may allow the coexistence of taxa that would otherwise be subjected to competitive exclusion. A second way in which microbiotas differ from other ecosystems is the existence of horizontal gene transfer. Frequent phage-mediated horizontal gene transfer between physically close microbes is known to spread mutant phenotypes such as antibiotic resistance across

bacterial communities (Huddleston, 2014), and this can greatly influence competitive relationships between bacterial taxa, since their competitive abilities can easily become locally "public property". Lastly, due to horizontal gene transfer and high recombination rates microbial evolution is fast and this makes their ecological niches more dynamic than in the macro-world. Specifically, microbes have much greater potential for fast local evolutionary adaptation in times of environmental change. In contrast with the macro-world, where rapid environmental change usually triggers plastic responses, i.e. shifting ranges, gut microbiotas are known to adapt to rapid changes (i.e. shifts in nutrient availability) both through ecological adaptation (whereby relative abundances of the best adapted taxa will increase) as well as evolutionarily (less adapted taxa will not necessarily perish but can rapidly evolutionarily adapt to the new food source).

1.4.1.2 Host-microbiota interactions

Paralleling how macroecological communities are shaped by abiotic attributes of each patch, gut microbial communities are continuously shaped by the nutritional and selective environment inside the host. Host attributes influencing the microbiota include diet (David et al., 2014) and physiology, especially immune (Round & Mazmanian, 2009) and endocrine systems (Neuman et al., 2015). Diet and physiological systems function as environmental selection processes, setting limits on which microbes can establish and sustain a population in the gut. Just like in macro-ecosystems, the microbes live in a lower-scale world where their success is primarily defined by interactions with other microbes in the community, while the environment (host) is a higher-scale entity, selecting for emergent traits of the whole microbial community (Foster et al., 2017).

Notably both of the key physiological systems regulating the microbiota (immune and endocrine systems) are also heavily influenced by the microbiota (Foster et al., 2017; Hooper et al., 2012; N. Shi et al., 2017), making host-microbiota cross-talk very much a two-way interaction. However, variation in immune and endocrine phenotypes also has a strong genetic basis (de Craen et al., 2005; Solberg et al., 2006) and consequently a great deal of genetic variation underlying microbiota heritability in humans is in fact driven by immune genes, such as MHC genes (Kubinak, Stephens, et al., 2015). In line with this,

the gut microbiota of wild mice was found to have some strong genetic determinants, including genes associated with immunity in humans (Suzuki et al., 2019). A recent study also found that host genotype had a weak but highly significant and remarkably uniform effect across microbes in the gut of wild baboons (Grieneisen et al., 2021). Similar to immune phenotypes and genotypes, both heritable and plastic variation in hormonal status can also influence the gut microbial community. For example, most animal species show partially heritable individual variation in responsiveness to stress, resulting in covariation between behavioural phenotype (e.g. "personality") and reactivity of the glucocorticoid "stress hormones" of the HPA-axis (Carere et al., 2010; Koolhaas et al., 2010) and notably glucocorticoids are further known to influence the gut microbiota. This interplay between stress hormones, behaviour and microbiota is a focus of much interest, since all three phenotypes are interrelated and link the internal physiology of an animal to processes happening in its external environment (Stothart et al., 2016).

On a proximate level, the host can control its microbiota with means such as eliminating certain microbes from the system through selective immune function (Kubinak, Petersen, et al., 2015), favouring the growth of others through selective nutrient provisioning ("*host feeding*"; Schluter & Foster, 2012; Tremaroli & Bäckhed, 2012) or changing the pH or other environmental attributes of gut through endocrine function (Jensen et al., 2020; Neuman et al., 2015). The composition of a microbiota can thus shift under host control, which can be adaptive, for example, in face of a seasonal change in diet (Davidson et al., 2020). It is important to note that rapid shifts in microbiota composition can happen through direct perturbations of the microbial community (e.g. antibiotic use), but also through perturbations that change the landscape of host control (e.g. via stress or other immunosuppressive forces) (Costello et al., 2012a). Following this, seemingly similar shifts in microbiota composition may reflect adaptive, "allostatic" changes under host control or stochastic "dysbiotic" fluctuations that are generally detrimental to the host. Separating these phenomena is an area of active research, in which researchers aim to measure the stochasticity vs determinism of compositional shifts (Shetty et al., 2017; Zaneveld et al., 2017).

1.4.1.3 Transmission

The mammalian gut microbiota is initially formed through maternal transmission during birth (Ferretti et al., n.d.; Korpela et al., 2018; Yassour et al., 2018), but mounting evidence implies that transmission remains a major force shaping microbiota composition later in life as well (Robinson et al., 2019). For example, sharing a living environment homogenises the microbiotas of co-habiting humans and this cohabitation effect has been repeatedly shown to dominate over effects of genetic relatedness on the microbiota (Song et al., 2013; Turnbaugh et al., 2009; Yatsunenko et al., 2012), either in terms of close familial relationships or racial differences (Rothschild et al., 2018). In line with this, a study comparing gut microbial profiles among wild mammals of the western hemisphere found that sympatric species communities hosted geographically restricted microbiotas: Compositional similarity between gut microbiotas declined exponentially with distance, independent of the phylogenetic relatedness and dietary niche variation between species (Moeller et al., 2017), implying that microbial transmission from the shared environment or between species can be a major force shaping the gut microbiota of wild mammals in general.

Microbes inhabit almost all surfaces on earth and thus transmission of microbes into the gut of a mammal may happen through various forms of contact the host has with its external environment.

For instance, the skin and gut microbiota of human children has been shown to be shaped by their varying contacts with the local ecosystem and natural soils. Specifically, rural children, or children with more green areas around their home, hosted more diverse microbiotas (and subsequently had more realistically reacting immune system) compared to urban children living in a microbially poor environment (Fyhrquist et al., 2014; Lehtimäki et al., 2017; Ruokolainen et al., 2015, 2017, 2020). Contact with soil has been further shown to diversify the gut microbiota of laboratory mice experimentally exposed to natural soils (Ottman et al., 2019; Zhou et al., 2016). Strikingly, local soil properties also explained more than half of variation in gut microbial profiles among sympatric populations of baboons, with microbial transmission from the soil one possible explanation (Grieneisen et al., 2019). The general environmental microbiota can also be affected by the host-associated microbiotas nested within it. For example, sharing a room was associated with gradual homogenisation of gut microbiota between students but also the

microbiota on the room surfaces became gradually more similar to that of the people living in it (Sharma et al., 2019).

While microbes can spread between hosts and the environment, transmission of microbiota members can also happen through direct contact with others, and recent research has emphasized the importance of social behavior in the spread of gut microbes among interacting animals. For example, many social mammals have been shown to harbour a social group-specific microbiota composition (Degnan et al., 2012; Grieneisen et al., 2017; Leclaire et al., 2014; Perofsky et al., 2017; Raulo et al., 2018; Theis et al., 2012; Wikberg et al., 2020). In red-bellied lemurs for instance, social-group membership explained up to 28% variation in microbiota profiles among 8 social groups inhabiting the same area (2-6 individuals per group) (Raulo et al., 2018). In addition, within-group pairwise social relationships were shown to predict gut microbiota similarity among group members in these same lemurs, as well as multiple other species of primate (Amato et al., 2017; Moeller, Foerster, et al., 2016; Perofsky et al., 2017; Tung et al., 2015) and other social mammals (Antwis et al., 2018). In humans, gut microbiota similarity was predicted by social relationships (e.g. strangers, friends, partners) independent of who lived with whom, and more intimate relationships had a stronger effect on microbiota similarity, with friends sharing more gut bacteria than strangers, and couples reporting a physically close social relationship sharing even more of their microbiota than friends or couples who reported a less close relationship (Dill-McFarland et al., 2019). Gut microbes can be shared between conspecific individuals with social behaviors involving physical contacts, such as grooming and huddling (Raulo et al., 2018; Tung et al., 2015), licking and regurgitating (Wilkinson, 1986, 2019) or kissing (Kort et al., 2014). In fact, multiple studies have suggested that some intimate physical contact behaviors, such as kissing in humans, might have evolved originally to foster microbial transfer between mothers and their infant (Lombardo, 2008; Sherwin et al., 2019). Many forms of physical contact behavior also occur between individuals of different species, and can foster microbial transfer across host species. For example, humans share more gut microbes with their own dogs than those of others (Song et al., 2013) and predator-prey relationships were found to predict gut microbiota similarity between sympatric mammalian species in an ecological network (Moeller et al., 2017).

Overall, there is evidence for microbiota transmission through both social and environmental contact. Importantly, these alternate transmission mechanisms may be influential for different subsets of the microbiota, because microbial species vary in their ability to survive outside the host. Specifically, some bacteria (e.g. aerotolerant and spore-forming) can readily survive outside the host, while others (e.g. strictly anaerobic, non-spore forming) cannot. Following this, aerotolerant taxa may be more likely to spread from and through the environment while anaerobic bacteria may require intimate physical contact to spread from one host to another. In line with this hypothesis, research on wild baboons has shown that the microbial taxa shared between individuals through social grooming were enriched in anaerobic or non-spore forming bacteria (Tung et al., 2015), while the microbial taxa differing most between baboon groups inhabiting different environments were enriched in aerobic lifestyles (Grieneisen et al., 2019). Paralleling this, microbes transmitted from mother to offspring were largely anaerobic while microbes spreading through shared environment were dominated by aerotolerant taxa in a study of laboratory mice (Moeller et al., 2018).

1.4.2 Microbiota transmission in host social networks – "The social microbiome"

The strong and widespread effects of social contact on the microbiota have motivated a view of the microbiota as a community spreading through the social network of the host population (Perofsky et al., 2017; Raulo et al., 2018; Tung et al., 2015). The ecological landscape of this "social microbiome" has been portrayed as a social archipelago of host islands (Sarkar et al., 2020), in which the connectedness between sub-communities is defined by social associations among hosts. This network approach provides a powerful generalization of social effects evident between pairs of individuals. Considering the microbiota in a transmission network allows modeling microbiota beyond processes within individual hosts and even beyond processes occurring between a pair of hosts. Examples of such processes are effects of indirect connectedness or overall network connectivity on the microbiota of individuals and populations of hosts, both phenomena that have not yet been considered by microbiota science. For example, an individual's position in a network of contacts can be described through various measures known in network science as centrality metrics (Newman, 2018). An individual can be central in a network for instance by having high degree (many connections), high eigenvector centrality (many

connections with many connections), high clustering coefficient (many interconnected connections) or high betweenness (connecting otherwise disconnected parts of a network). Depending on the transmission ecology of microbes, these centrality measures can be expected to have different effects on emergent community properties of the microbiota, such as its alpha diversity. For instance, whether the richest microbiota accumulates in the most sociable individuals has been a question receiving considerable interest, but the evidence has remained inconsistent (Johnson, 2020; Moeller, Foerster, et al., 2016; Perofsky et al., 2017; Raulo et al., 2018), due in part to lack of a network perspective on microbiota transmission. For example, (Raulo et al., 2018) and (Perofsky et al., 2017) found seemingly contradictory evidence on the effect of sociability on microbiota diversity in lemurs, with the former documenting lower microbiota diversity in more sociable individuals and the latter documenting the opposite trend. However, "sociability" in the former study was defined as the total amount of social interaction an individual exhibited, whereas the latter study defined sociability quite differently, as the number of unique social relationships an individual had. Both of these can be considered aspects of social personality, but they reflect very different positions in a transmission network of microbes.

1.4.3 Combining network theory and metacommunity theory to explain microbiota transmission

While the social network approach to microbial transfer is not a new idea but a standard method in epidemiological research, standard transmission models designed to trace the movements of one organism across a network of host have needed to be considerably updated to account for the process of microbiota transmission (Brito & Alm, 2016). This is because microbiota exchange in a (static) network of contacts is best described as a symmetrical process in which infectious organisms are by default exchanged in both directions, a phenomenon perhaps mathematically more similar to information flow than epidemics. Novel analytical methods for such processes have been developed (See Box 1), but despite these advances I would argue that conceptually this network approach has not yet been optimally combined with metacommunity theory, especially newer microbiome-tailored models (Miller et al., 2018; Miller & Bohannan, 2019). This is for two reasons. First, the "social microbiome" approach (the analysis of how social networks predict microbiota similarity, see above) ignores to a large extent the

transmission dynamics of microbes that can persist outside the host (e.g. aerobic, spore-forming taxa) and thus spread through the environment in the absence of close host contact. Second, the recent attempts to update metacommunity theory for microbiotas (Miller et al., 2018; Miller & Bohannan, 2019) considers microbial transmission as a process shaped by connectedness between hosts as defined by their distance from each other in space, like in macroecological metacommunities, rather than number of steps between them, like in epidemiological networks**.** In other words, the social microbiome approach is blind to the effects of environmental microbiota transmission while the updated metacommunity framework is blind to the emergent effects of social contact transmission, such as indirect transmission routes between hosts. The key goal of this thesis is to try and separate out effects of microbiota transmission via social contacts, from those arising via shared space use and environmental transmission in modifying microbiota composition. I aim to do this by combining methods from social network analysis and metacommunity ecology and attempting to model the host-associated microbiota as a *metacommunity nested inside the social network of their hosts,* separated by a matrix that is more or less hostile to different subsets of the microbial community (Figure3). This leads to two separate transmission ecologies potentially working in parallel: the microbial metacommunity can be structured both by social transmission through the host contact network, and also by environmental transmission from hosts' exposures to the geographical space they live in.

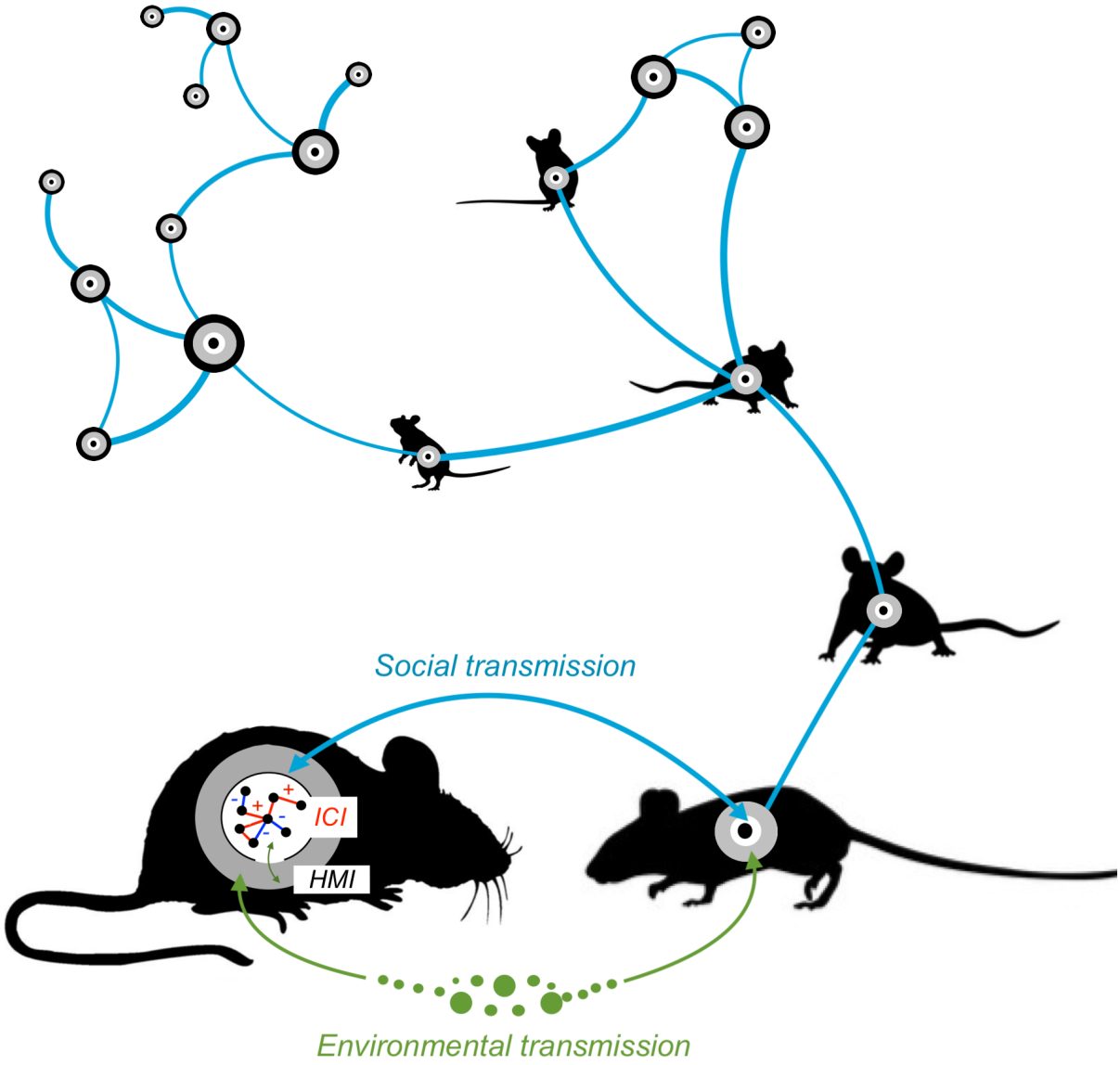

Figure 3. A Metacommunity-Network framework for microbiota assembly in a population of hosts. The composition of a microbiota in a given host is defined by i) microbial intra-community interactions (ICI) happening in a selective environment created by ii) host physiology (host-microbe interactions HMI) and ultimately defined by iii) transmission. This transmission can happen through two independent pathways creating two separate ecological landscapes for microbes across hosts: Environmental transmission (green) homogenises the microbiota of hosts that overlap more, or are closer in geographical space while social transmission (blue) creates transmission flux between microbiotas that are directly or indirectly connected in the less continuous "social space" defined by the social network among hosts (black mice and circles).

1.4 Study system

To separate the effects of social and environmental contact transmission on microbiota, a study species is needed that lives in a microbially natural environment and whose space use and social relationships can be characterised in detail, and thoroughly teased apart from each other and any individual variation in diet. The wood mouse (Apodemus sylvaticus) is a suitable species to study these questions in the wild, because they are abundant, territorial (easy to recapture) and their omnivorous diet, while shifting seasonally, is markedly homogenous among individuals co-existing in the same habitat withn the same season (Marsh, 2020). As a semi-social species, their social relationships are somewhat independent of their space-use patterns. Specifically, wood mice are not group-living, but have pairwise social associations, with the propensity to co-nest in underground burrows varying seasonally and between individuals and sexes (Lambin, 1988; Montgomery, 2009; Wolton, 1985; Zgrabczynska & Pilacinska, 2002). Individuals have small, stable, partially overlapping home ranges, and yet vary in their level of social contact, making them a particularly suitable species in which to study both social and environmental transmission of microbes. Because of these small and overlapping territories, a large number of mice can be tracked simultaneously, especially with passive tracking systems like RFID-tags, and in parallel individuals can be frequently trapped for faecal sample collection. Furthermore, the small and stable home ranges also mean that the microhabitat within each individual's home range is relatively easy to characterize in detail. These aspects together should make it possible to collect data on social associations, spatial overlap and microhabitat similarity across a large number of individual mice in their natural habitat, and link these pairwise values with their microbiota sharing patterns to disentangle and assess the relative importance of social and environmental transmission processes on gut microbiota. Furthermore, these transmission processes can be relatively confidentially separated from any diet-effects on microbiota because wood mouse diet is known to vary markedly across time but not so much among individuals of the same population. Specifically, wood mice have been shown to shift from insect-based to seed-based diet in the late summer and this dietary shift is accompanied by a predictable seasonal shift in gut microbiota composition (Maurice at al., 2015, Marsh et al., 2020). A recent study found that the diet effects on wood mouse microbiota were in fact solely explained by temporal variation in diets,

and there existed no significant variation in individual diets among mice living in the same general habitat (Marsh, 2020). Thus, by controlling for the effect of time on microbiota variation, we can account for any significant diet variation effects as well.

Social behaviours that might facilitate gut microbe transmission in wood mice include contact behaviours such as allogrooming, licking, anogenital inspections, huddling (Lambin, 1988; Zgrabczynska & Pilacinska, 2002), nest-sharing (Wolton, 1985) or aggressive interactions (Gurnell, 1978; Lambin, 1988) (Figure 4A-D). Individual variation in space use that might facilitate differential exposure to environmental transmission of microbes include variation in movement behaviour (Benhamou, 1991; Jamon, 1994), home range area (Attuquayefio & Wolton, 1986; Godsall, 2015) and differences in microhabitats between home ranges (Figure 4E-I). Transmission could also occur through non-contact social behaviours, such as scent-marking and investigation of faecal cues (Brouard et al., 2015; Stoddart & Smith, 1986), or possibly coprophagy as seen in other mouse species, though not documented in wood mice to date.

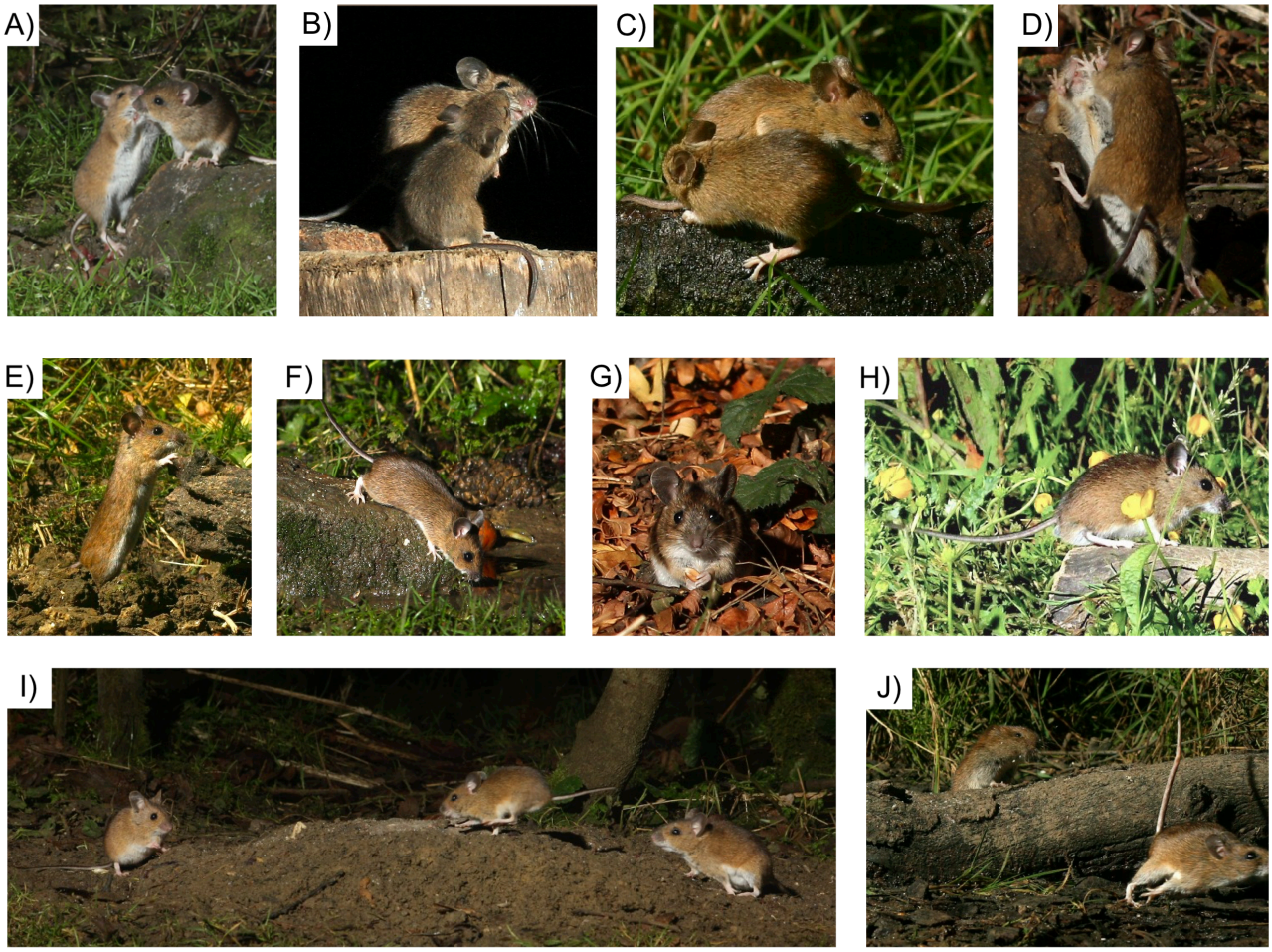

Figure 4. Examples of social and environmental contact behaviours in wood mice. Social contact behaviours that could transmit gut microbes include licking and grooming (A-B), anogenital inspections (C) and aggressive interactions (D). Individual variation in environmental contacts that could transmit gut microbes include ranging behaviour and exposure to different microhabitats (E-H), and more broadly, exposure to the same soils (I). Gut microbes might also be transmitted during close interactions between sympatric species individuals (see bank vole and wood mouse meeting in photo J). These photos were taken with a motion sensor camera trap (with a light) during natural wood mouse activity hours (night) from a natural wood mouse habitat in Milton Keynes, UK. Photo credit Roy and Marie Battell. See Supplementary Appendix.2 for more.

The work in this thesis is conducted using two separate study populations of wild wood mice, both inhabiting similar deciduous woodland habitats in England: Silwood Park in Berkshire and Wytham Woods in Oxfordshire. In both sites, similar field work procedures were carried out, including fortnightly overnight trapping of individuals for 1) faecal sample collection for microbiota analysis and 2) injection of all newly captured individuals with a Passive Integrated Transponder (PIT) tag for permanent identification and 3) continuous passive tracking using a set of novel RFID technology -based devices (loggers) which record the time-stamped presence of any tagged rodents within 1 square meter of their reading coil. These loggers produced fine-scale data on the social interactions and space use of mice across the study periods (12 months in Silwood and 10 months in Wytham), which allowed detailed description of social networks and space-use patterns of mice to be linked with their gut microbiota composition.

1.6 Study questions and overview of chapters

Using these data, I set out to explore four major study questions, with one chapter dedicated to each question:

1) Is the wild mouse gut microbiota shaped by transmission through social contacts?
2) Do environmental exposures and host behaviour shape the gut microbiota of wild mice?
3) What are the *relative* influences of social and environmental transmission on the gut microbiota, and do they spread the same, or different, bacterial taxa?
4) Are gut microbes transmitted between individuals of different sympatric rodent species?

In Chapter 2, I explore the first study question, "Is the wild mouse gut microbiota shaped by transmission through social contacts?", using data from the Silwood study population. I combine sequence data on the gut microbiota profiled from faecal samples with tracking data collected using a pilot set of 9 RFID-logger tracking devices to construct various different social networks that explore the links between social contacts and the gut microbiota. To extract the signal of social transmission on the microbiota, I

also use a genetic pedigree of the captured mice based on genotyping from tissue samples, and matrices of spatial proximity based on logger data to control for potential effects of shared space and kinship on the microbiota. I show that social association strength in a social network is a strong predictor of microbiota similarity among mice, manifold stronger than effects of other covariates such as spatial proximity or genetic relatedness, and that socially well-connected mice have richer microbiotas. I also show that the social effect on microbiota similarity is driven by male-male and male-female social relationships. Further, I demonstrate the power of the network approach by showing how an individual's wider indirect social contact landscape can influence their microbiota. Specifically, I find that well-connected individuals with bridge-like positions in the social network (those connecting otherwise poorly connected subparts of the network, i.e. with high betweenness) have higher microbial diversity, whereas well-connected individuals with highly interconnected contacts (e.g. individuals with high eigenvector centrality) do not. These results suggest that variation among individuals in their social contact landscape has pervasive effects on both gut microbiota composition and diversity, even in this relatively asocial species.

In Chapter 3, I explore the second study question, "Do environmental exposures and host behaviour shape the gut microbiota of wild mice?", with data from the Wytham study population. Here I use gut microbiota data profiled from faecal samples together with much higher resolution data on mouse movements (collected with a much larger set of 120 RFID loggers of new and improved design), and explore links between gut microbiota composition and individual variation in environmental contact and movement behaviour. Specifically, I assess whether individual variation in home range size, microhabitat diversity or locomotor behaviour influences gut microbiota composition and diversity. Additionally, I compare gut microbial and local soil microbiota profiles for mice trapped in various different habitats within the same woodland. With this data I first show that across different sites within the same woodland, mice from the same site had more similar microbiota to those from the same site than to those from others, and shared more microbial taxa with their local soil than with soil from other sites within the same woodland, but these effects were weak. Using more detailed data on environmental contacts, I then show that fine-scale individual variation in environmental exposure did not predict overall gut microbial composition, but did predict microbiota alpha diversity. Specifically, individuals with larger

home ranges had higher microbiota diversity but against my expectations, local microhabitat diversity was negatively associated with gut microbiota diversity across mice. Overall, these effects suggest that at this spatial scale individual variation in environmental exposure has relatively weak effects on gut microbiota composition and diversity.

In Chapter 4, I explore the third study question, "What are the *relative* influences of social and environmental transmission on the gut microbiota, and do they spread the same, or different, bacterial taxa?", with data from the Wytham study population. Here, I use the same dataset used in Chapter 3 to assess the relative effects of environmental and social transmission on microbiota composition. I construct social networks alongside pairwise measures of home range overlap and microhabitat similarity and in a fully dyadic Bayesian regression framework, link these with pairwise measures of gut microbiota similarity between mice. I show that social association, home range overlap and habitat similarity all significantly and independently predict microbiota similarity between pairs of mice, but that the effect of social association is many times stronger, implying that at this scale, social transmission is a more influential force than environmental transmission in driving individual variation in gut microbiota composition. Furthermore, I explore which microbial taxa are driving these social, spatial and habitat signals on microbiota composition, and show that social signal is driven more by anaerobic bacteria whereas spatial and habitat signals are driven more by aerotolerant bacteria. This suggests that different sets of microbes with distinct transmission ecologies are spread through social vs environmental pathways in mice, and that bacteria that cannot readily persist outside the host rely strongly on social contact to be transmitted, whereas aerotolerant taxa have greater indirect transmission through environment.

In Chapter 5, I explore the last study question, "Are gut microbes transmitted between individuals of different sympatric rodent species?", using comparable data from both Silwood and Wytham study population in parallel. Here, I use RFID-tracking data to construct multi-species social networks for from the same three sympatric rodent species tagged and monitored in both study sites: wood mice (*Apodemus sylvaticus*), yellow-necked mice (*Apodemus flavicollis*) and bank voles (*Myodes glaerolus*). I link these networks with gut microbiota profiles analysed from faecal samples to explore whether social association

or spatial proximity to conspecific vs interspecific individuals differentially affects the gut microbiota of wood mice. I first show that in both study populations independently, social associations predict microbiota sharing only between conspecific pairs while space-sharing predicts microbiota similarity between pairs of rodents belonging to different species. However, further analyses that focused specifically on host species-indicative bacterial taxa did provide evidence of cross-species transmission among sympatric rodents; I show that in both populations, direct and indirect connectedness to yellow-necked mice predicts the richness and abundance of microbes typically found in yellow-necked mice in the guts of wood mice, while same is not true for connectedness to bank voles. These results highlight the potential for individual-level variation in behaviour to drive processes of gut microbial spread across species boundaries, especially between closely related species with overlapping niches. This final chapter also emphasizes the importance of repeatability and the power of repeating the same study twice in different study populations to separate spurious trends from consistent ones.

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Supplementary Material S1 for

Chapter 1

Introduction

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Supplementary Appendices

Appendix S1.1. Opinion articles over microbiome-mediated behaviour published in Trends in Ecology and Evolution (TREE) 2020-2021

Three articles published in TREE in order of publication, together forming a debate over microbiome-mediated behaviour in wild animals. We first wrote the opinion piece (1) and this piece received a response from Nguyen et al. (2) to which we then wrote a reply (3).

1. *"Identifying microbiome-mediated behaviour in wild vertebrates"*

By Gabrielle Davidson, Aura Raulo & Sarah Knowles
Published in Trends in Ecology and Evolution, 2020, Vol. 35, No. 11

2. *"Disentangling the Environment in Wildlife Microbiome–Behaviour Interactions: Response to Davidson et al. "*

By Hanh Nguyen, Penelope Jones, Dave Kendal, Emily Flies
Published in Trends in Ecology and Evolution, 2021, Vol. 36, No. 4.

3. *"Response to Nguyen et al. 'Laboratory- Inspired Manipulations Hold Value for Wild Microbiome-Behaviour Research"*

By Gabrielle Davidson, Aura Raulo & Sarah Knowles
Published in Trends in Ecology and Evolution, 2021, Vol. 36, No. 4.

CellPress
REVIEWS

Opinion

Identifying Microbiome-Mediated Behaviour in Wild Vertebrates

Gabrielle L. Davidson,[1,*,@] Aura Raulo,[2,@] and Sarah C.L. Knowles[2,@]

Recent research in laboratory animals has illuminated how the vertebrate gut microbiome can have diverse and powerful effects on the brain and behaviour. However, the ecological relevance of this microbiome–gut–brain (MGB) axis outside the laboratory remains unexplored. Here we argue that understanding behavioural and cognitive effects of the gut microbiome in natural populations is an important goal for behavioural ecology that may shed light on the mechanisms and evolution of behavioural plasticity. We outline a toolkit of approaches that could be applied in this endeavour and argue that beyond collecting observational data on the microbiome and behaviour from free-living animals, the incorporation of manipulative approaches tailored to such systems will be a key next step to progress understanding in this area.

Highlights

Laboratory studies in model organisms have shown that the gut microbiome can strongly affect host behaviour and cognition through the microbiome–gut–brain (MGB) axis. However, the importance of such mechanisms in wild animals remains unknown.

Associations of the gut microbiome with diet as well as with social interactions have been documented in wild animals. While these are usually interpreted to reflect the effect of host behaviour on the microbiome, effects in the opposite direction are also plausible.

Measuring microbiota-mediated behavioural and cognitive plasticity in the wild will be necessary to identify the MGB axis's role in ecological and evolutionary processes.

Impacts of the Microbiome on the Brain

A surge of studies has demonstrated how the **gut microbiome** (see Glossary) can affect the central nervous system (CNS) through the so-called **MGB axis** and the consequences of such effects for behaviour and cognition. Biomedically focussed studies in laboratory rodents have used germ-free animals, faecal transplantation, and antibiotic treatment to document the impact of an altered gut microbiome on a range of behavioural traits, including social behaviours [1], anxiety [2], and **cognitive performance** [3,4]. While the generality of such findings has to some extent been explored through observational studies in humans and the mental health literature, the wider ecological relevance of the MGB axis across vertebrates remains unknown, despite its likely importance for evolutionary and behavioural ecology.

The microbiome can affect the CNS, and consequently behaviour, through a number of pathways [5] (Box 1). Although it has sometimes been suggested that such effects reflect evolved microbial strategies of host manipulation, for sound evolutionary theoretical reasons this is unlikely [6]. Rather, behavioural impacts of the microbiome more likely reflect either incidental effects of bioactive microbial metabolites (e.g., short-chain fatty acids, neurotransmitters) or the host's integration of microbial signals into regulatory networks that control physiology and behaviour, over evolutionary time. We suggest that the gut microbiome may constitute an important mediator of behavioural plasticity, either through effects in early life (**developmental plasticity**) or throughout an organism's lifespan (**reversible behavioural plasticity**) (Box 2) and thus modulate the ability of organisms to respond adaptively to environmental change [7,8].

To date, research on the microbiome's behavioural impacts have focussed heavily on laboratory-based model organisms (mostly rodents) and humans [9]. However, to understand the ecological and evolutionary relevance and the taxonomic breadth of such effects, studies in laboratory animals and humans alone are insufficient. While laboratory animals are highly tractable and easy to manipulate, their **gut microbiota** often differs markedly from that of their wild counterparts [10], their behaviours are highly constrained (by both a captive environment and historical artificial

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Box 1. Mechanistic Understanding of the MGB Axis

Several landmark studies have revealed key mechanistic pathways underlying the MGB axis in captive rodent models. In seminal work, Sudo and colleagues [50] demonstrated how the gut microbiota shapes the development of appropriate stress responses, by programming the hypothalamic–pituitary–adrenal (HPA) axis in early life and mediating the plasticity of the brain. In this study, it was observed that germ-free mice had exaggerated stress responses, elevated levels of corticotropin-releasing factor mRNA in the hypothalamus, and decreased expression of brain-derived neurotrophic factor in the cortex and hypothalamus. These effects did not emerge if germ-free mice were reconstituted with a probiotic (*Bifidobacterium infantis*) at birth or by faecal transplantation from a normal donor within an early developmental window [50]. Subsequent research further demonstrated that an absence of gut microbes in germ-free mice also causes reductions in anxiety-like behaviours. These mice showed differential expression of several genes in the hippocampus, cortex, and striatum as well as high turnover rates in neurotransmitters known to be associated with anxiety [2]. Plasticity in behaviour associated with the MGB axis has also been demonstrated in adult mice. The repeated administration of *Lactobacillus rhamnosus* to conventional adult mice with an intact microbiota has been shown to improve anxiety and depressive-like behaviours and stress hormone release and alter the levels of GABA mRNA expression in the hippocampus, prefrontal cortex, and amygdala [18]. An important discovery in this study was that the effects on host phenotypes were no longer observed if the vagus nerve was severed, pointing to a key physical route of communication between the gut microbiota and the brain [18].

Similar studies across model vertebrates suggest that MGB axis mechanisms are taxonomically widespread. For example, in adult zebrafish (*Danio rerio*) anxiety behaviour was reduced and GABA receptor and serotonin transporter genes expression in the brain were upregulated following administration of a probiotic (*Lactobacillus plantarum*) [51]. In Japanese quail (*Coturnix japonica*), memory was improved in birds given the probiotic *Pediococcus acidilactici* [52]. Moreover, germ-free quail were more emotionally reactive than quail with normal microbiomes [53]. An important next step will be to test the translatability of these laboratory-based findings to natural systems. One method to do so would be to focus on model species where MGB axis mechanisms are well known from the laboratory (e.g., house mice, zebrafish) and to pair laboratory-based experiments with field studies of their wild counterparts to investigate whether similar MGB mechanisms occur in the wild.

selection), and natural microbial transmission and fitness effects are impossible to capture in the laboratory. Conversely while humans constitute a 'natural' free-living system, controlled manipulations and studies of fitness impacts are both extremely challenging for ethical and logistical reasons. We therefore argue there is a need to expand research on behavioural impacts into a broader range of non-model free-living animals and adapt our approaches to do this effectively.

Box 2. The Gut Microbiome and Behavioural Plasticity in the Wild

The microbiome has been proposed to mediate adaptive phenotypic plasticity or environmental acclimation [7], and this has already been demonstrated for some physiological traits, such as the modulation of energy expenditure, host thermogenesis, and gut physiology in response to cold temperatures [54]. We predict that the microbiome could also mediate reversible plasticity in key behavioural phenotypes such as migration, sociality, mate-seeking, or foraging behaviour. These behaviours are also likely to in turn shape the microbiome, by changing microbial exposures (e.g., through altered environmental or social transmission) or which microbes can flourish inside the host (e.g., by changing diet through foraging behaviour) [55]. Such feedback loops create the potential for microbes to act as environmental sensors in regulatory circuits that modulate behaviour. The microbiome may enable two types of regulation in this context: homeostatic and allostatic regulation [56]. Homeostatic regulation involves only negative feedback and buffers phenotypic change, returning the host to an optimal setpoint. As a behavioural example, the microbiome may drive foraging choices, resulting in host nutritional status (and microbiome composition) returning to an optimal state. Alternatively, the microbiome may function as an allostatic regulator, enabling hosts to rapidly, even predictively, adapt to changing phenotypic optimums [56,57]. Allostatic regulation can involve negative and/or positive feedback, with the net result facilitating phenotypic change toward a new optimum phenotype suitable for a new environment. For example, the microbiome may modulate host behaviour in a way that helps it acclimate to a fluctuating environment, such as over a seasonal or migratory cycle.

Laboratory-based studies have shown that the microbiome can mediate both developmental and reversible forms of behavioural plasticity, depending on the trait in question. For example, the characteristic reduced anxiety of germ-free mice can be reversed by microbial colonisation in early life (e.g., up to 3 weeks) [2,58], but not by colonisation at 10 weeks [44]. By contrast, dietary manipulation [22], faecal transplants [3] and antibiotics [4] cause reversible behavioural plasticity in fear conditioning and memory in adult mice. For any given behavioural trait, which of these pathways is expected to be more important (developmental or reversible plasticity) should guide the design of both observational and experimental microbiome–behaviour studies in wild populations. For some behaviours, and in most non-model species, there may be no pre-existing information about microbiome effects to guide such expectations, but we may know when in life they are most labile or responsive to the environment. For example, social learning strategies and social network position in zebra finches (*Taeniopygia guttata*) are known to be strongly affected by early life stress [59,60], whereas spatial memory performance in food-storing birds varies seasonally throughout life [61].

Glossary

16S rRNA amplicon sequencing: amplification and sequencing of the conserved prokaryotic 16S rRNA gene containing hypervariable regions that allows taxonomic assignment of previously described, undescribed, cultured, and uncultured operational taxonomic units.
Allostasis: physiological regulation based on feedforward mechanisms that enable physiological parameters to adaptively and predictively fluctuate between different environment-dependent optima.
Alpha diversity: alpha diversity indices measure the average microbial diversity (e.g., species richness) within a single community.
Beta diversity: measures differences in composition among communities. Typically this is captured by estimating community dissimilarity indices, which vary in whether or not they account for quantitative differences in microbial abundance, and the phylogenetic relatedness of constituent microbes.
Cognitive performance: a quantification of a putative cognitive ability inferred from the subject's observable behaviour. For example, in a task aiming to measure spatial memory, cognitive performance could be measured as the number of correct spatial locations in which the subject searches for food following a retention interval.
Developmental plasticity: when a single genotype displays alternative behaviours in adulthood as a result of differing environments experienced during development. Responses to the developmental environment tend to have persistent effects on the phenotypes of mature organisms.
Gut microbiome: the gut microbiota together with its host environment.
Gut microbiota: the community of microbes (bacteria, archaea, fungi, and viruses) present in the gut.
Homeostasis: physiological regulation that promotes the stability of an internal state despite a fluctuating environment.
Metabolomics: analysis of the metabolites (the small-molecule substrates, intermediates, and products of metabolism) present in a tissue or sample.
Metagenomics: analysis of the full genetic material recovered from a particular sample. From such data, microbial genomes can be

A growing literature describes the gut microbiota of **wild vertebrates** and has revealed strong associations with diet [11], season [12], habitat [13], and social contact patterns [14–16]. While such findings provide clues to how and why microbiota composition varies in nature, the underlying causal processes, and the consequences of microbiome variation for the behaviour of wild animals, remain unexplored. For behavioural ecology, two key outstanding questions are therefore: when and how does the gut microbiome drive changes in host behaviour and cognition through the MGB axis in wild animals; and what relevance do such effects have for our understanding of behavioural plasticity and evolution in these systems? Inferring causality will be key to understanding the microbiome's role in driving behavioural variation (Box 3). This is no easy task in wild systems, where observational data often lack temporal resolution, experimentation is not straightforward, and there may be a trade-off between performing manipulative interventions and maintaining ecological validity.

Which Behavioural Traits Should We Focus on in the Wild?

In the large body of research on microbiome–behaviour links in laboratory rodents, well-established behavioural assays have been applied to address biomedical questions related to human disease [17]. This work has revealed several behavioural traits that are microbially modulated (in some cases paired with knowledge about the mechanistic pathways [18]) that make good candidates to explore in wild settings. These include social behaviour [1], anxiety [2], fear conditioning, and memory [3]. In the wild, similar effects could influence behaviours like memory for routes and food locations, the propensity to join social groups or participate in prosocial or aggressive social interactions, predator avoidance, and dietary and technical innovation (Figure 1). Behaviours that can affect microbial transmission (e.g., social behaviour, foraging strategy) perhaps warrant particular attention as they have the potential to be involved in feedback loops, in which the microbiome and behavioural phenotype affect each other reciprocally through **homeostasis** or **allostasis** (Box 2). We may also look to behavioural ecology

Box 3. Challenges in Identifying the Drivers of Microbiome–Behaviour Associations in the Wild

The identification of causal pathways between the gut microbiome and host behaviour in wild settings faces unique challenges compared with laboratory settings. First, confounding factors may generate spurious links between the microbiome and behaviour where no causal relationships exist. Environmental factors such as diet and seasonal changes are likely to affect the microbiome [11,12] and behaviour [62] simultaneously, such that microbiome variation can strongly correlate with behavioural variation despite no causal link (Figure IA). Second, when causal relationships between the microbiome and behaviour do exist, their directionality may be hard to infer as effects in both directions, and feedback, may be expected. For example, the gut microbiome could affect a cognitive trait such as memory, while the same cognitive trait could affect microbiome composition if, for example, host memory of food locations alters diet and therefore the microbiome (Figure IB). Finally, the microbiome is just one component of several interlinked physiological pathways that together shape host behaviour (Figure IC). Such pathways include the HPA axis, which regulates the release and down-regulation efficiency of glucocorticoids and underpins, for example, fear conditioning acquisition and recall [63]. HPA axis programming and regulation is also in part influenced by the gut microbiome [32]. The complexity of such interlinked physiological systems may make identification of the microbiome's role more challenging. Finally, behavioural effects may not be generated by the microbiome present when a behaviour is measured but by the microbiome that colonised the host at an earlier time point, such as during a critical developmental window (Figure ID).

While inferring causality from observational data has limitations, statistical estimation of causality in microbiome–behaviour covariation should be possible, particularly because the microbiome typically has high variability. The direction of causality could be estimated in a structural equation modelling framework [64], using inference methods depending on the underlying structure of the microbiome variation. With known temporal structure in microbiome variation, Granger causality methods [65] could help to estimate whether the microbiome is driving a behaviour. Invariance methods [66] allow similar use of other forms of variability, including spatial variability in the microbiome (e.g., as observed in [67]), spatiotemporal variation [68], or variation associated with host traits such as age or sex, providing these traits can be assumed to have invariant (fixed slope) effects on the microbiome. Further, genetic structure known to shape the microbiome can be used as an instrumental variable [69], paralleling Mendelian randomisation methods used to infer causality between the microbiome and disease [70].

reconstructed or the functional potential of a whole microbial community assessed.

Metatranscriptomics: analysis of expressed RNA in a sample. Metatranscriptomics provides information on which genes are being expressed in a given sample/microbial community, not just which genes are present.

Microbiome–gut–brain (MGB) axis: a general term describing the bidirectional communication network that links the gut and its resident microbial community and the central nervous system. Through this network, conditions in the gut and gut microbes can affect brain activity and host behaviour and vice versa. The pathways by which signalling is thought to occur from the gut to the brain include stimulation of the vagus nerve as well as via endocrine and immune pathways.

Microbiome transfer experiments: experiments in which the microbiome of one host individual is experimentally transplanted into another host individual.

Prebiotic: a substrate that is selectively utilised by symbiotic microorganisms conferring a health benefit.

Probiotic: live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Probiotics can, for example, stabilise gut PH, outcompete undesirable microbes, improve immune responses, and increase growth.

Relative abundance: the proportion of a community that belong to a specified taxon (e.g., a specific family or genus).

Reversible behavioural plasticity: when a single genotype displays different behaviours in response to different environments.

Wild vertebrates: free-living animals that are wild born (not born in a captive environment). An important feature of wild vertebrates is that their long-term and recent evolutionary history will have been shaped by their natural habitat.

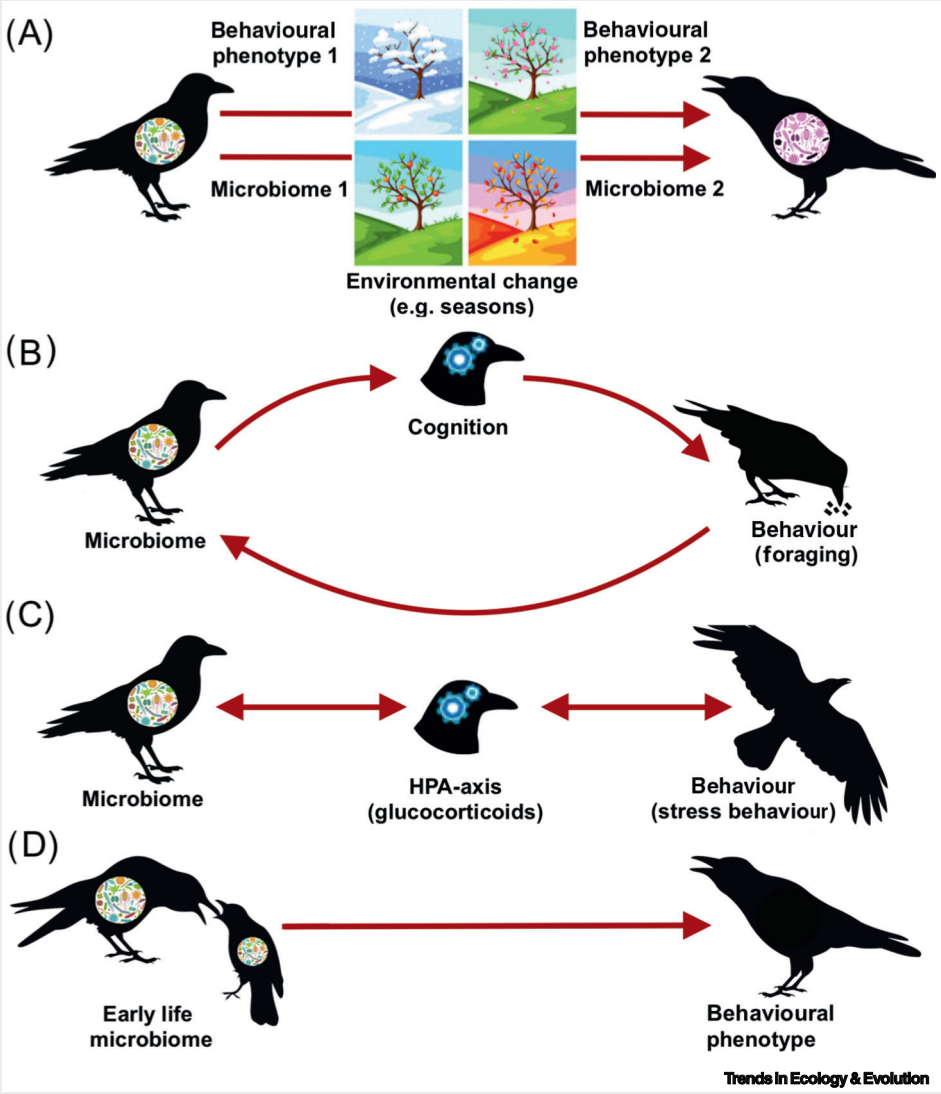

Figure I. Challenges in Identifying Behavioural Effects of the Microbiome in Wild Animal Systems. (A) Confounding environmental factors (e.g., seasonality, diet) may shape both the microbiome and behaviour, driving spurious associations in wild systems without causal links. (B) Causal links between the microbiome and behaviour may be bidirectional and involve feedback loops. (C) The effect of the microbiome on host behaviour may be indirect and potentially complex, involving multiple physiological pathways associated with that behaviour, such as the hypothalamic–pituitary–adrenal (HPA) axis. (D) Behavioural effects of the microbiome may occur across developmental timescales and involve time lags, such as when the microbiome in early life affects behavioural phenotypes measured in adulthood.

research for candidate behavioural traits of longstanding interest that vary among and within individuals, such as home range size [19], competitive ability [20], and boldness [21] (Figure 1). For a given trait, the life history stage at which microbial influences could arise should be considered carefully. Behavioural traits vary in the extent to which they are programmed by events early in life or can change in response to the environment throughout adulthood. Such differences will guide not only predictions about links with the microbiome, but also methodological approaches to study this (Box 2).

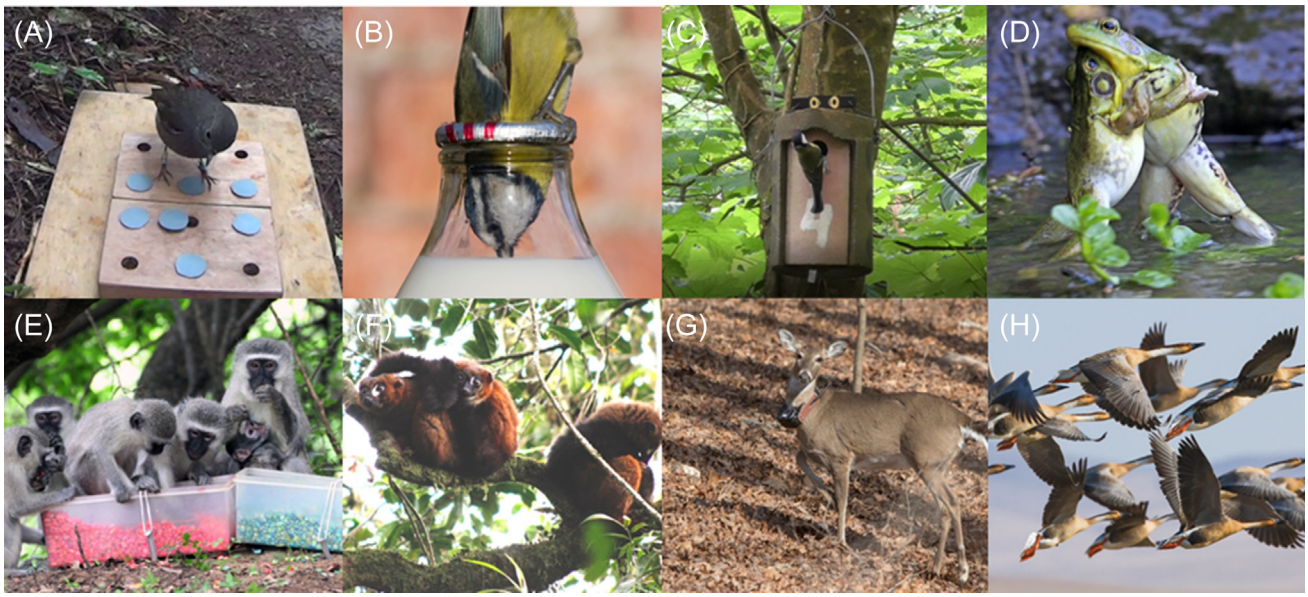

Figure 1. Candidate Behavioural and Cognitive Traits in Vertebrates That May Be Influenced by the Gut Microbiome. (A) Memory for food locations; (B) technical and dietary innovations; animal personality traits such as (C) boldness; social behaviours such as (D) aggression; (E) social learning and (F) grooming; movement traits such as (G) home range size and (H) migratory behaviour. Photographs with permission from: (A) Rachael Shaw; (B) Paul Sawyer; (C) Gabrielle Davidson; (D) Adrian Binns, Wildside Nature Tours; (E) copyright Erica van de Waal; (F) Velontsara Jeanne Baptiste and Long-Term Red-Bellied Lemur Project; (G) MDC Staff, courtesy Missouri Department of Conservation; (H) Jargal Lamjav.

What Features of the Microbiome Are Expected to Influence Host Behaviour?

Links between behaviour and microbiome metrics such as **alpha diversity**, **beta diversity**, and the **relative abundance** of specific microbial taxa have been identified in rodent and human studies [3,22,23]. While **16S rRNA amplicon sequencing** can be used as a starting point to characterise the microbiota's broad taxonomic composition to study links with behaviour, we advocate the incorporation of complementary functional analyses of the microbiome that have the potential to provide mechanistic insight. These include **metabolomic** [24], **metagenomic** [25], and **metatranscriptomic** analyses (e.g., on faecal, tissue, or serum samples). A range of microbial metabolites and pathways are already known to be involved in modulating behavioural phenotypes. For instance, gut microbes produce short-chain fatty acids (the end-products of fibre fermentation in the colon), neurotransmitters (e.g., serotonin) and their precursors (e.g., tryptophan) that have been implicated in altering behaviour in rodents [26]. Using these methods, metabolites or gene functions found to associate with a given behaviour or experimental response can be cross-referenced against molecules known to be neuroactive, such as gamma-aminobutyric acid (GABA), dopamine, and noradrenaline [27,28]. Such approaches have already proved useful in elucidating the neuroactive properties of specific gut flora and their associations with depression and quality of life in humans [29]. In wild animal systems, researchers could thus explore which (microbial or host) metabolic pathways are involved in observed behavioural associations or experimental responses, and generate mechanistic hypotheses for further investigation.

What Can Be Gained from Observational Data?

The collection of observational data is prominent in field-based ecology and advantageous for understanding natural host–microbe relationships because it involves minimal interference with naturally evolved gut microbiomes. Descriptive data on microbiome–behaviour relationships remain

scant and are often interpreted as showing that behaviour drives the microbiome. For example, a recent study showed that two types of social behaviour (grooming and scent marking) are positively associated with microbiota alpha diversity in Verreaux's sifaka (*Propithecus verreauxi*), thought to arise because these behaviours drive microbial transfer among individuals [14]. However, a reverse causality is also possible in which the microbiome shapes social behaviour, as has been documented in rodent studies [30]. Such correlative findings therefore set the stage for further work to test the true direction of effect. Longitudinal studies (in which individuals are followed across seasons or years) are likely to provide more powerful inference than cross-sectional studies, by allowing one to observe how both the microbiome and behaviour change under shifting environmental conditions and to test for within-individual, time-lagged associations that are more likely to reflect causal links. While such methods cannot provide definitive evidence of causal relationships, statistical approaches such as structural equation modelling [31] can be used to improve causal inference from correlational data (Box 3). Overall, characterising how microbiome variation and host behaviours covary in natural settings is an important first step that would ideally be followed up with manipulative approaches capable of more definitive hypothesis testing.

Microbiome Manipulations to Infer Causality

A major challenge lies in finding ways of manipulating the microbiome in non-model species, either in the field or in captive settings, to ask how natural microbiome variation affects behavioural phenotypes. This is far from easy, reflected by the fact that all studies investigating microbiome–behaviour links in wild animals to date have been purely correlational. Experimental approaches from laboratory-based microbiome research provide a starting point for inspiration but will need adapting to fulfil this brief. A common approach in laboratory studies is to completely remove the microbiome and test how behaviour is altered in 'germ-free' animals. For instance, several studies have reported cognitive deficits, reduced anxiety, and hyperactive adrenal responses in germ-free rodents compared with conventional controls [9,32]. However, since most questions about the microbiome's behavioural effects in wild animals concern how naturally occurring microbiome variation might shape behaviour, the relevance of germ-free comparisons is questionable. However, other available manipulative approaches hold promise, and include the use of antibiotics, **prebiotics** or **probiotics**, and **microbiome transfer experiments**.

Antibiotic treatment constitutes one tool to manipulate the gut microbiome and examine effects on behaviour and has been shown to negatively affect cognition in laboratory rodents [4]. Controlled antibiotic treatment experiments could be performed in wild populations (or in semi-natural setups that allow natural behaviours) in which the microbiome is perturbed (either during a developmental window or in adulthood) and effects on behavioural traits are monitored before, during, and after treatment. One limitation is that antibiotics are a blunt manipulative tool and cannot be used to target very specific microbial taxa, as most are broad spectrum. Thus, they may also treat pathogens or commensal eukaryotes with potential effects on host behaviour. Some antibiotics can also cause mitochondrial dysfunction and increase oxidative stress in mammals [33,34], which may directly affect cognitive abilities or behaviours associated with energy expenditure. Additionally, concerns about the spread of antibiotic resistance may preclude the use of antibiotics in free-living populations.

Probiotics and prebiotics present another potential manipulative tool and in laboratory rodents have been used to influence behaviour, physiology, and/or protein expression in the brain [18,35,36]. Compared with prebiotics, probiotics offer the advantage of providing direct evidence for causal effects of specific microbes. Prebiotics generally contain some form of indigestible fibre such that their major impact is expected to occur through the microbiome [37], although other

pathways cannot be entirely ruled out. In principle, one could use field-based pro- or prebiotic treatment experiments to test whether strains shown to affect behaviour in laboratory animals also affect behaviour in their wild counterparts. While there is at least one commercially available probiotic strain licenced for use with wild birds (*Bacillus subtilis* C-3102), most probiotics have been designed in commercial and biomedical research contexts for use in humans or farm or laboratory animals and their relevance, safety, and efficacy in wild vertebrates are far from certain. We therefore suggest a more tailored approach to using these tools. Here, once a candidate microbe has been identified as associated with a behavioural trait (e.g., in some insect systems [38]), one could isolate it and perform probiotic supplementation experiments in natural or semi-natural contexts to observe behavioural impacts.

Manipulation of diet (e.g., food availability, nutrient content) may be a useful approach when studying behaviours that are already known to respond to diet, where the goal is to examine whether the microbiome might mediate this. One example of a candidate phenotype from wild animals is divergent migratory behaviour across bird populations, which is associated with dietary specialisations [39]. In such cases, one could examine whether diet-induced microbiome changes predict diet-induced behavioural differences. Although food supplementation in the wild would not exclude access to alternative food sources, supplementation could also be performed in captivity. Where diet manipulation induces parallel changes in both the microbiome and behaviour, follow-up microbiome transfer experiments (see below) could test definitively whether microbiome changes play a causal role. More complex experiments combining diet manipulation (e.g., specific nutrient supplementation) with microbiome manipulation (e.g., transfers, antibiotic treatment) could also provide an effective way to explore the mechanisms underpinning diet-driven microbiome effects on behaviour [40].

Microbiome transfer experiments are a powerful inferential tool, commonly used to test whether the microbiome causally drives a given phenotype in laboratory studies. Such experiments have shown how the microbiome can mediate phenotypes from senescence in fish [41] to diet-induced anxiety, fear-conditioning, and memory in laboratory mice [3]. Gut microbiota transfers from wild mice into laboratory strains (creating so-called 'wildling' mice) have recently shown how such experiments can make use of naturally occurring microbiome variation to address important questions in immunology [10,42]. This approach could be adapted in MGB research by sourcing the gut microbiome from members of a wild population divergent in a behavioural trait of interest that shows correlative associations with the gut microbiome. If the microbiome plays a causal role in driving behavioural variation, microbiome transfer should lead to recapitulation of the donor's behavioural phenotype in recipient animals. Recipients could be either laboratory-reared germ-free animals or wild-caught individuals treated with antibiotics to deplete the native microbiota. The microbial state of recipient animals (i.e., germ-free vs non-sterile) is an important variable to consider, as there is a trade-off between clean manipulation (germ-free) and ecological reality when testing the microbiome's phenotypic effects [43]. For instance, an important consideration here is that some behavioural deficits in germ-free mice are already known to be irreversible on microbial colonisation [44]. Recipient animals could be kept in a captive or seminatural setting for behavioural testing or observation. If captive recipients are used, behavioural assays in captivity should strive to capture phenotypes in wild donor animals as accurately as possible. This is feasible, as laboratory-based proxies for several different wild behavioural traits have been successfully validated, such as migratory restlessness as a proxy for migratory behaviour [45] and mate choice assays to capture wild mating behaviour [46]. Assays have also accurately characterised avian exploration behaviour in both wild and captive environments [47]. Cognitive phenotypes may be best captured by adopting a psychometric approach using a test battery to measure multiple putative cognitive traits and by establishing test validity across wild and captive environments [48]. For transfer experiments to

accurately infer behavioural effects of the microbiome and have maximal validity, robust experimental design is of paramount importance [49], as is maintaining as much ecological reality as possible in terms of mimicking how host–microbiome interactions occur in nature [43].

Concluding Remarks

The discovery that the gut microbiome shapes host behaviour has changed the way the biomedical field understands human health. Equally, MGB axis research has the potential to yield important insights in ecology and evolution, shedding light on the role of microbial symbionts in phenomena such as rapid adaptation via behavioural plasticity, the dynamics and evolution of trophic relationships, the evolution of complex behaviours, and speciation. However, if we are to make progress in this field, it is critical that studies can distinguish between microbiome-dependent and microbiome-independent effects on host behaviour (see Outstanding Questions). Although challenging in wild systems where the potential for manipulation is limited, utilising the experimental tools we have outlined we can advance our understanding of how and when the gut microbiome drives behavioural variation and plasticity in nature.

Outstanding Questions

What features of gut microbial communities most influence cognition and behaviour, and can we detect these influences in wild animal systems?

Can we disentangle microbiome effects on cognition and behaviour from other host and environmental variables?

Do behavioural impacts of microbiome perturbations discovered in laboratory animal studies translate to similar effects of natural microbiome variation in wild settings?

What is the relative importance of early life effects versus environmental variation in adulthood on behavioural plasticity via the gut microbiome?

How common are feedback loops between the gut microbiome and host behaviour and what are their evolutionary implications?

To what extent are behavioural phenotypes transmitted among individuals through gut microbe transmission, and might this shape collective behaviour?

How often do microbiome effects on behaviour transcend generations; for example, the effect of the maternal microbiome on offspring behaviour?

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Letter

Disentangling the Environment in Wildlife Microbiome–Behaviour Interactions: Response to Davidson *et al.*

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There is growing evidence that the gut microbiome strongly influences animal physiology and behaviour. In their recent article in *TREE*, Davidson *et al.* [1] call for research into the relationship between the gut microbiome and behaviour in free-living wildlife to better understand the mechanisms and evolution of behavioural plasticity. They provide a framework for investigating microbiome-mediated behaviour, including microbiome manipulation to infer causality. While the authors recognise that the environment influences both gut microbiomes and behaviours, we suggest that their proposed framework does not adequately capture the complexity and multiplicity of environment–microbiome–behaviour links. As we argue, any examination of the links between the gut microbiome and behaviour in free-living wildlife demands a more holistic perspective of the role of the environment in shaping gut microbiomes, behaviours, and their interactions.

When discussing the 'environmental factors' relevant to gut microbiome–behaviour interactions, Davidson *et al.* [1] largely focus on diet and season. Yet, there are other important pathways through which the biotic and abiotic features of the natural environment affect both the gut microbiome and behaviours of animals. Most importantly, the environmental microbiome (i.e., the microbes found in soil, air, water, and surfaces of the environment) shapes the composition of the gut microbiome of vertebrate animals, including humans [2]. This was demonstrated experimentally; for example, pigs (*Sus scrofa domesticus*) [3] and mice (*Mus musculus*) [4] exposed to soil had more diverse gut microbiomes compared with animals exposed to traditional bedding. In natural systems, characteristics of habitats, independent of diet, have also been demonstrated to influence the composition of gut microbiomes in wild animals, for example, in swan geese (*Anser cygnoides*) and American white ibis (*Eudocimus albus*) [5,6]. Finally, environmental microbiomes have been shown to impact both the gut microbiome and behaviour in mice [7]. Any explorations of the gut microbiome–behaviour pathway in artificial environments are unlikely to translate easily to wild animal populations, where environmental microbiomes impact both the presumptive effector and response variables. These studies provide compelling evidence that realistic environmental microbiomes must be considered when investigating gut microbiome–behaviour links.

Animal behaviour, animal microbiomes, environmental microbiomes, and habitats are interdependent. An approach that recognises this complexity is needed to disentangle these interactions in biologically meaningful ways. Many of the approaches proposed by Davidson *et al.* [1], such as pre- and probiotic treatments, and diet manipulation, are important for identifying mechanisms linking gut microbiomes and behaviours in wild animals. Yet, to address the complexity of the interactions between habitats, environmental microbiomes, gut microbiomes, and behaviour with full ecological relevance, experiments must take place in natural habitats of free-living animals that offer real-world conditions, including realistic diets, social interactions, and habitat and microbiome variation. As Davidson *et al.* [1] point out, controlled laboratory experiments are less messy than natural environments, and there are trade-offs when living laboratories are used to explore questions about microbiome-mediated behaviour. However, with careful study design and appropriate statistical techniques, studies undertaken in natural systems on different species of free-living animals, which measure and incorporate fluctuations in environmental microbiomes and heterogeneity in habitats, can generate answers that are rich, evolutionarily relevant, and translatable [8].

Urban areas are ideal settings for such natural experiments, because they offer variation in environmental microbiomes, and a range of ecosystem characteristics, including habitat fragmentation, noise, light and toxin pollution, temperature and biogeochemical cycle changes, and food supplementation [9], which have been shown to affect behavioural plasticity and eco-evolutionary processes, including animal behaviour [10]. Within cities, there is a mosaic of habitats and considerable fine-scale spatial and temporal heterogeneity [11]. These replicated gradients of change create opportunities to unpack the interrelated ways that biophysical environments influence external and internal microbial communities and behaviour. Studying the microbiomes and behaviour of cosmopolitan species [e.g., house sparrows (*Passer domesticus*) or black rats (*Rattus rattus*)] across cities and along urbanisation gradients, or locally endemic species along urbanisation gradients, could generate new insights into these inter-relationships. Importantly, urban areas are also extremely dynamic and provide the opportunity to work with planners and managers to develop experimental and quasi-experimental manipulations of habitat and environmental microbiomes [12]. Studies of wildlife microbiomes and behaviours before and after interventions occur (and compared with control sites) could help isolate the influence of wildlife microbiomes on behaviour in the context of habitat and environmental microbiome heterogeneity.

In sum, the environment simultaneously constrains and influences wildlife microbiomes, behaviours, and their interactions. Studies that transplant lab-based techniques to the field, while important in furthering understanding of mechanisms, do not adequately address environmental constraints on evolutionary responses and behavioural plasticity and, therefore, may generate unrealistic predictions. Studies that incorporate multiple dimensions of the environment, including environmental microbiomes, are vital for disentangling the environment–microbiome–behaviour links. Novel environments, such as cities, are promising sites for these studies due to their environmental variations, and the possibility of experimental manipulation of habitats and environmental microbiomes that could further improve predictions.

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Letter

Response to Nguyen *et al.* 'Laboratory-Inspired Manipulations Hold Value for Wild Microbiome-Behaviour Research'

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Identifying causal effects of the gut microbiome on host behaviour in wild vertebrates is no easy task, and our opinion piece offers observational, manipulative, and statistical tools to differentiate causal relationships from spurious ones [1]. Given the infancy and complexity of such research, we welcome dialogue on how best to progress this field. Nguyen *et al.* [2] argue that due to strong interdependence between the environment, the gut microbiome, and behaviour, field-based studies that explicitly consider the natural environment, particularly the 'environmental microbiome' (microbes in soil, water, etc.) are vital to understand microbiome–behaviour links. The authors state that our examples of seasonal and dietary variations are too restrictive and the manipulative techniques we suggest are too artificial to generate realistic predictions about the gut microbiome's role in behavioural plasticity. We agree that understanding the nature of environment-driven microbiome differences is fundamental to generating meaningful hypotheses about microbiome-mediated behavioural plasticity. However, while the environmental microbiome can have prominent effects on animal gut microbiomes, there is currently insufficient evidence to suggest studies outside a natural context cannot generate ecologically valid inferences. Moreover, we view the question of the environment's role in shaping animal gut microbiomes and behaviour as related to, but distinct from that of understanding the microbiome's role in shaping behaviour. Studying the causes and consequences of variation in the same trait (here, the microbiome) separately can be a valid and informative approach. We maintain that experiments which directly manipulate the gut microbiome can provide the most compelling evidence that these microbial communities, rather than independent effects of the environment, causally affect behaviour.

Methods that enable researchers to unpick the microbiome's specific role in generating behavioural variation, a key aim in our original article, can be adopted in the context of any relevant biotic or abiotic environment that can change the microbiome. We used season and diet as illustrative examples because they are environmental factors well known to influence both behaviour [3] and the microbiome [4,5]. We welcome Nguyen *et al.*'s [2] suggestion of urbanisation as another valuable environmental context in which to investigate microbiome–behaviour links, which can capitalise on large-scale 'natural experiments' and help elucidate the microbiome's potential significance in organismal responses to important gradients of anthropogenic change. We agree that appropriate statistical tools (such as those described in [1]) will be critical when attempting to infer the microbiome's role in any patterns observed.

However, even the best designed environmental manipulations aiming to uncover the microbiome's effect on host behaviour will likely fall short of robust causal evidence, because they are indirect. If an environmental factor is manipulated and both the microbiome and behaviour respond, our ability to infer that the microbiome mediates behavioural change relies on there being sufficient variability in the microbiome response, covariation between this response and behavioural change, and enough statistical power to detect this. It is for this reason that we suggested adapting inferential tools from laboratory-based microbiome science, especially direct methods of microbiome manipulation like transfer experiments, when exploring the microbiome's significance for behavioural variation in wild vertebrates. An example from our own recent work illustrates the value, and limitations, of indirect microbiome manipulations. An environmental (diet) manipulation on wild-caught great tits (*Parus major*) in winter revealed that an all-insect diet reduced gut microbiota diversity and problem solving performance (PSP) and that higher alpha diversity correlated with better PSP [4]. While these findings are consistent with the idea that ecologically relevant (in this case seasonal) diet-induced microbiome changes might modulate PSP, diet could also be altering PSP through other mechanisms. Follow-up direct microbiome manipulations of the sort we suggested offer a powerful way to test the microbiome's precise role in these effects.

It is undeniable that direct, meaningful manipulation of the microbiome in non-model organisms and natural populations presents a significant challenge, even more so when trying to measure behaviour in an ecologically relevant way. Direct manipulations could conceivably be field-based such as administering antibiotics or a microbial supplement to wild animals, either actively or passively through computerised 'selective feeders' that restrict access to particular individuals [6]. But researchers may also choose a hybrid research model that combines field and laboratory-based approaches. We do not perceive the addition of 'artificial' manipulations in captive settings as undesirable if the advantage is improved causal inference. Indeed, even the example cited by Nguyen *et al.* [2] to illustrate how the natural environment affects both the microbiome and behaviour was carried out in captivity [7].

Box 1. The Value of Captive Experiments C for Research on Adaptive Phenotypic Plasticity NP

If we turn to the related field of developmental plasticity for inspiration, both field- and laboratory-based studies have made complimentary contributions to identifying how the natural environment affects animal phenotypes, the adaptive value of plasticity across environmental gradients, and its underlying biological mechanisms [8,9]. One specific example comes from a long-term study of free-living song sparrows (*Melospiza melodia*). Glucocorticoid stress responses were found to correlate with both song complexity and fitness in the wild (see [10] and references therein). Complimentary studies in the laboratory then manipulated developmental stress both indirectly (using food restriction) and directly (through corticosterone administration), confirming that developmental stressors are capable of generating covariation between glucocorticoid levels and song complexity, as observed in the wild [10]. Laboratory work further revealed how and when developmental stress impaired specific brain regions associated with song learning, while fieldwork revealed that fitness effects associated with stress responses were dependent on environmental variability [10]. Thus, an approach that hybridises field studies and laboratory-based experiments can assemble more pieces of a research puzzle, to provide a more complete view of how a given process might operate in natural settings. Granted, the microbiome is a living community that is more challenging to manipulate than hormone levels, but ultimately, we cannot know whether microbiome–behaviour experiments in captivity might miss critical elements of what is happening in natural settings or not, until we try them.

Many fields have benefitted from combining laboratory and field-based approaches to answer key questions (for an example, see Box 1). Rather than viewing field- and captivity-based studies as competing options, we suggest they offer researchers alternate ways to make different, complimentary inferences. Overall, our view is that a multipronged approach, involving both field-based studies and more controlled experiments in captivity will maximise our ability to understand microbiome-mediated behavioural plasticity in the natural world.

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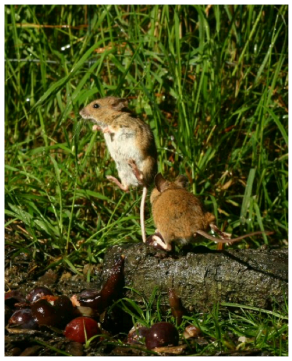
Appendix S1.2. Additional photo-evidence of wood mouse social behaviour

As part of this work, it was also found that a previously undocumented form of social behaviour in wood mice is flying or hovering in the air.






2

Social networks strongly predict the gut microbiota of wild mice

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List of contributions

- *Aura Raulo* designed the study questions, completed all laboratory analyses on gut microbiota profiling prior to sequencing, developed analytical methods, analysed the data and wrote the manuscript
- *Bryony Allen* helped collect and clean the tracking data set from Silwood
- *Tanya Troitsky* completed the genotyping of the mouse population and provided feedback on the manuscript.
- *Arild Husby* supervised and helped with the genotyping of the mouse population and provided feedback on the manuscript.
- *Josh Firth* supervised the research project, developed social network analysis methods and provided feedback on the analyses and the manuscript
- *Tim Coulson* supervised the research project and provided feedback on the analyses and the manuscript
- *Sarah Knowles* supervised the research project, helped develop the tracking technology and design the study, collected data from Silwood, planned and supervised laboratory methods, developed analytical methods and provided feedback on analyses and the manuscript.

Abstract

The mammalian gut teems with beneficial microbes, yet how hosts acquire these symbionts remains poorly understood. Research in primates suggests that microbes can be picked up via social contact, but the role of social interactions in non-group-living species remains unexplored. Here, we use a passive tracking system to collect high resolution spatiotemporal activity data from wild mice (*Apodemus sylvaticus*). Social network analysis revealed social association strength to be the strongest predictor of microbiota similarity among individuals, controlling for factors including spatial proximity and kinship, which had far smaller or nonsignificant effects. This social effect was limited to interactions involving males (male-male and male-female), implicating sex-dependent behaviours as driving processes. Social network position also predicted microbiota richness, with well-connected hub individuals having the most diverse microbiotas. Overall, these findings suggest social contact provides a key transmission pathway for gut symbionts even in relatively asocial mammals, that strongly shapes the adult gut microbiota. This work underlines the potential for individuals to pick up beneficial symbionts as well as pathogens from social interactions.

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ARTICLE

Social networks strongly predict the gut microbiota of wild mice

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Abstract

The mammalian gut teems with microbes, yet how hosts acquire these symbionts remains poorly understood. Research in primates suggests that microbes can be picked up via social contact, but the role of social interactions in non-group-living species remains underexplored. Here, we use a passive tracking system to collect high resolution spatiotemporal activity data from wild mice (*Apodemus sylvaticus*). Social network analysis revealed social association strength to be the strongest predictor of microbiota similarity among individuals, controlling for factors including spatial proximity and kinship, which had far smaller or nonsignificant effects. This social effect was limited to interactions involving males (male-male and male-female), implicating sex-dependent behaviours as driving processes. Social network position also predicted microbiota richness, with well-connected individuals having the most diverse microbiotas. Overall, these findings suggest social contact provides a key transmission pathway for gut symbionts even in relatively asocial mammals, that strongly shapes the adult gut microbiota. This work underlines the potential for individuals to pick up beneficial symbionts as well as pathogens from social interactions.

Introduction

Symbiotic microbes are increasingly recognised as key modulators of host phenotypes. This is particularly true for the mammalian gut microbiota, whose metabolism is intimately entwined with that of the host. Among their many roles in host physiology, mammalian gut microbes modulate host energy metabolism [1, 2], regulate fat accumulation and thermal homoeostasis [3], and provide protection against pathogenic infection [4, 5]. They are also in constant dialogue with the host immune system, activating innate immune responses and tuning acquired immune responses to distinguish enemies from allies [6–8]. As such, alterations to these microbial communities can have significant impacts on host health and have been associated with major metabolic and immune-related health conditions in humans [1, 9, 10].

Despite gut microbiota'swell-established role in host biology, we know surprisingly little about the forces that shape microbiota composition within and between individuals in nature. Community composition is notoriously variable among individuals, and is affected by a number of processes that can be viewed within a metacommunity framework [11]: transmission processes (microbial dispersal) first determine which microbes colonise an individual host. Subsequently, aspects of the nutritional and immunological environment inside the host (e.g. host diet, genetics), as well as ecological interactions with resident microbes, selectively filter colonising microbes that can persist and thrive. In mammals, the microbiota is initially established through maternal transmission at birth [12], with community composition then further shaped by transmission from family members and the broader environment [13–15] as well as selective processes within the host [16].

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A key question is to what extent ongoing transmission throughout life shapes the microbiota. Accumulating evidence suggests the gut microbiota is affected by a host's environment, such as diet [17, 18] and contact with soil [15, 19, 20]. The microbiota can also be shaped by a host's social environment, since a special form of microbial transmission can occur through social contact. Intimate social contact, such as the many forms of prosocial touch common in mammals (e.g. grooming, licking, huddling), may function as an important transmission route for microbes. This is particularly true for microbes not easily transmitted via the environment, including strict anaerobes and non-spore-forming bacteria [21]. Moreover, if less transmissible microbes are more likely to positively impact host fitness [22], social interactions could constitute a key pathway (alongside vertical transmission) by which symbionts of high functional significance are transmitted in mammals. Laboratory rodent studies have repeatedly shown that cohousing drives convergence in microbiota composition [23–25], indicating that social interaction and close proximity facilitate microbial transmission under captive conditions.

In highly social group-living mammals, the host social environment seems to have important effects on the gut microbiota. Social group membership has been shown to predict gut microbiota composition in several species of primates [26–31] and other group-living mammals [32–34]. Social group effects also occur in humans, as unrelated individuals living in the same household were found to have a more similar microbiota than relatives living in different households [35]. However, the mechanisms underlying these observations remain unclear, and may include not only direct social transmission but also shared environmental exposures like diet. In some cases, social group effects on the microbiota have been found while controlling for kinship or shared diet, supporting the idea that social transmission homogenises the gut microbiota. For example, sifakas (*Propithecus verrauxii*) were found to have a social group-specific gut microbiota composition that was not explained by diet similarity, or habitat overlap, nor genetic relatedness [28]. Further support comes from individuals observed to switch social groups, for example immigrant male baboons [36], whose microbiota composition converged on that of their new social group.

Some evidence also suggests social interactions affect microbiota similarity at a dyadic level, within groups or populations. Several primate studies have shown the intensity of social interaction between group members to predict similarity in their microbiota [26–28, 30]. Baboons that groomed each other more were found to share more gut microbes, and these shared bacteria were enriched in anaerobic and non-spore-forming taxa [26]. Similar patterns were found in humans, with couples who reported having a "physically close relationship" sharing more gut microbes than less close couples or friends [37]. However, socially interacting primates often experience strong overlap in their environments, and thus it remains difficult to distinguish social transmission from shared environmental exposures [21]. Species that are not group-living (sensu Wilson, [38]) arguably provide more powerful systems in which to clearly distinguish effects of social interaction from confounding shared environmental exposures, as social interactions are more limited in time and space. However, the role of social transmission in shaping the microbiota in such species has yet to be explored.

Here, we use wild mice as a model system (wood mice, *Apodemus sylvaticus*) to assess how social interactions shape gut microbiota similarity among sympatric individuals, in comparison to effects of host kinship, spatial proximity, and other factors. These mice are not group-living, but can be considered a semi-social species, with the propensity to co-nest in underground burrows varying seasonally and among individuals [39, 40]. Individuals have stable, partially overlapping home ranges, and yet vary in their level of social contact, making them a particularly suitable species in which to study social transmission. Social behaviours that might facilitate gut microbe transmission in wood mice include contact behaviours such as allogrooming [41], huddling [42], nest-sharing [40] or aggressive interactions [41]. Transmission could also occur through non-contact social behaviours, such as scent-marking and investigation of faecal cues [43, 44], or possibly coprophagy as seen in other mouse species, though coprophagy has not been documented in wood mice to date. Using a tracking system based on passive radio-frequency identification (RFID) tags, we intensively followed a population of mice for one year and used social network analyses to test two specific hypotheses about social transmission of microbiota. First, we test the prediction that if social interactions drive microbial transmission, dyadic microbiota similarity will be positively predicted by proximity in the social network, independent of other potential confounders. Second, individuals that are more connected in the social network are predicted to have higher microbiota diversity, as they are exposed to more extensive social transmission.

Materials and methods

Field data collection

Data were collected over a one-year period (Nov 2014–Dec 2015) from a wild population of wood mice (*Apodemus sylvaticus*) in a 2.47 ha mixed woodland plot (Nash's Copse) at Imperial College's Silwood Park campus, UK

(Fig. S1A). Live traps were set for one night every 2–4 weeks in an alternating checkerboard design, to ensure even coverage. At first capture, all mice were injected subcutaneously with a passive integrated transponder tag (PIT-tag) for permanent identification. At each trapping, demographic data on captured animals was recorded and samples for gut microbiota analysis and mouse genotyping collected (see Appendix 1 in Supplementary material).

Data on rodent space use and social associations was collected in parallel to trapping using a set of nine custom-built PIT-tag loggers (described in [45] and Appendix 2; Figs. S1), distributed across the trapping grid. Loggers consisted of a box with entrance tubes, that recorded the time-stamped presence of any rodent that entered. Loggers were rotated systematically around the plot throughout the study period, using a sampling design that ensured even spatial coverage, with each 100 m^2 grid cell covered on average 5.49 (SD 1.61) times (Fig. S1C). Between logging nights, loggers were thoroughly cleaned with 70% ethanol (see Appendix 2). After data cleaning and filtering (Fig S2), 83 of the 93 mice tagged during study period were present in the logger data.

Kinship analysis

To derive estimates of host genetic relatedness, ear tissue samples were used to genotype all captured mice at eleven microsatellite loci (Tables S1 and S2; detailed in [39]) and build a pedigree in COLONY 2.0.6.5 [46]. Full details of genotyping methods and pedigree reconstruction are provided in Appendix 3. After sample failures, genetic relatedness could be inferred for 70 of the 83 monitored mice.

Constructing social networks

All analyses were conducted in R version 3.6.1 (R-Core-Team 2019). To capture patterns of spatiotemporal coincidence among wood mice, social networks were constructed from logger data using the package *asnipe* [47] and plotted using *igraph* [48]. Individual mice were nodes, and edges described the number of instances two individuals were observed associated, i.e. observed at the same logger during the same night (12 h period, 6 pm to 6 am), within a specific time window of each other. To measure association strength, we used an adjusted version of the Simple Ratio Index (SRI), that accounted for variable overlap in individual lifespans (i.e. time between first and last logger observation) [49], hereafter "Adjusted SRI". Adjusted SRI is defined as follows for two individuals, A and B:

$$I = \frac{X}{[X + y_{AB} + y_A + y_B]}$$

where X is the number of instances (night-location combinations) in which A and B were observed associated (at the same location within a specified time window of each other), y_{AB} is the number of instances in which A and B were both observed, but not associated, y_A and y_B are the number of instances in which both were known to be alive but only A or B were observed respectively. By taking lifespan overlap into account we could incorporate data from all 83 individuals across the entire year into one static social network.

To examine how the definition of social association might affect social network-microbiota relationships, we constructed a series of networks using increasingly intimate definitions of social association, by applying a sliding time window criterion of variable length to define social association (i.e what counts as X in the formula), from 12 h (as above) down to a 2 min period (12 h, 4 h, 1 h, 30 min, 10 min, 2 min). We also calculated a parallel set of networks with binary social association indices (BI), where '1' indicated the dyad were observed associated at least once, and '0' indicated they were not.

Gut microbiota characterisation

The gut microbiota was successfully characterised from 239 faecal samples belonging to 75 individual wood mice (covering 90% of the monitored mice, mean = 3.2 samples/mouse, range = 1–9). Full details of library preparation, sequencing and bioinformatics are given in Supplementary Material (Appendix 4; Figs. S3–S5). Briefly, microbiota profiling involved amplicon sequencing of the 16S rRNA gene (V4-region). Sequence data were processed through the DADA2 pipeline v1.6.0 [50], to infer amplicon sequence variants (ASVs) and taxonomy assigned using the GreenGenes Database (Consortium 13.8). Using package *phyloseq* [51], ASV-counts were normalised to proportional abundance within each sample [52] and singleton ASVs as well as those belonging to non-gut microbial taxa (Cyanobacteria, Mitochondria) were removed. Lastly, we used package iNEXT [53] to estimate asymptotic richness and Shannon diversity for each sample.

Statistical analyses

To describe compositional microbiota variation, package *vegan* [54] was used to calculate Jaccard distances and Bray–Curtis dissimilarities among samples (Fig. S6). We used the Jaccard Index (1-Jaccard distance, the proportion of shared ASVs between sample pairs) as our primary measure of microbiota similarity, as we considered this metric most relevant for investigating microbial transmission among hosts. However, for robust inference, we repeated key analyses using Bray–Curtis dissimilarity (an

abundance-weighted metric less sensitive to potential sequencing artefacts), and repeated analyses on both Jaccard Index and Bray–Curtis dissimilarity with a rarefied dataset, to confirm beta diversity results were not affected by read depth variation among samples.

General predictors of gut microbiota composition

We performed permutational analysis of variance (PERMANOVA) in *vegan* to (1) test the repeatability of gut microbiota composition among individuals sampled multiple times, (2) identify non-social effects on the microbiota to be controlled for in subsequent analyses and (3) estimate how much individual variation was independent of these covariates. We tested effects of time (month, as a factor), host age (juvenile/adult), sex, plot region, habitat type, and individual identity on Jaccard distance. Plot region and habitat type for each individual were defined from logger data, as the most common logger territory (no.1–9) and habitat type (rhododendron, open woodland/bluebell, bamboo or mixed; Fig. S1A) they were detected in.

Associations between social association strength and microbiota similarity

To test whether dyadic microbiota similarity was predicted by social association strength, we performed Bayesian regression models in package *brms* [55]. These models are well-suited for this as they permit random effect structures able to account for the types of dependence inherent to dyadic data, and repeat sampling of individuals [56]. We constructed *brms* models that included all dyadic sample comparisons except within-individual comparisons. Microbiota similarity (Jaccard Index) was used as the response, with social association strength (adjusted SRI, or BI index) as the main predictor. As the Jaccard Index is a proportion, a logit link function was used. To control for potential confounding variables as far as possible, we fitted several dyadic covariates: spatial distance between hosts, sampling interval (time in days between samples taken), kinship, sex and age similarity (0/1 for different/same). Spatial distance was calculated as the distance between individuals' mean spatial coordinates from logger records (minimum 34 logger records per mouse). All covariates either naturally ranged from 0 to 1 or were scaled to do so, to make model estimates for all terms comparable. To control for non-independence in the dataset arising from a dyadic response variable and repeat samples per mouse, both the model intercept and slope (social association strength effect) were allowed to vary as defined by two random effects: (i) a multi-membership random effect capturing the individuals in each dyad (Individual A + Individual B) and (ii) a multi-membership random effect capturing the samples in each dyad (Sample A + Sample B).

To test for sex-dependence in the effect of social association (e.g. arising from specific sexual behaviours) on the microbiota, the main model (12 h edge definition) was also run including dyad sex category (male-male, male-female or female-female) and its interaction with social association strength. In this model, only a multi-membership random intercept was fitted (not a random slope) to help ensure there was enough power to estimate the interaction effect. Finally, to check our results were robust to the chosen statistical approach, we confirmed key results with two alternative statistical modelling frameworks: (1) *MCMCglmm*, an alternative R package for Bayesian regression [57] and (2) a matrix permutation-based method common in social network analyses, Multiple Regression Quadratic Assignment procedure (MRQAP; [58]), with a data subset including one randomly selected sample per individual (Appendix 5; Fig. S7).

Social network position and microbiota diversity

We hypothesised that an individual's social network position might affect gut microbiota (alpha) diversity. Depending on the transmission ecology, different types of network position might best predict diversity. To explore this, we calculated six different metrics of network position, that capture different aspects of social connectedness (Fig. 1). If the sheer amount of social interaction or number of social partners can diversify the microbiota, we expect diversity to be predicted by measures of general network centrality (Fig. 1). Alternatively, if diversity is driven by the distinctness of transmission sources, and if this is reflected in their social distance, we expect diversity to be predicted by measures of bridge-type centrality (Fig. 1). To test the relationship between each centrality measure and gut microbiota diversity, we used Bayesian regression models in *MCMCglmm* with either asymptotic ASV richness or asymptotic Shannon diversity as the response. We first explored how several covariates predicted diversity: host age, sex, sampling month (as a factor), plot region, habitat type, read count, and PCR plate (4-level factor), and simplified models to include only covariates with $p < 0.1$. We then added into this model one of our six measures of social centrality (Fig. 1), derived from either the 12 h or 2 min network. Individual identity and PCR plate were fitted as a random factors. A node permutation test was used to verify that significant effects were not driven by network structure. The observed posterior mean estimates for network position were compared with those derived from 1000 models in which network positions were randomised across individuals.

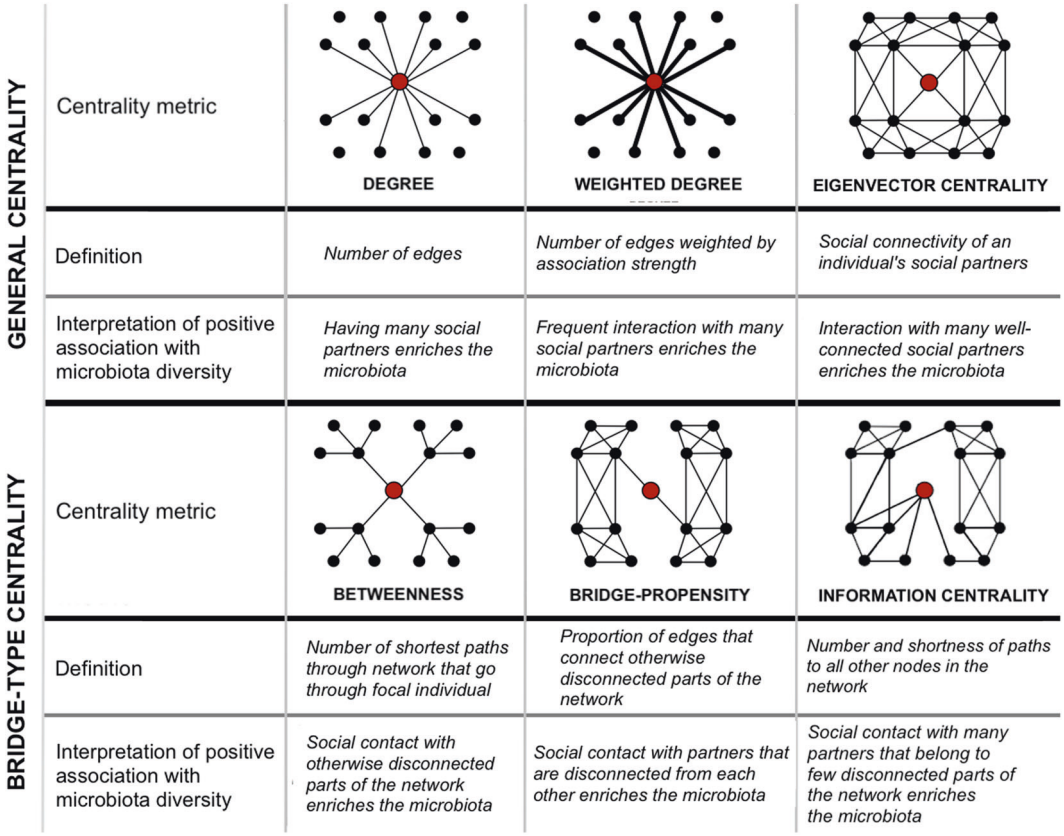

Fig. 1 Six measures of network centrality and interpretation of a positive relationship with microbiota diversity. Images depict focal individuals (red circles) whose social interactions (lines) with other individuals (black circles) give them a high value of each centrality metric.

Identifying which bacterial taxa associate with social interaction

To identify candidate socially transmitted bacterial taxa, we tested how each bacterial family affected the strength of correlation between social association strength and microbiota similarity. We recalculated the Jaccard Index excluding each bacterial family in turn, then compared (both 12 h and 2 min) social network effect sizes and credible intervals from MCMCglmm models using these indices (full model details in Appendix 5).

Results

Factors predicting gut microbiota composition

The mean Jaccard Index across the whole population of mice was 0.17 (sd = 0.6). In a marginal PERMANOVA on data from repeat-sampled mice, individual identity explained 33% compositional variation in the microbiota, while temporal fluctuations (month) explained 6%, with similar results for both Jaccard Index and Bray–Curtis dissimilarity (Table S3, See Fig. S8 for more thorough description of the temporal fluctuations). When other individual-level attributes were included (age, sex, plot region and habitat type), 27% variation in microbiota composition remained attributable to individual identity (Table S4), indicating the microbiota showed consistent individual variation that was not explained by measured host factors. No other variables predicted microbiota composition, except for a weak effect of habitat type (marginal PERMANOVA on data with one sample per individual, Table S5). Among the subset of hosts (70 of 75) with kinship information, kinship and microbiota similarity (Jaccard Index) were unrelated (Mantel test: $r = 0.001$, $p = 0.520$).

Wood mouse social structure

The wood mouse social network showed marked variation in edge weights (social association strength) but no clear clustering (Fig. 2). Social association strength did not differ significantly among female-female, female-male and male-male pairs (Table S6). Global network density declined as increasingly intimate edge definitions were used (Fig. 2). The correlation among social networks with different edge definitions also decayed as the difference in time windows

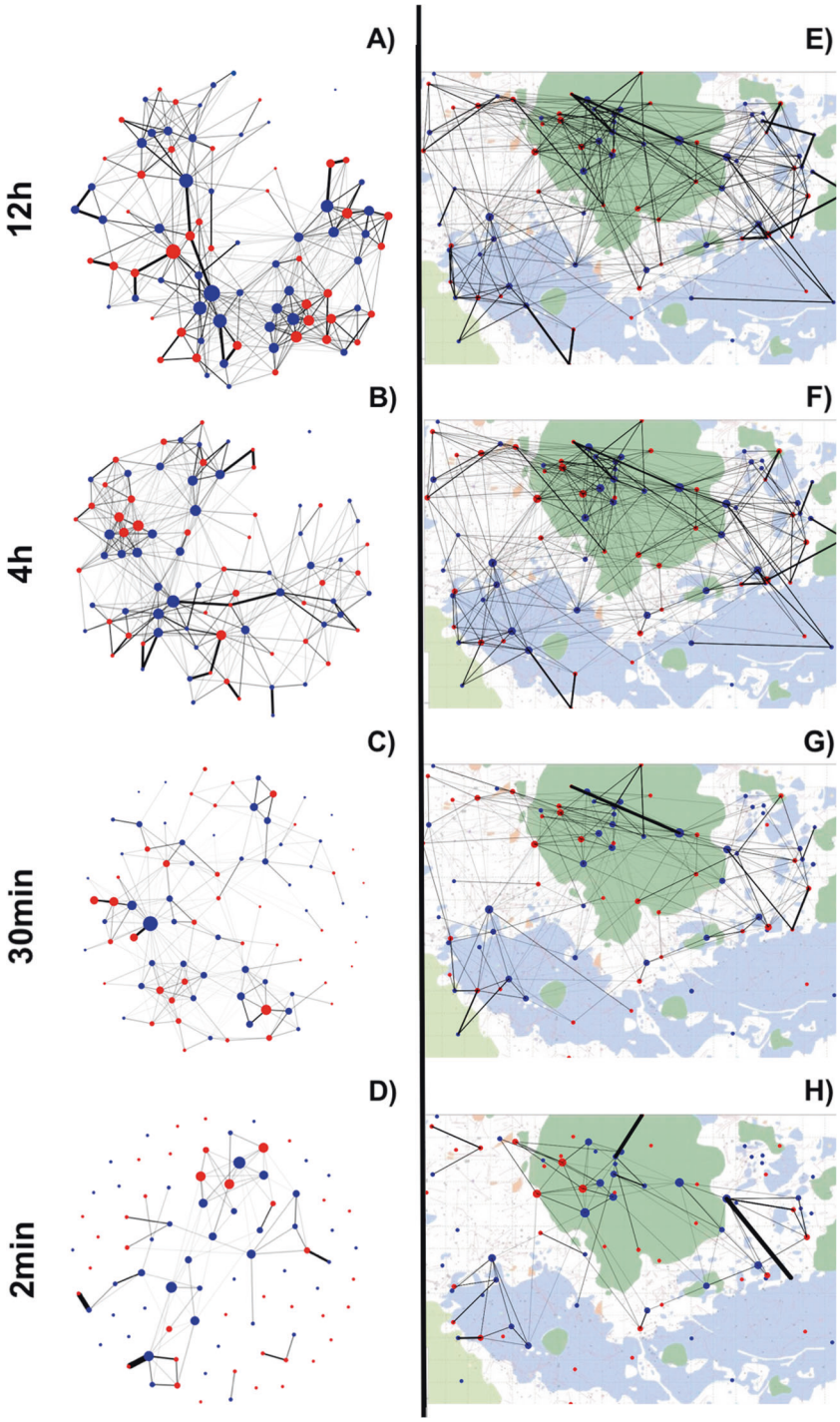

Fig. 2 Wild wood mouse social networks with different edge definitions (2min-12h), plotted in either social space (A–D) or geographical space (E–H). In **A–D** networks are plotted using a standard weighted spring layout that minimises the sum of edge lengths and overlap across the network (*igraph*, [43]), and in **B–H** mice are positioned at their mean spatial coordinates recorded from logger data, superimposed on a habitat map of the study area. Background colours reflect habitat types (dark green = rhododendron, light green = bamboo, blue = bluebell, white = open woodland). Red and blue circles represent female and male mice respectively, and line thickness is proportional to social association strength.

increased (Table S7). As expected, social association strength was to an extent predicted by spatial proximity in all networks (MRQAP $p < 0.001$, Table S6), though this spatial effect weakened as more intimate edge definitions were used (Fig. S9, Table S6). Even in the least intimate (12 h) social network, mice clearly did not solely associate

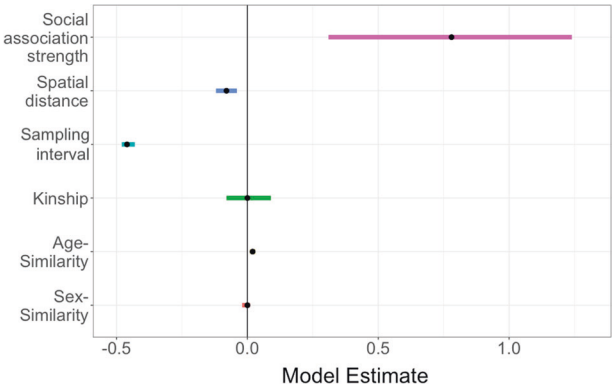

Fig. 3 Social association strength predicts gut microbiota similarity more strongly than spatial distance, kinship and other effects. Effect size estimates (points) and their 95% credible intervals (coloured lines) are plotted from Bayesian regression (*brms*) models with pairwise microbiota similarity among hosts (Jaccard Index) as the response. Where credible intervals do not overlap zero, a variable significantly predicts microbiota similarity. Social association strength in the 12 h network has a strong positive effect on microbiota similarity, that is larger than that of other variables.

with their nearest neighbours, as distances to the closest social partner (mean 25.6 m; sd = 15.3 m) were on average over three times greater than those to the nearest neighbour (mean = 8.4 m; sd = 5.5 m). Some strong social associations were observed between individuals whose mean spatial locations were over 60 m apart (Fig. 2E–H). As such, the social structure of this population was only partially determined by spatial location, and this spatial influence on social contact was weakest in the 2 min network.

Social association strength predicts microbiota similarity

Among pairs of individuals, the strength of social association strongly and positively predicted similarity in gut microbiota composition (in 12 h network: posterior mean 0.78, CI = 0.34–1.24; Fig. 3). Specifically, the proportion of ASVs shared within dyads (Jaccard Index) was positively predicted by their social association strength in all networks, even when controlling for effects of sex, age, kinship, sampling interval, and spatial distance (Table S8). Other variables also predicted microbiota similarity, including the spatial distance between hosts (posterior mean −0.08, CI = −0.12 to −0.04) and the time interval over which they were sampled (posterior mean −0.46, CI = −0.48 to −0.43), but the size of these effects was consistently smaller than that of social association strength (Fig. 3, Table S8). Consistent results were obtained with models using alternative statistical frameworks, when using Bray–Curtis dissimilarity, and when using indices derived from a rarefied microbiota ASV dataset (Appendix 5; Tables S9 and S10). Even binary social networks predicted microbiota similarity (Table S11), albeit less strongly than association strength.

The relationship between social association strength and microbiota similarity became stronger as networks with increasingly intimate edge definitions were analysed (Fig. 4A), while spatial and temporal effects remained comparable across networks (Table S8). As such, the effect of social association increased from 1.7 times as large as the next strongest (sampling interval) effect in the 12 h network, to over 13 times as strong in the most intimate (2 min) network. Accordingly, the mean predicted Jaccard Index among mice with a weak (0 ≤ Adjusted SRI < 0.1) vs. strong (SRI > 0.9) level of social association increased modestly from 0.23 to 0.30 in models using the 12 h network, but approximately doubled from 0.29 to 0.59 in models using the 2 min network (Fig. 4C). Since more intimate networks also had fewer edges (i.e. lower density, Fig. 2), we also tested whether variation in network density alone might drive this trend. To do this, we ran a set of null models (described fully in Appendix 6) in which the least intimate (12 h) network was thinned to have the same number of edges as seen in each real network. In contrast to the real networks, social network effect sizes remained relatively constant in null models using artificially thinned networks (Fig. 4B).

Sex-dependent effects of social association on microbiota similarity

We further found that the effect of social association strength on microbiota similarity depended on the sex of interacting individuals. In a model including an interaction between social association strength and dyadic sex combination, social association strength predicted microbiota similarity strongly in male-male pairs (posterior mean 0.28, CI = 0.01–0.56; Table S12) and male-female pairs (posterior mean 0.30, CI = 0.04–0.56) but not significantly in female-female pairs (posterior mean 0.10, CI −0.15 to 0.35; Fig. 5, Table S12).

Social network position and microbiota diversity

Both microbiota diversity metrics (richness and Shannon diversity) were predicted by plot region, habitat type, and month (Table S13). Both diversity estimates were also associated with PCR plate, and richness was also predicted by read count. Four measures of network position positively predicted gut microbiota richness: degree and information centrality predicted richness in both 12 h and 2 min networks, and betweenness and bridge propensity additionally predicted richness in the 2 min network (Table 1). No measures of network position predicted Shannon diversity when controlling for covariates (Table S14).

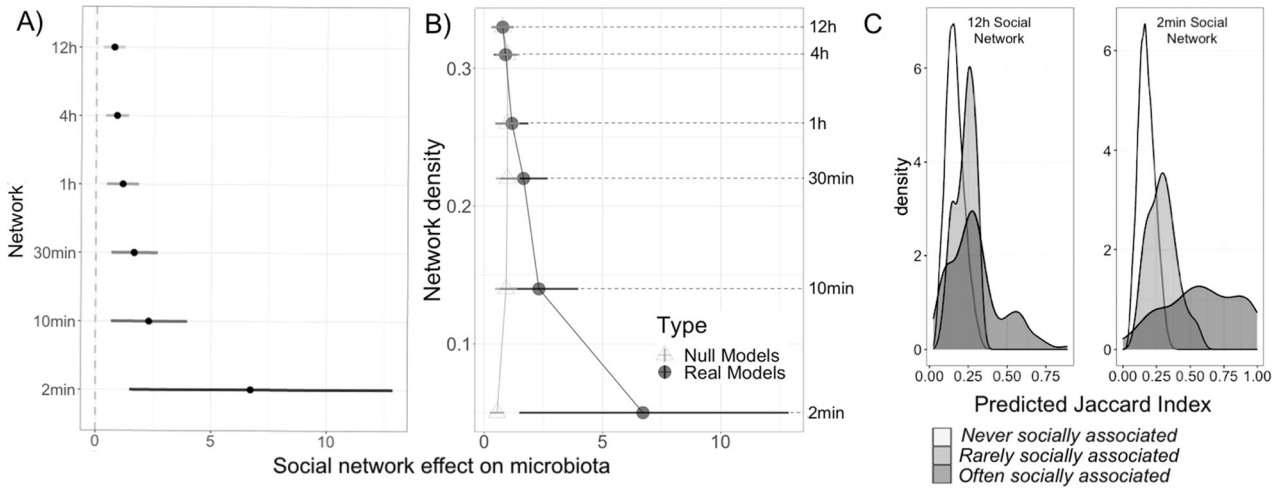

Fig. 4 Social association strength predicts microbiota similarity more strongly in networks that use a more intimate edge definition. **A** The effect of social association strength on microbiota similarity (Jaccard Index) is stronger in networks with more intimate edge definitions. Social network effect sizes (estimated slope of the relationship between social association strength and microbiota similarity, the Jaccard Index) and their 95% credible intervals are plotted from Bayesian regression (*brms*) models that included the same covariates shown in Fig. 3. **B** Differences in effect size across networks are not due to variation in network density, as effect size did not change in null models where the 12 h network was artificially thinned by removal of the weakest edges to have the same density as each real network of differing edge definition. **C** The distribution of predicted microbiota similarity (Jaccard Index) values in pairs of mice who were observed socially associated either never (white, SRI = 0), rarely (light grey, $0 < SRI \leq 0.1$) or often (SRI > 0.9, dark grey) in either 12 h or 2 min social networks (columns).

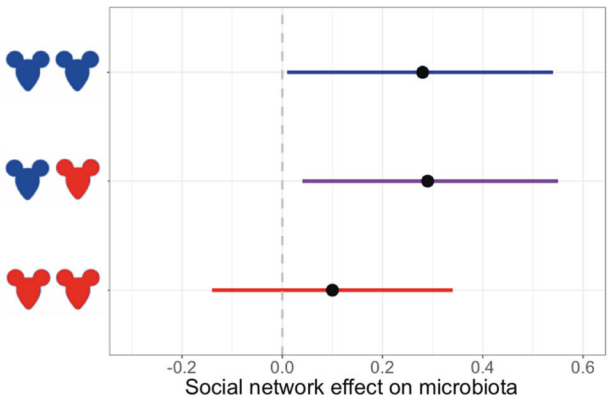

Fig. 5 Social association strength predicts microbiota similarity only among dyads involving males. Estimated social network effects on the microbiota (slope of the relationship between social association strength and Jaccard Index) and 95% credible intervals are plotted from a Bayesian regression (brms) model using the 12 h social network that included an interaction term between social association strength and dyad sex-category (male-male, male-female or female-female). Females are depicted in red and males in blue respectively. Social association strength has a significant positive association with microbiota similarity in dyads involving males, but not in female-only dyads.

Identifying bacterial taxa that drive social network effects

The social network effect we identified did not depend entirely on any single bacterial family, since it remained statistically significant in all models where a single bacterial family was excluded (Fig. 6). For some of the more diverse bacterial families, effect size did shift slightly when they were excluded, but not in a way that directly related to their diversity. Excluding the family S24-7 made the social network effect somewhat weaker and almost nonsignificant when using the most intimate (2 min) edge definition (taking the p.MCMC-value from $p < 0.001$ to $p = 0.012$), a pattern that was similar but weaker in the 12 h network. Conversely, excluding Lachnospiraceae, the most diverse family, if anything slightly strengthened the social network effect in both networks (Fig. 6). Excluding Lactobacillaceae also slightly weakened the social network effect size, but only when using the less intimate (12 h) edge definition.

Discussion

Recent studies have shown that the social environment can strongly affect gut microbiota composition in group-living species, such as primates living in large groups [26, 29] or smaller family units [27, 28, 30]. Here, we provide the first evidence for similar effects in a non-group-living species. The social network of wood mice showed no clear clustering, as those of group-living species do. Yet, the social network strongly predicted similarity among individuals in gut microbiota composition, and this effect was far stronger than effects of spatial or temporal proximity, kinship, and similarity in other host attributes (age, sex). In short, mice who were observed at the same location within the same short timeframe, shared more gut bacterial taxa than mice who were observed

Table 1 Social network centrality metrics predict individual gut microbiota richness.

	12 h network			2 min network		
	Posterior mean (95% CI)	p.MCMC	p.perm	Posterior mean (95% CI)	p.MCMC	p.perm
Degree	**0.005 (0.001, 0.009)**	**0.042**	0.004	**0.02 (0.001, 0.041)**	0.042	0.004
Weighted degree	0.038 (−0.097, 0.180)	0.556	n/a	−0.012 (−0.157, 0.118)	0.832	n/a
Eigenvector centrality	0.119 (−0.010, 0.262)	0.092	n/a	−0.0073(−0.147, 0.139)	0.968	n/a
Betweenness	0.008 (−0.087, 0.109)	0.866	n/a	**0.018 (0.004, 0.033)**	0.016	0.002
Information centrality	**0.017 (0.001, 0.035)**	**0.050**	0.004	**0.021 (0.004, 0.039)**	0.024	0.004
Bridge propensity	−0.007 (−0.236, 0.189)	0.500	n/a	**0.017 (0.002, 0.031)**	0.020	0.004

Posterior means and 95% credible intervals are shown from MCMCglmm models including the covariates shown in Table S13 and a single centrality metric. Significant effects are shown in bold. Significance was inferred from two *p* values: If the Bayesian model *p* value calculated from posterior distribution (p.MCMC) < 0.05, the result was further tested by calculating a permutational *p* value (p.perm). p.perm represents the probability of generating the observed posterior mean given the data, based on 1000 node-based permutations in which the centrality values of nodes are randomly shuffled before running the model.

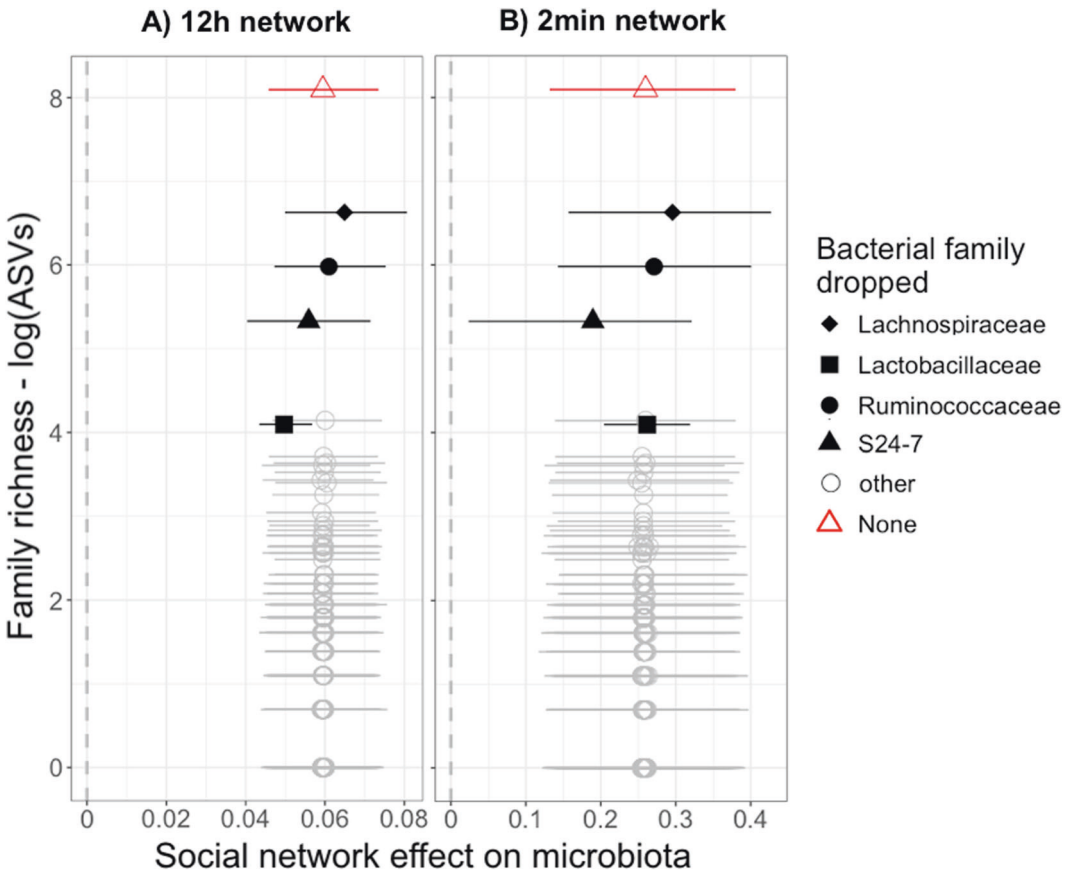

Fig. 6 The influence of specific bacterial families on social network effect size. Social network effect sizes (slope of the relationship between social association strength and microbiota similarity, Jaccard Index) and 95% credible intervals are plotted from 146 Bayesian regression (MCMCglmm) models, in which a single bacterial family was excluded from the calculation of microbiota similarity. Effects are plotted against the species richness of each dropped family (logged number of ASVs, *y*-axis). Results are shown from models using **A** the least intimate, 12-h network and **B** the most intimate, 2-min network.

together less often. This social effect was sufficiently strong that mice who were observed together even once shared more bacterial taxa than mice who were never observed together. This co-occurrence can be seen as a proxy for a more nuanced social relationship, perhaps involving close physical contact behaviours like allogrooming or huddling, that serve as routes for social microbiota transmission.

Social effects on the microbiota can result from social partners having more similar environmental exposures, and previous studies have struggled to separate such influences

from the effect of social transmission. Here, several findings suggest the social effect we see is likely driven by social transmission rather than shared exposures. First, we find a strong social network effect even when controlling for host spatial and temporal proximity as well as kinship, reducing the likelihood it is driven by shared traits or exposure to microbes from common environmental sources, such as diet or soil. Second, more intimate definitions of social association (mice co-occurring within a two-minute period, rather than simply during the same night) predicted microbiota similarity more strongly, suggesting close interaction between hosts is important in driving the effect. Finally, the strength of the social network effect varied according to which bacterial families were included in the analysis, in ways that are consistent with a social transmission explanation. When members of the anaerobic, non-spore-forming bacterial family S24-7 (Bacteroidales, Muribaculaceae; [59]) were excluded, the social network effect weakened. Conversely, when members of the spore-forming family Lachnospiraceae were excluded (which are able to survive outside the host and have been found in soil; [60, 61]), the social network effect became slightly stronger. These observations suggest that microbial transmission during close host contact is an important driver of the social effect, allowing hosts to share microbes that cannot persist in the external environment. Previous work in hominids has also shown high host fidelity and even cospeciation with the host among members of the Bacteroidales, while Lachnospiraceae members showed low host fidelity and frequent host switches [62]. Taken together, these findings are consistent with the idea that microbes unable to persist outside the host are more reliant on transmission by close contact (e.g. social behaviour or birth), and perhaps in part because of this, may evolve increased host specificity.

Besides shared environmental exposures, another factor that could have contributed to the social effect we observe here is "artificial" transmission of microbes at the logger boxes we used to monitor mouse behaviour. While impossible to rule out entirely, we think logger contamination is unlikely to have played a significant role in generating the social effects observed here, for several reasons. First, logger boxes were thoroughly sterilised between logging nights (see Appendix 2). Second, our data suggested mice did not spend long periods of time in logger boxes (mean minutes logged per mouse per night when observed was 3.8 +/− sd 2.1), nesting inside loggers was never observed, and faecal pellets inside were also rare. Finally, and importantly, the observed effect of social association on the microbiota was sex-dependent, which is inconsistent with transmission occurring solely at loggers. Specifically, social association strengths derived from logger co-occurrence data were similar in magnitude for all pair types, yet only significantly predicted microbiota similarity for dyads involving males, but not female-female pairs. This implies that the effect of social association on microbiome similarity is driven by behaviours outside logger boxes that are differentially expressed between the sexes.

Indeed, our finding of a sex-dependent effect indicates the link between social interactions and the gut microbiota might be more nuanced than previously thought. It suggests that behaviours which vary in type, frequency or strength according to the sex of social partners are involved in gut microbial transmission. In wood mice, home range overlap is much greater among male-female and male-male dyads than among female-female dyads [39, 63] and observations in captivity suggest allogrooming may be more common between males and females [41]. Limited data has also suggested that co-nesting may be more common for male-female than same-sex pairs [40]. Female wood mice are therefore expected to socially interact with one another less often, and female-female links in our social networks may reflect actual social contact to a lesser extent than male-female and male-male links. In line with our findings, a recent study found that interactions involving males were more important for the transmission of a herpesvirus pathogen in wood mice [64]. It is therefore possible that in this species, the spread of infectious agents more broadly is dominated by interactions involving males. Our findings seem to constitute a mirror image of the common trend in primates, where female-female social bonds are often physically closer than male-male bonds [65], and where social interactions among females have been shown to predict microbiota similarity more strongly than those among males [66, 67]. In pair-bonding species like humans, the strongest microbiota-homogenising effects of social interaction may occur in close sexual relationships [37]. Interestingly, in wood mice (which do not pair-bond), we find no evidence that male-female associations predict microbiota similarity more strongly than male-male associations. This might be because sexual relationships are not well-captured by our measure of social association, or because other social behaviours prevalent among males are more important in transmission of gut microbes than behaviours specific to mixed-sex pairs.

In addition to social contact homogenising the gut microbiota, we also found that the diversity of an individual's microbiota is predicted by their position in the social network. Individuals with a central position in the social network, particularly with many contacts or in positions that bridged different parts of the network, carried more bacterial taxa in their gut. Of all network metrics, the strongest predictor of microbiota richness was the number of others an individual was connected to in the network (their degree). Similar trends were previously reported in sifakas [28] and chimpanzees [29], and humans self-reporting more social relationships also had greater gut microbial diversity [67]. However, effects in the opposite direction have also

been found. In barn-swallows, the extent of same-sex social interaction was negatively correlated with microbiota diversity [68] and in red-bellied lemurs, the most sociable individuals had the lowest gut microbiota diversity [27]. Perhaps a more careful consideration of social connectedness patterns may help in understanding how sociability might shape microbiota diversity. For example, the sheer amount of social interaction (the definition of sociability in [27]) might be less important in diversifying the microbiota than the number of transmission sources (the definition of sociability in [28]). We find that social interactions predict both alpha- and beta-diversity of the gut microbiota – social network position predicted community richness, and social partners had more similar community compositions. Metacommunity theory predicts that connectivity among local communities (hosts) is critical to explaining overall patterns of diversity. On average, dispersal (microbial transmission through host social interaction) is expected to diversify local communities up to a point, by providing novel colonists and rescuing rare species from extinction, but then cease to be enriching as high dispersal begins to homogenise communities and the best competitors at a regional scale come to dominate and exclude others [69]. In other words, local diversity is expected to be maximal at intermediate average levels of dispersal [70]. If social connectivity is uneven among hosts (as is common in social networks, including ours), a metacommunity could also maintain both diversifying flux and a level of local community uniqueness, that allows competing microbial species to coexist within the metacommunity. In such a network, hosts that interact with many others, especially those likely to harbour distinct microbes, may experience the most diversifying effects of social transmission, compared to those interacting with the same or similar individuals. Consistent with this idea, we found that hosts interacting with others from different parts of the network (with high bridge-type centrality) had more diverse microbiotas, while this was not true for highly connected individuals with more interconnected partners (i.e. with high eigenvector centrality).

Overall, our findings suggest the social environment is an underestimated force shaping the gut microbiota among free-living animals. An important future question then is what role this "social microbiome" [21] plays in host fitness. Besides the pathogenic challenges arising from social contact, which have been acknowledged for some time [71–73] there may also be benefits. Our results suggest social transmission affects microbiota attributes that have potential relevance for host health: microbiota diversity, similarity among interacting individuals, and transmission of bacteria that cannot readily persist outside the host. While exact relationships between microbiota diversity and beneficial functions remain poorly understood [74, 75], a diverse microbiota might bring benefits in terms of resisting pathogenic infection [11, 76] or increasing metabolic capacity [1, 77]. Immunological benefits may also result from microbiota similarity among closely interacting individuals. Since symbiotic microbes can be pathogenic in an unaccustomed individual [78, 79], sharing a set of familiar microbes with social partners might help maintain diversity, while minimising the threat of opportunist pathogens [20, 80]. Lastly, if anaerobic, non-spore-forming microbes are less likely to be harmful [22] and more likely to be beneficial, social interactions may facilitate the sharing of functionally important, and perhaps more host-specialist symbionts, such as members of the Bacteroidales [62, 81]. Since such benefits of social behaviour could be present even without any others (e.g. benefits of cooperative behaviour), it is possible that the social transmission of gut microbes could represent an underappreciated force in the early evolution of sociality.

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Author contributions SCLK conceived and designed the study, BA and SCLK carried out fieldwork, collected samples and cleaned field data for analysis, AR conducted microbiome laboratory work, analysed the data and led writing of the manuscript. TT and AH performed mouse genotyping and built the pedigree, JF helped with social network analysis, TC and SCLK provided guidance during analyses, and all authors contributed to and reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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Supplementary Material S2 for

Chapter 2

Social networks strongly predict the gut microbiota of wild mice

Index

Note that in the published article text, these supplementary materials are referred with an S and a single number (e.g. “Figure S1”), emitting the “S2.” referring to this thesis chapter.

Supplementary Materials and Methods

Supplementary Figures

Supplementary Tables

Appendix S2.1. Additional trapping and sampling methods

Data for this study was collected as part of a longer-term rodent capture-mark-recapture study. While several rodent species were caught (*Apodemus sylvaticus*, *Apodemus flavicollis* and *Myodes glareolus*), we focus on the most abundant species, wood mice (*Apodemus sylvaticus*). Trapping was performed every 2-4 weeks, using 122 small folding Sherman traps (5.1 x 6.4 x 16.5cm, H. B Sherman). Traps baited with eight peanuts, a slice of apple and sterile cotton wool for bedding were set at dusk and collected at dawn, with all animals processed, sampled and then released inside the 100m^2 grid cell they were captured in. As part of processing, captured individuals were identified to species, sexed, weighed, and aged (juvenile or adult) based on size and pelage characteristics. Ear punches were collected from all mice and stored in ethanol at -20°C to provide genetic material for host genotyping. Faecal samples for gut microbiota analysis were collected from the trap and frozen at -80^{o}C within 8 hours of collection. Utensils used for faecal pellet collection (e.g. tweezers) were sterilized using 70% ethanol between each sample processed, and all traps showing evidence of rodent contact were washed in bleach solution and autoclaved between trapping sessions.

Appendix S2.2. Behavioural data collection using PIT-tag loggers

PIT-tag loggers took the form of a large plastic box with two entrance tubes (Figure S2.1B) leading to a central wooden box containing sawdust and a single peanut (Figure S2.1B). An RFID coil surrounding the entrance to the central box recorded the PIT-tag ID of any tagged rodent present under it every 0.3 seconds. Peanut oil around the tube entrances, renewed at each logger rotation, was used as a minor lure. Upon each logger rotation, each logger box and entrance tubing was thoroughly cleaned with 70% ethanol and the sawdust was replaced. Cleaning was aided by the box's removable plastic floor that could be cleaned thoroughly. Loggers were powered using 12V lead acid rechargeable batteries. Further details about the loggers can be found in (Godsall et al., 2014). Initially 9 loggers were distributed across the plot, though for a period of 6 weeks (April-May 2015) one logger was broken and only 8 were used during this time. We designed a logger rotation plan such that each logger had an assigned "territory" (plot region) including a constant number of contiguous 100m^2 grid cells, and was moved between these in order to achieve even spatial coverage of the plot throughout the study period. With 9 loggers (as used for the vast majority of the study), each logger had a territory of 27 contiguous 100m^2 grid cells (Figure S2.1A). At each rotation, an R script was used to select the new locations to which loggers would move: each logger was moved to a new grid cell selected randomly without replacement from those in its territory, until all grid cells had been monitored, before the selection process was repeated. The precise (1m^2) position of each logger within a grid cell was also randomly selected at each rotation.

Loggers were always rotated between 10am and 2pm when wood mice (which are strongly nocturnal) are least active. Rotations were performed on average 3.54 times per week throughout the year (range 2-7 times per week). When loggers were left for several consecutive nights in a location for logistical reasons (e.g. over a weekend), only data from the first of these nights was used in subsequent analyses, to maintain even spatial coverage in the final dataset (Figure S2.1C). Loggers were deliberately not moved to a new location just after a trapping night, as logger data from trapping nights (when many mice may be in traps and therefore not recorded) was also intentionally filtered out of the data before analysis. Logger data rows containing corrupt tag reads was also removed during data cleaning, prior to analysis (See Figure S2.2 for details on how much data was lost in each step).

Appendix S2.3. Mouse genotyping and pedigree reconstruction

Genotyping

Genomic DNA was extracted from tissue samples (ear clips) using QIAamp DNA Micro Kits (Qiagen). Twelve microsatellite loci were selected for *Apodemus sylvaticus* from the literature (2,3). Target regions were amplified in four multiplex PCRs designed according to target region size range and annealing temperature (Table S2.1). PCR conditions were as follows: initial denaturation at 95 °C for 15min, 30 cycles involving 95 °C for 30s, the annealing temperature (Table S2.1) for 90s, and extension at 72 °C for 1min, followed by a final extension at 60 °C for 10min. PCR reaction volume was 10µl containing 1µl template DNA, 0.5µl each primer (at 10 µmol concentration), 5µl Qiagen Multiplex PCR Master Mix , 1µl Q-solution and 2µl PCR-grade water. Each PCR plate included one negative (H_20) control which was run on a 2% agarose gel to verify lack of contamination. PCR products were prepared for sequencing by diluting to equal concentration (measured using a Qubit fluorometer) and mixing with a solution of deionized 95-100% formamide and size standard (Genescan 500 ROX) before sequencing on a 3730 DNA Analyzer (Applied Biosystems) and scoring in GeneMapper Software (version 5). Genotyping results were evaluated with Cervus 3.0.7 (Kalinowski et al., n.d.) and Micro-checker 2.2 (VAN OOSTERHOUT et al., 2004), after which one microsatellite marker (GACAB3A) was discarded as it showed significant deviation from Hardy-Weinberg Equilibrium. Summary characteristics for the remaining 11 loci used for pedigree reconstruction are shown in Table S2.2.

Pedigree reconstruction

A pedigree was constructed using COLONY 2.0.6.5 (Wang et al., n.d.) a program for parental and sibship inference from genotype data. This software was chosen because it can perform analyses for polygamous species and accounts for genotyping error. It divides samples into family clusters, in which individuals are related either via sibship or shared parentage. The likelihood of a cluster is calculated based on Mendelian inheritance rules (JONES & WANG, 2010). COLONY was run multiple times to get the most accurate estimates for sibship/parentage, adjusting parameter expectations to take into account that wood mice are polygamous (half-sibs are common) and may inbreed (genotype frequencies may be biased towards homozygosity). We compared kinship results from COLONY with trapping data, aided with visualization with Pedigree Viewer to identify impossible relationships (based on age and time trapped). We found 11 impossible mother-pup and 4 impossible father-pup pairs, which were excluded after which the model was re-run, this time estimating kinship without any conflicts with the trapping data. The resulting pedigree contained 17 mother-pup pairs, 14 father-pup pairs, 13 full-sibling-pairs and 26 half-sibling-pairs. This pedigree was used to create dyadic numeric kinship matrices, where kinship was transformed into a numeric distance variable, with for example parent-offspring pairs and full siblings both assigned a kinship value of 0.5 and half-siblings/cousins 0.125.

Appendix S2.4. Gut microbiota analysis using 16S rRNA profiling

Library preparation and sequencing

DNA was extracted from samples using Zymo Quick-DNA™ Fecal/Soil Microbe 96 kits (Zymo) according to manufacturer's instructions. For extractions as well as subsequent PCRs, samples were randomized across batches/plates to avoid confounding biological and technical effects on downstream microbial data. An approximately 240bp V4 region of the 16S rRNA gene was amplified using primers N515F and N806R (Caporaso et al., 2011) in two-step (tailed-tag) approach with dual-indexing (D'Amore et al., 2016). In addition to samples, we included one extraction control (PCR-grade water subjected to the DNA extraction method) and one PCR control (PCR-grade water) per 96-well plate as well as 2 extractions of a mock community (ZYMOBiomics Microbial Community Standard cat. No. D6300) to evaluate extraction, PCR and sequencing accuracy.

For all samples a test first round PCR was performed, to check sample amplification and decide on an appropriate number of PCR cycles for the final library preparation. For this test PCR, the following were combined in a 20µl reaction volume: 10µl KAPA 2x Mastermix (KAPA Biosystems), 0.25µl each primer at 10µM, 4.5µl PCR-grade water and 5µl undiluted DNA extraction. Cycling conditions were as follows: denaturation at 98°C for 2min, 35 cycles of 95°C for 20s, 65°C for 15s,

70°C for 30s, followed by a final extension at 70°C for 5min and a 4°C hold. Products were visualized on 2% agarose gels, and no amplification was observed in extraction or PCR controls. Subsequently the first round PCR for use in subsequent sequencing was performed, using exactly the same conditions as described above but with fewer cycles (20 instead of 35).

Unpurified first round PCR products were sent to the Centre for Genomic Research (Liverpool, UK) for indexing PCRs, purification, pooling, amplicon size selection and sequencing. First round PCR products were purified using Agencourt Ampure XP beads (Beckman Coulter, Brea, CA, USA), by adding 20µl beads at room temperature to samples, before washing twice with 200µl 80% ethanol and eluting in 10µl H_20. For the second round PCR (addition of indices), the following were combined in a 20µl reaction volume: 10µl KAPA 2x Mastermix (KAPA Biosystems), 0.5µl of each primer at 10µM and 9µl of clean PCR product. For indexing, eight forward primers (i5) and twelve reverse (i7) primers were used, each containing a unique barcode, creating 96 unique combinations (barcode sequences are reported in the Illumina Nextera Protocol (10). Second round PCR products were purified using Agencourt Ampure XP beads as described above, but eluted in 20µl H_20. Each second round PCR product (library) was analyzed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and up to 96 libraries pooled per sequencing run, at equimolar concentration of fragments in the expected size range. Each pool was size-selected using a Pippin Prep, by excluding fragments outside the expected range. Libraries were sequenced using 2x250bp paired-end sequencing on an Illumina MiSeq, with samples from different extraction batches randomly allocated to four different runs. Visualization of the composition of the mock samples revealed that our laboratory- and bioinformatics pipeline successfully captured the microbial diversity present in mock samples (Figure S2.3).

Bioinformatics

Sequence data were processed through the DADA2 pipeline (version 1.6.0) (Callahan et al., 2016). *cutadapt* (journal & 2011, n.d.) was used to determine optimal trimming parameters for removal of leading primer and adapter sequences (27 base pairs from the beginning of each read), and trimming was performed using the trimLeft argument in DADA2. Following visual inspection of sequence quality, low-quality tails were also trimmed (leaving 230 bp for forward and 180 bp for reverse reads). Sequences were dereplicated, and amplicon sequence variants (ASVs) inferred using the DADA2 algorithm. Forward and reverse paired-end reads were merged after which reads that could not be merged, were putative chimeric sequences or were of abnormal length (<238 bp or > 245 bp) were removed from the dataset.

Within the R package *phyloseq* (McMurdie & Holmes, 2013) taxonomy was assigned to ASVs using the GreenGenes Database (GreenGenes Database Consortium 13.8). One sample with exceptionally low read count was removed from the dataset (n=300 reads, while read count >10 000 for all other samples). We then used package iNEXT (Hsieh et al., 2016) to confirm that sample completeness estimates plateaued and diversity estimates stabilized at read counts around 2500-4000 reads, well below the read counts for all samples in the remaining dataset (Figure S2.4) We also used iNEXT to derive sample-level asymptotic estimates of microbial richness and Shannon diversity, which are corrected for the modelled effect of read count. After asymptotic correction, Shannon diversity estimates were not significantly predicted by read count but richness estimates were (linear regressions, $p<0.01$), and thus effect of read count was controlled for in all models predicting richness. Singleton reads were filtered out of the dataset, to avoid residual bias from possible contaminants or sequencing errors, as well as ASVs assigned to taxa known to not be gut bacteria (Cyanobacteria, Xanthomonadales, Mitochondria). Since the distribution of abundances for remaining taxa showed no obvious tail of rare taxa (Figure S2.5). further abundance filtering was not performed. Finally, ASV counts were normalized by calculating proportional abundance within each sample (McKnight et al., 2019).

Appendix S2.5. Alternative statistical frameworks for predicting microbiota composition with social association strength

To check our results were robust to the statistical approach used, we used two alternative statistical methods to verify key results from the model predicting microbiota composition with the 12h social network. First, another Bayesian regression model from the R package *MCMCglmm* (Hadfield, n.d.) was used with the same data. Model structure was identical to that used in *brms* models, except that, since this package does not allow beta-regression and since the distribution of response variable values was not skewed, the model used a Gaussian link function. As with *brms* models, the MCMCglmm model was run first with Jaccard Index as the response, and then with Bray-Curtis dissimilarity as the response, to assess whether results remained the same when using an abundance-weighted community similarity metric. Second, an alternative matrix permutation-based modelling framework was used, to further verify results with a contrasting analytical method. Here we used multiple regression quadratic assignment procedure (MRQAP, 17), to test the relationship between social proximity and gut microbiota similarity while controlling for the same set of covariates as above. MRQAP is a null model-based matrix permutation test commonly applied in social network analyses (e.g. 15,16). MRQAP was run on a dataset including only one microbiota sample per individual, selected at random in each of 100 different iterations. Finally, to confirm no single dyadic

interaction disproportionately influenced results, each dyad was dropped one at a time from the MCMCglmm model, and the effect on coefficients and statistical significance examined. Results from these analyses showing a consistent effect of the social network on microbiota composition are presented in Table S2.9, Table S2.10 and Figure S2.7 respectively.

Appendix S2.6. Null models describing the effect of reduced network density on estimates of models predicting microbiota with social association

In order to accurately compare social association-microbiota effect sizes across networks, we had to account for decreasing density in networks with more intimate edge definitions (Figure 2), since it is possible that differences in network density alone could influence effect size differences. We therefore compared observed social association effect size estimates with those derived from artificially thinned versions of the least intimate (12h) network. Six artificial versions of the 12h social network were made by stepwise thresholding (converting the weakest edges to zero), to achieve the same percentage of non-zero-links (=density) as each of our real social networks. In this way, we created a set of artificial networks that had the same density as each of our real networks made using edge definitions of varying intimacy, but that were in fact subsets of stronger links from the 12h network.

Supplementary Figures

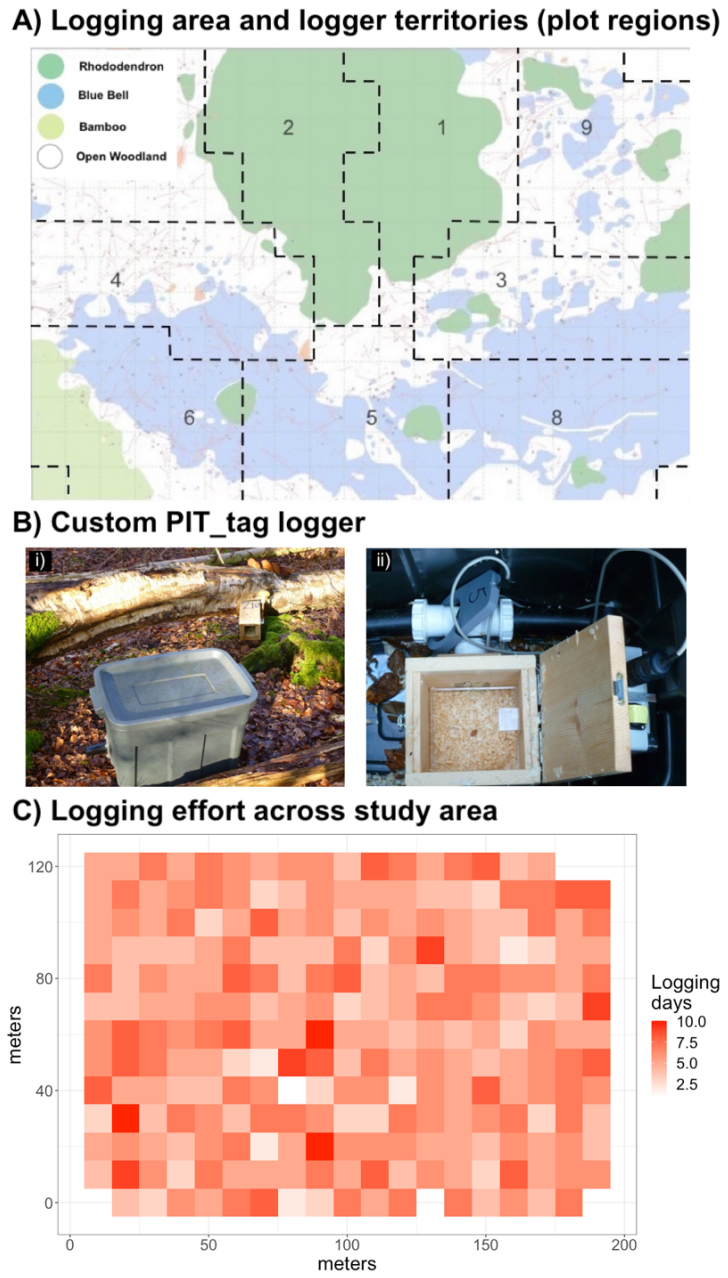

Figure S2.1: The Logger, the study design and logging effort across the grid. (A) Map of the 2.47 ha study plot (Nash's Copse) showing nine numbered logger territories (plot regions) coloured with habitat type (rhododendron=dark green, bamboo=light green, open woodland=white or blue). (B) Custom PIT-tab logger with i) external view showing two entrance tubes on opposite sides and ii) Internal view showing RFID reader (labelled '5') around the entry tubes, and central box containing sawdust and a provisioned with a peanut. (C) Map of study plot showing logging effort (total number of logging days performed for each 100m^2 grid cell).

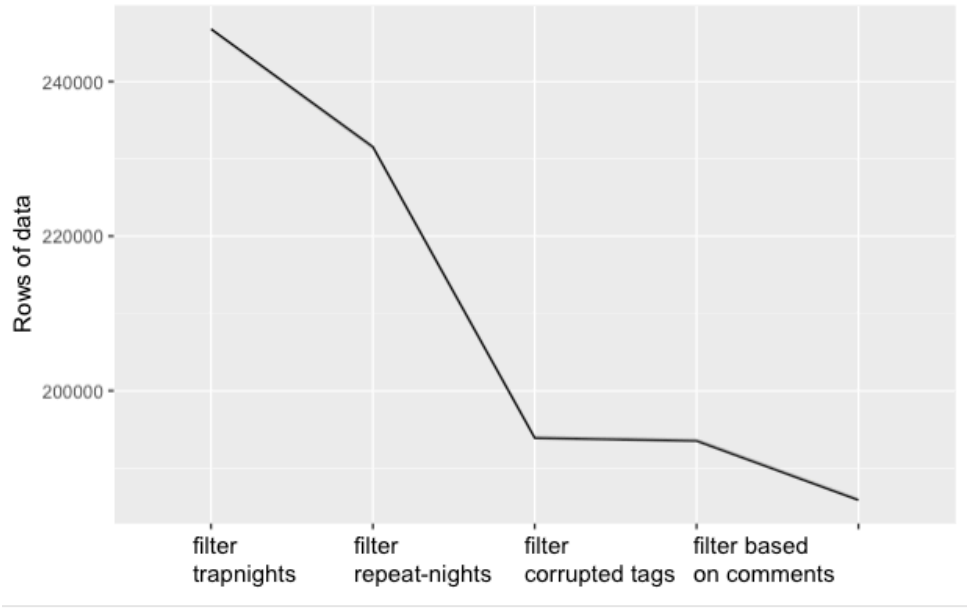

Figure S2.2: Logger data filtering. The effect of each filtering step (x-axis) on the number of logger data rows (y-axis)

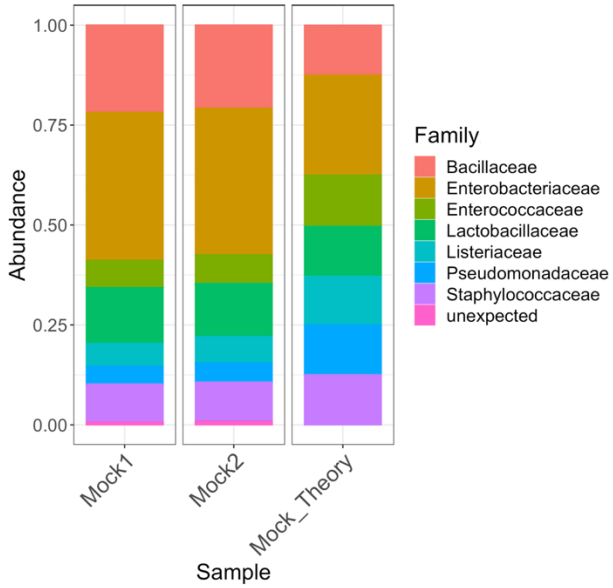

Figure S2.3: Microbial standard (mock) community profiles. Community composition in sequenced mock community aliquots (Mock1, Mock2) compared to the expected composition (Mock_Theory).

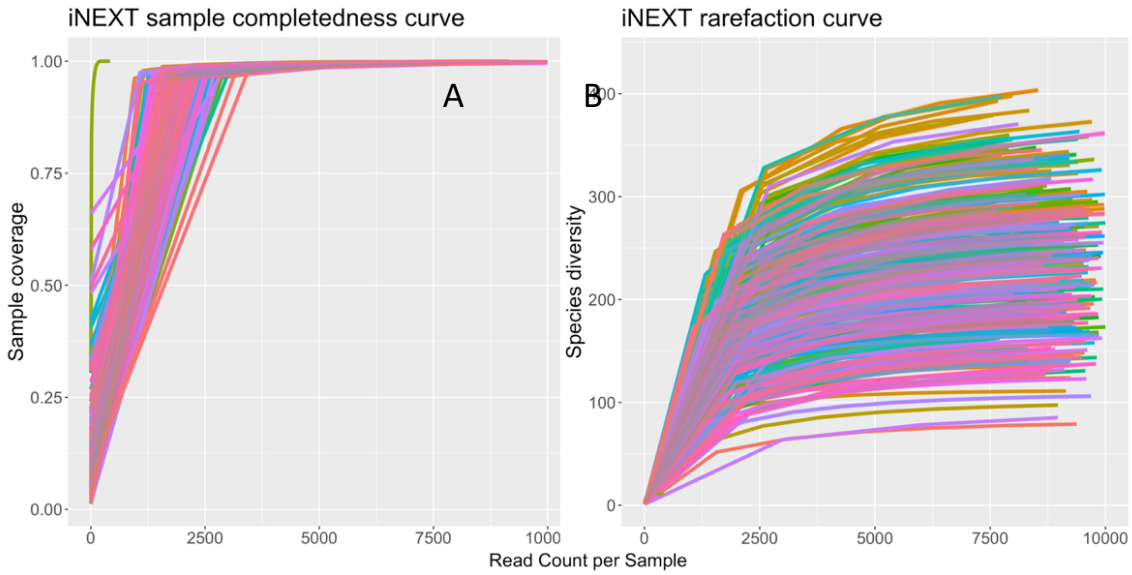

Figure S2.4: iNEXT analysis results A) Sample completeness curve, showing completeness plateaus above read counts of approximately 4000. B) Rarefaction curve, showing diversity estimates stabilize at read counts above approximately 2500.

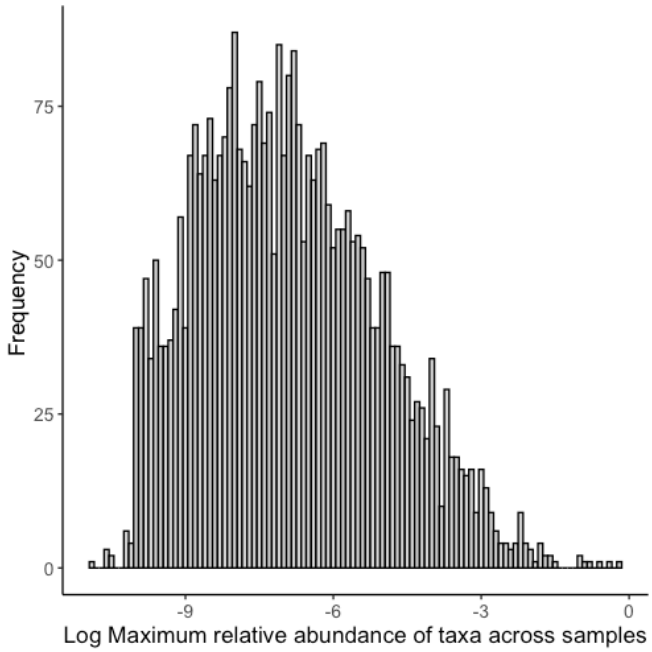

Figure S2.5: Distribution of bacterial taxon (ASV) relative abundances across all samples.

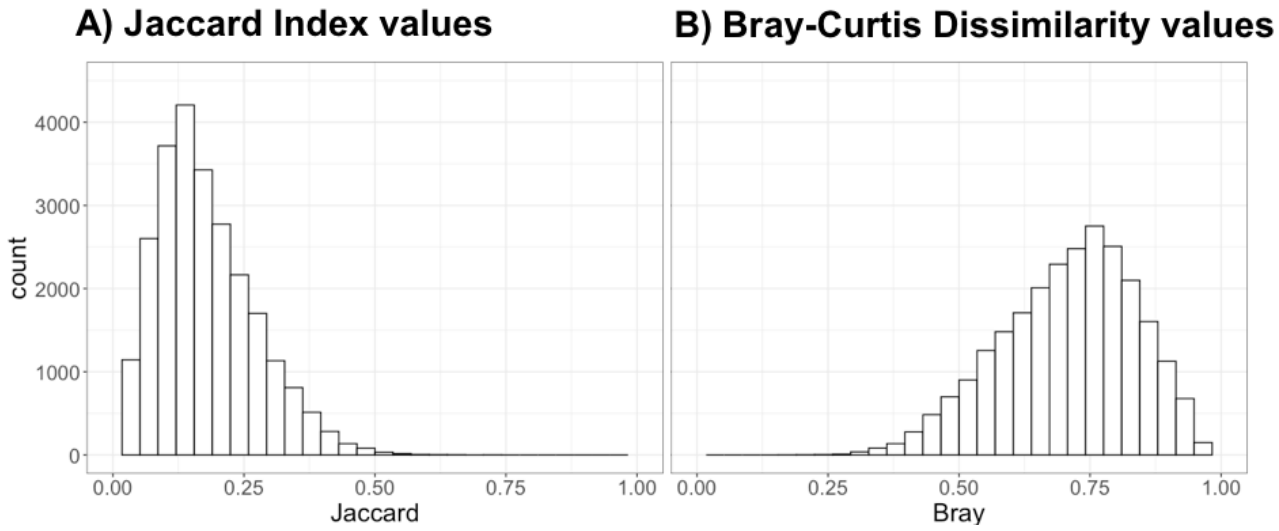

Figure S2.6: Distribution of microbiota similarity metrics A) Jaccard Index of similarity B) Bray-Curtis index of dissimilarity.

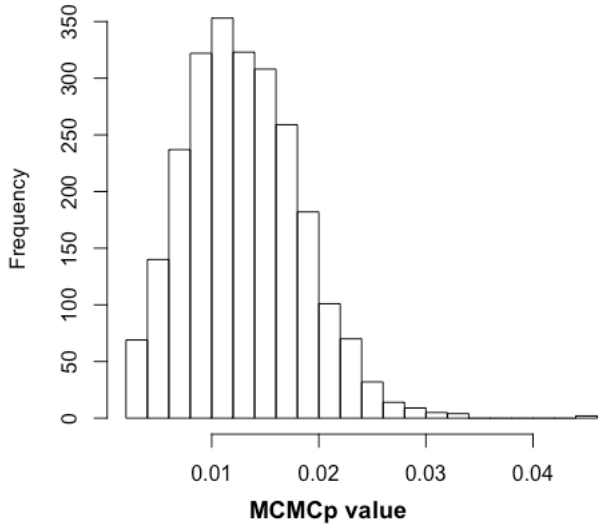

Figure S2.7: No single dyad drives the effect of social network on microbiota similarity. Distribution of p-values for the social network effect from a series of MCMCglmm models that excluded a single dyad from the model. Models used the 12h network to predict microbiota similarity (Jaccard Index). Since p-values remain <0.05 in all models, no single dyad drives the significance of the social network effect.

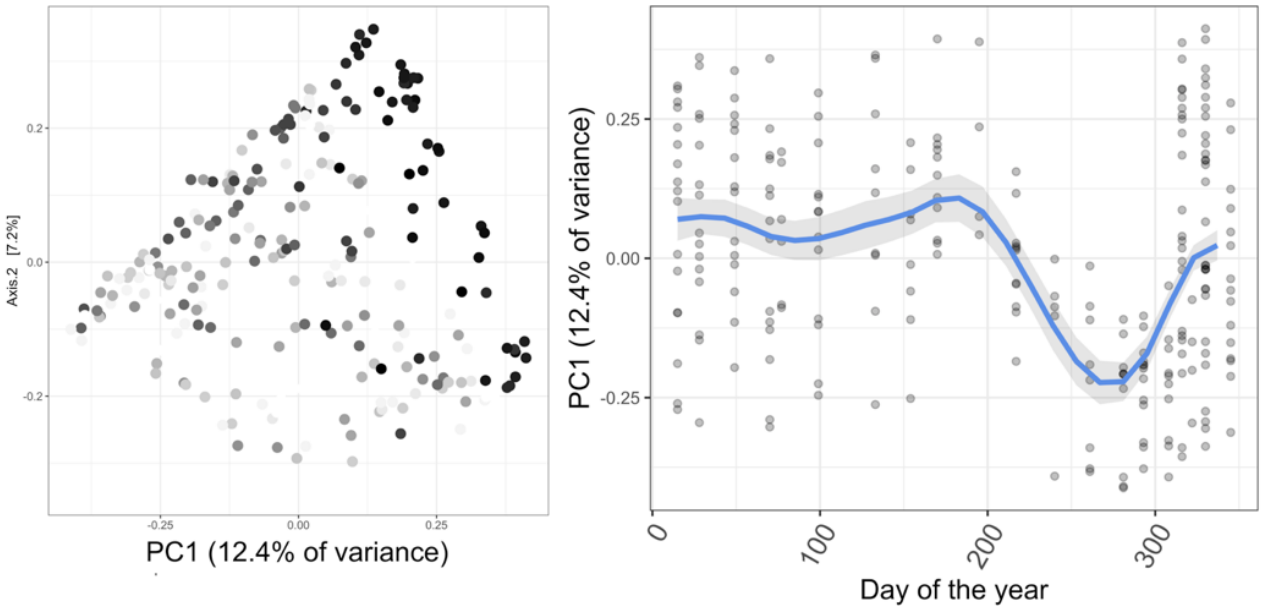

Figure S2.8: Temporal dynamics of the wood mouse gut microbiota A) PCoA on Jaccard distance shows how temporal (seasonal) variation in microbiota composition (darker colour = later in the year) is largely captured by the first axis of variation (PC1). B) PC1 shows marked variation during the year, with a major fluctuation between July and October. A model fit from a generalised additive mixed model (GAMM) is plotted, with the blue line indicating fitted values and shaded grey areas the approximate 95% confidence intervals. The GAMM fits a flexible smoothed function for microbiota variability (PC1 of Jaccard Index) across days of the year.

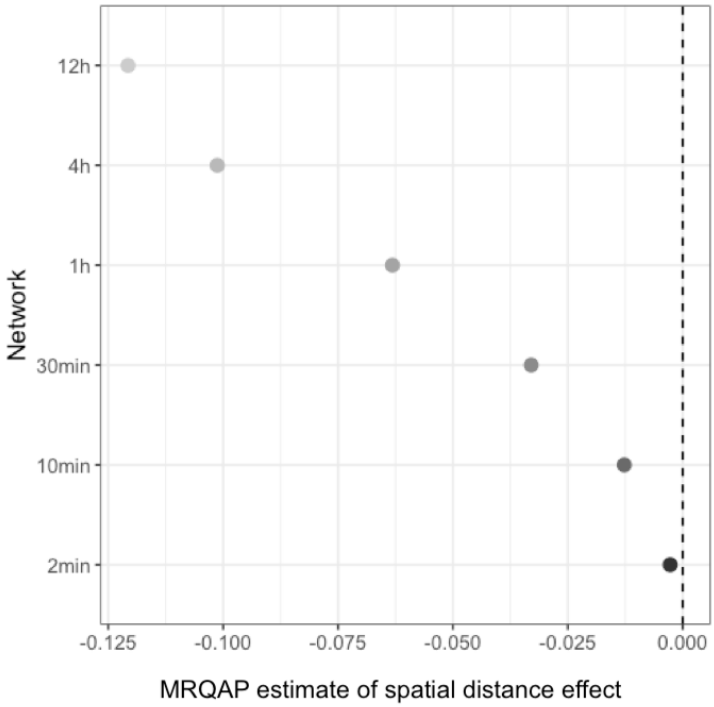

Figure S2.9: MRQAP model estimates for the effect of spatial distance on social association strength across networks. Networks (y-axis, point colour) with increasingly intimate edge definition have lower model estimate predicted by spatial distance. In other words, more intimate social associations are less strongly predicted by spatial proximity between individual mice. Model covariates and results in Table S2.6.

Supplementary Tables

Table S2.1: **PCR protocols and multiplexing for 12 microsatellite loci used to genotype wild-caught wood mice**

Locus	*Fluorescent label*	*Primer Concentration (µmol)*	*Multiplex*	*Annealing temperature*	*Size standard*	*Source*
AS-7	FAM	0.1	A	50	ROX	Godsall 2015
MSAf-8	HEX	0.1	A	50	ROX	Godsall 2015
AS-11	TAMRA	0.1	A	50	ROX	Wilson 2014
As-20	FAM	0.1	B	57	ROX	Godsall 2015
GCATD7S	HEX	0.1	B	57	ROX	Godsall 2015
As-34	TAMRA	0.1	B	57	ROX	Godsall 2015
Apfl_BF6	FAM	0.1	C	57	ROX	Godsall 2015
GACAA12A	HEX	0.1	C	57	ROX	Godsall 2015
TNF-CA	TAMRA	0.1	C	57	ROX	Godsall 2015
As-12	FAM	0.1	D	Touchdown 56-53	ROX	Godsall 2015
CAM-13	HEX	0.1	D	Touchdown 56-53	ROX	Godsall 2015
GACAB3A	TAMRA	0.1	Singleplex	55	ROX	Wilson 2014

Table S2.2: Summary data for the final 11 microsatellite loci used in pedigree reconstruction. Observed (Hobs) and expected (Hexp) heterozygosity values, deviation from Hardy-Weinberg Equilibrium (HWE, NS = non-significant), null allele and scoring error rates are shown. Null alleles, estimated using COLONY, use the expected distribution of homozygosity under HWE to describe the expected proportion of alleles missed by technical issues, from all alleles in a locus. Scoring error is a measure of total error, due human and technical variation, in repeatability when genotyping the same individual.

Locus	*No. Alleles*	*Size range*	*Successfully genotyped samples*	*Hobs*	*Hexp*	*HWE*	*Null allele*	*Scoring Error*
Apfl_BF6	9	340-410	138	0.717	0.780	NS	0.0370	0.001
AS-7	22	80-140	138	0.783	0.862	NS	0.0449	0.000
AS-11	20	230-280	137	0.920	0.911	NS	-0.0067	0.001
As-12	23	220-270	138	0.862	0.942	NS	0.0426	0.062
As-20	20	120-170	138	0.775	0.913	NS	0.0802	0.003
As-34	22	150-210	138	0.862	0.876	NS	0.0056	0.001
CAM-13	3	180-200	138	0.391	0.361	NS	-0.0451	0.023
GACAA12A	7	230-260	136	0.765	0.723	NS	-0.0321	0.001
GCATD7S	11	170-230	138	0.855	0.852	NS	-0.0047	0.015
MSAf-8	26	160-230	138	0.935	0.900	NS	-0.0227	0.002
TNF-CA	20	100-150	138	0.906	0.889	NS	-0.0122	0.003

Table S2.3: The relative effects of individual identity, temporal change and methodological factors on gut microbiota compositional variation. Results are from a marginal PERMANOVA on data from mice sampled more than once, with either Jaccard distance or Bray-Curtis dissimilarity as the response. A marginal PERMANOVA was used such that the variance explained by each term does not depend on the order of explanatory variables. Significant terms ($p<0.05$) are shown in bold.

JACCARD				
	Df	**F**	**p**	**R^2**
Read count	1	0.956	0.526	0.003
PCR plate	**3**	**1.285**	**0.032**	**0.013**
Individual ID	***74***	***1.321***	***0.001***	***0.334***
Month	**12**	**1.471**	**0.001**	**0.060**
Residual	150			0.512
Total	240			1.000
BRAY-CURTIS				
	Df	**F**	**p**	**R^2**
Read count	1	0.993	0.437	0.003
PCR plate	**3**	**1.501**	**0.029**	**0.014**
Individual ID	***74***	***1.533***	***0.001***	***0.342***
Month	**12**	**1.813**	**0.001**	**0.066**
Residual	150			0.452
Total	240			1.000

Table S2.4: The extent of individual-level variation (repeatability) in gut microbiota after controlling for known individual-level covariates. Results are from a sequential PERMANOVA including all samples. A sequential PERMANOVA was used, in order to test the effect of individual identity once other (temporal and host) effects were accounted for. Terms are listed in the order they appear in the model, and significant terms ($p<0.05$) are shown in bold.

JACCARD				
	Df	**F**	**p**	**R^2**
Month	**12**	**3.254**	**0.001**	**0.134**
Age	**2**	**1.492**	**0.013**	**0.010**
Sex	1	1.390	0.067	0.005
Plot Region	**8**	**1.705**	**0.001**	**0.047**
Habitat type	**3**	**1.713**	**0.001**	**0.018**
Individual ID	***62***	***1.247***	***0.001***	***0.265***
Residual	152			0.521
Total	240			1.000
BRAY-CURTIS				
	Df	**F**	**p**	**R^2**
Month	**12**	**5.076**	**0.001**	**0.185**
Age	**2**	**1.821**	**0.011**	**0.011**
Sex	1	1.639	0.053	0.005
Plot Region	**8**	**2.118**	**0.001**	**0.051**
Habitat type	**3**	**2.295**	**0.001**	**0.021**
Individual ID	***62***	***1.405***	***0.001***	***0.265***
Residual	152			0.462
Total	240			1.000

Table S2.5: The effect of demographic individual-level variables on gut microbiota composition. Results are from a marginal PERMANOVA on a dataset including one randomly selected sample per individual, with either Jaccard distance or Bray-Curtis dissimilarity as the response. Only one sample per ID was included so that the variance explained by individual-level factors could be directly compared (in a marginal PERMANOVA) without pseudoreplication. Significant terms ($p<0.05$) are shown in bold.

JACCARD INDEX				
	Df	**F**	**p**	**R^2**
Month	**11**	**1.511**	**0.001**	**0.200**
Age	2	1.222	0.090	0.029
Sex	1	1.037	0.332	0.012
Region	8	1.103	0.125	0.104
Habitat type	**3**	**1.227**	**0.045**	**0.044**
Residual	49			0.576
Total	74			1.000
BRAY-CURTIS				
	Df	**F**	**p**	**R^2**
Month	**11**	**1.858**	**0.001**	**0.221**
Age	2	1.380	0.072	0.030
Sex	1	1.037	0.372	0.011
Region	8	1.132	0.151	0.098
Habitat type	**3**	**1.371**	**0.050**	**0.045**
Residual	49			0.531
Total	74			1.000

Table S2.6: Variables predicting social association strength (Adjusted SRI) across networks. Results of MRQAP models predicting Adjusted SRI for different social networks. Significant terms ($p<0.05$) are shown in bold. Spatial distance significantly predicted social association strength in all networks, whereas kinship, age and sex similarity predicted social association strength in some, but not all, networks. More related mice were observed more often together in the 30min and 10min social network, adult mice were observed more often together than with juveniles in the 12h network, and opposite sex mice were more likely to be observed together in 30min network. Further exploration on the effect of sex shows that social association strength in 12h and 2min social networks was not significantly affected by the sex category of the dyad (1= Female-female, 2= Female-Male, 3=Male-Male).

12h Social Network				
	Estimate	**P(β>=r)**	**P(β<=r)**	**P(|β|<=|r|)**
Intercept	0.064	1.000	0.000	0.000
Sex-similarity (0/1)	-0.003	0.141	0.859	0.281
Age-similarity (0/1)	**0.009**	0.983	0.017	**0.044**
Spatial distance	**-0.121**	0.000	1.000	**0.000**
Kinship	0.038	0.924	0.076	0.086
4h Social Network				
	Estimate	**P(β>=r)**	**P(β<=r)**	**P(|β|<=|r|)**
intercept	0.056	1.000	0.000	0.000
Sex-similarity (0/1)	-0.004	0.111	0.889	0.202
Age-similarity (0/1)	**0.006**	0.934	0.066	**0.157**
Spatial distance	**-0.101**	0.000	1.000	**0.000**
Kinship	0.037	0.945	0.055	0.058
1h Social Network				
	Estimate	**P(β>=r)**	**P(β<=r**	**P(|β|<=|r|)**
intercept	0.037	1.000	0.000	0.000
Sex-similarity (0/1)	-0.004	0.051	0.949	0.095
Age-similarity (0/1)	0.001	0.546	0.454	0.807
Spatial distance	**-0.063**	0.000	1.000	**0.000**
Kinship	0.033	0.944	0.056	0.056

30min Social Network				
	Estimate	**P(β>=r)**	**P(β<=r)**	**P(|β|<=|r|)**
intercept	0.018	1.000	0.000	0.000
Sex-similarity (0/1)	**-0.003**	0.007	0.993	**0.010**
Age-similarity (0/1)	0.003	0.957	0.043	0.118
Spatial distance	**-0.033**	0.000	1.000	**0.000**
Kinship	**0.039**	0.990	0.010	**0.010**
10min Social Network				
	Estimate	**P(β>=r)**	**P(β<=r)**	**P(|β|<=|r|)**
intercept	0.008	1.000	0.000	0.000
Sex-similarity (0/1)	-0.001	0.285	0.715	0.584
Age-similarity (0/1)	-0.000	0.291	0.709	0.757
Spatial distance	**-0.013**	0.000	1.000	**0.000**
Kinship	**0.036**	0.980	0.020	**0.020**
2min Social Network				
	Estimate	**P(β>=r)**	**P(β<=r)**	**P(|β|<=|r|)**
intercept	0.001	0.978	0.022	0.022
Sex-similarity (0/1)	-0.000	0.058	0.942	0.114
Age-similarity (0/1)	0.000	0.968	0.032	0.193
Spatial distance	**-0.003**	0.000	1.000	**0.000**
Kinship	-0.001	0.299	0.701	0.418
12h Social Network				
	Estimate	P(β>=r)	P(β<=r)	P(|β|<=|r|)
intercept	0.058	1.000	0.000	0.000
Sex-category (1=FF, 2=FM, 3=MM)	0.007	0.941	0.059	0.132
Age-similarity (0/1)	0.008	0.968	0.032	0.079
Spatial distance	**-0.121**	0.000	1.000	**0.000**
Kinship	0.038	0.947	0.053	0.058
2min Social Network				
	Estimate	P(β>=r)	P(β<=r)	P(|β|<=|r|)

intercept	0.000	0.903	0.097	0.119
Sex-category (1=FF, 2=FM, 3=MM)	0.000	0.915	0.085	0.261
Age-similarity (0/1)	0.000	0.930	0.070	0.246
Spatial distance	**-0.003**	0.000	1.000	**0.000**
Kinship	-0.001	0.342	0.658	0.482

Table S2.7: Correlations among social networks with varying edge definitions. Results are shown from Mantel tests assessing the correlation between social networks varying in the edge definition used.

Network edge definition (time window)	Correlation (r) with 12h network	p-value
12h	1	0.000
4h	0.96	0.001
1h	0.82	0.001
30min	0.59	0.001
10min	0.43	0.001
2min	0.16	0.003

Table S2.8: Results of *brms* models testing the effect of social association strength and covariates on microbiota similarity (Jaccard Index). Significant terms (where 95% credible intervals do not include zero) are shown in bold. Est.Error indicates the standard deviation of the posterior distribution.

12h Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.46	0.06	-1.57	-1.35
Sex	-0.00	0.01	-0.02	0.01
Age	**0.02**	**0.01**	**0.01**	**0.03**
Kinship	0.00	0.04	-0.08	0.09
Spatial distance	**-0.08**	**0.02**	**-0.12**	**-0.04**
Sampling interval	**-0.46**	**0.01**	**-0.48**	**-0.43**
Social Network	**0.78**	**0.23**	**0.31**	**1.24**
4h Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.45	0.05	-1.55	-1.34
Sex	-0.00	0.01	-0.02	0.01
Age	**0.02**	**0.01**	**0.01**	**0.03**
Kinship	-0.01	0.04	-0.09	0.08
Spatial distance	**-0.09**	**0.02**	**-0.13**	**-0.04**
Sampling interval	**-0.47**	**0.01**	**-0.49**	**-0.44**
Social Network	**0.90**	**0.26**	**0.40**	**1.40**
1h Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.44	0.05	-1.55	-1.33
Sex	-0.00	0.01	-0.02	0.01
Age	**0.02**	**0.01**	**0.01**	**0.04**
Kinship	0.01	0.04	-0.07	0.10
Spatial distance	**-0.09**	**0.02**	**-0.13**	**-0.05**
Sampling interval	**-0.48**	**0.01**	**-0.50**	**-0.46**
Social Network	**1.17**	**0.35**	**0.47**	**1.86**

30min Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.43	0.05	-1.54	-1.33
Sex	-0.00	0.01	-0.02	0.01
Age	**0.02**	**0.01**	**0.01**	**0.04**
Kinship	0.01	0.04	-0.08	0.09
Spatial distance	**-0.09**	**0.02**	**-0.13**	**-0.05**
Sampling interval	**-0.49**	**0.01**	**-0.51**	**-0.46**
Social Network	**1.66**	**0.51**	**0.67**	**2.68**
10min Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.41	0.05	-1.52	-1.30
Sex	-0.00	0.01	-0.02	0.01
Age	**0.02**	**0.01**	**0.01**	**0.04**
Kinship	0.01	0.05	-0.08	0.10
Spatial distance	**-0.12**	**0.02**	**-0.15**	**-0.08**
Sampling interval	**-0.50**	**0.01**	**-0.52**	**-0.48**
Social Network	**2.31**	**0.85**	**0.67**	**3.97**
2min Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.40	0.05	-1.51	-1.29
Sex	-0.00	0.01	-0.02	0.01
Age	**0.02**	**0.01**	**0.01**	**0.04**
Kinship	-0.00	0.04	-0.09	0.09
Spatial distance	**-0.15**	**0.02**	**-0.18**	**-0.11**
Sampling interval	**-0.51**	**0.01**	**-0.53**	**-0.49**
Social Network	**6.71**	**2.91**	**1.48**	**12.87**

Table S2.9: **Results of MCMCglmm models testing the effect of social association strength and covariates on microbiota similarity/dissimilarity.** Microbiota beta diversity is described alternatively by either the Jaccard Index (a similarity index) or Bray-Curtis dissimilarity. To ensure

results are not skewed by variation in sample read depth, models were additionally run with indices calculated from microbiota data rarefied to an even read depth of 16866 reads per sample. Significant terms (MCMCp<0.05) are shown in bold. post.mean indicates the mean of the posterior distribution of effect estimates, while l-95% CI and u-95% CI show the lower and upper limits of 95% credible intervals. Effective sample size (eff.samp) is the number of samples taken, adjusted for autocorrelation in the chain. An effect is considered significant if 95% credible intervals do not overlap zero, which is also indicated by MCMCp-values. Specifically, MCMCp values indicate the proportion of posterior samples (out of 1000) that fall on the other side of zero from the majority. The effect is considered significant when MCMCp<0.05.

Jaccard Index					
	post.mean	**l-95% CI**	**u-95% CI**	**eff.samp**	**MCMCp**
(Intercept)	0.206	0.192	0.220	889.6	<0.001
Sex	-0.000	-0.002	0.002	1000.0	0.670
Age	**0.003**	**0.001**	**0.005**	**1000.0**	**0.004**
Kinship	0.002	-0.011	0.015	1000.0	0.762
Spatial distance	**-0.014**	**-0.019**	**-0.008**	**1000.0**	**<0.001**
Sampling interval	**-0.079**	**-0.082**	**-0.075**	**1000.0**	**<0.001**
12h Social Network	**0.063**	**0.050**	**0.078**	**1000.0**	**<0.001**
Bray-Curtis dissimilarity					
	post.mean	**l-95% CI**	**u-95% CI**	**eff.samp**	**MCMCp**
(Intercept)	0.664	0.652	0.693	397.1	<0.001
Sex	0.001	-0.002	0.003	1000.0	0.560
Age	**-0.005**	**-0.008**	**-0.002**	**1000.0**	**0.004**
Kinship	-0.001	-0.021	0.015	1000.0	0.942
Spatial distance	**0.020**	**0.012**	**0.027**	**1000.0**	**<0.001**
Sampling interval	**0.109**	**0.104**	**0.114**	**1000.0**	**<0.001**
12h Social Network	**-0.086**	**-0.105**	**-0.068**	**1141.2**	**<0.001**
Rarefied Jaccard Index					
	post.mean	**l-95% CI**	**u-95% CI**	**eff.samp**	**MCMCp**
(Intercept)	0.205	0.191	0.220	1000	<0.001
Sex	-0.000	-0.002	0.001	1000	0.614
Age	**0.003**	**0.001**	**0.005**	**1000**	**<0.001**

Kinship	0.001	-0.011	0.014	1000	0.882
Spatial distance	**-0.014**	**-0.020**	**-0.008**	**1000**	**<0.001**
Sampling interval	**-0.078**	**-0.081**	**-0.074**	**1000**	**<0.001**
12h Social Network	**0.062**	**0.049**	**0.076**	**1370**	**<0.001**
Rarefied Bray-Curtis dissimilarity					
	post.mean	**l-95% CI**	**u-95% CI**	**eff.samp**	**MCMCp**
(Intercept)	0.671	0.651	0.689	1000.0	<0.001
Sex	0.001	-0.002	0.003	1000.0	0.510
Age	**-0.005**	**-0.007**	**-0.002**	**1000.0**	**0.002**
Kinship	-0.000	-0.017	0.018	1147.2	0.976
Spatial distance	**0.020**	**0.012**	**0.028**	**935.4**	**<0.001**
Sampling interval	**0.108**	**0.103**	**0.113**	**1000.0**	**<0.001**
12h Social Network	**-0.086**	**-0.105**	**-0.068**	**1000.0**	**<0.001**

Table S2.10: **Results of MRQAP models testing the effect of social association strength and covariates on microbiota similarity (Jaccard Index)**. Significant terms (mean p<0.05 across 100 iterations, each including a single randomly selected sample per individual) are shown in bold. Results are show both for the subset of individuals with kinship data, as well as the full dataset.

Model term	Individuals with kinship data (n=70)		All individuals (n=75)	
	Mean Estimate	Mean p-value	Mean Estimate	Mean p-value
Sex-similarity (0/1)	-0.0022	0.53	-0.0022	0.50
Age-similarity (0/1)	-0.0004	0.50	-0.0008	0.44
Kinship	-0.0216	0.40		
Spatial distance	-0.0000	0.41	-0.0000	0.41
Sampling interval	**-0.0002**	**0.00**	**-0.0002**	**0.00**
12h Social Network	0.0624	0.06	**0.07**	**0.02**

Table S2.11: Results of *brms* models testing the effect of binary social association (BI) and covariates on microbiota similarity (Jaccard Index). Significant terms (where 95% credible intervals do not include zero) are shown in bold.

12h Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.47	0.06	-1.58	-1.36
Age	0.01	0.01	0.00	0.03
Kinship	0.01	0.04	-0.08	0.10
Sex	-0.00	0.01	-0.01	0.01
Spatial distance	**-0.10**	**0.02**	**-0.13**	**-0.06**
Sampling interval	**-0.43**	**0.01**	**-0.45**	**-0.40**
12h Social Network	**0.16**	**0.04**	**0.08**	**0.24**
4h Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.46	0.06	-1.58	-1.35
Age	0.01	0.01	0.00	0.03
Kinship	-0.00	0.04	-0.09	0.08
Sex	-0.00	0.01	-0.02	0.01
Spatial distance	**-0.10**	**0.02**	**-0.13**	**-0.06**
Sampling interval	**-0.44**	**0.01**	**-0.47**	**-0.41**
4h Social Network	**0.15**	**0.04**	**0.07**	**0.23**
1h Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.45	0.06	-1.56	-1.34
Age	0.02	0.01	0.00	0.03
Kinship	-0.01	0.05	-0.10	0.08
Sex	-0.00	0.01	-0.02	0.01
Spatial distance	**-0.09**	**0.02**	**-0.13**	**-0.05**
Sampling interval	**-0.47**	**0.01**	**-0.49**	**-0.44**
1h Social Network	**0.14**	**0.04**	**0.06**	**0.21**

30min Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.44	0.05	-1.55	-1.34
Age	**0.02**	**0.01**	**0.01**	**0.03**
Kinship	0.00	0.05	-0.09	0.09
Sex	-0.00	0.01	-0.02	0.01
Spatial distance	**-0.09**	**0.02**	**-0.12**	**-0.05**
Sampling interval	**-0.48**	**0.01**	**-0.50**	**-0.45**
30min Social Network	**0.14**	**0.04**	**0.06**	**0.21**
10min Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.42	0.05	-1.52	-1.32
Age	**0.02**	**0.01**	**0.01**	**0.04**
Kinship	0.03	0.04	-0.06	0.11
Sex	-0.00	0.01	-0.01	0.01
Spatial distance	**-0.11**	**0.02**	**-0.15**	**-0.07**
Sampling interval	**-0.50**	**0.01**	**-0.52**	**-0.48**
10min Social Network	**0.13**	**0.04**	**0.06**	**0.20**
2min Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.40	0.05	-1.51	-1.30
Age	**0.03**	**0.01**	**0.01**	**0.04**
Kinship	0.00	0.04	-0.09	0.09
Sex	-0.00	0.01	-0.01	0.01
Spatial distance	**-0.13**	**0.02**	**-0.17**	**-0.10**
Sampling interval	**-0.51**	**0.01**	**-0.53**	**-0.49**
2min Social Network	**0.14**	**0.05**	**0.05**	**0.25**

Table S2.12: Results of *brms* models testing whether the effect of social association strength on microbiota similarity (Jaccard Index) varies according to the sex of interacting individuals.

12h Social Network				
	Estimate	**Est.Error**	**l-95% CI**	**u-95% CI**
Intercept	-1.51	0.09	-1.69	-1.34
Age	**0.02**	**0.01**	**0.01**	**0.04**
Kinship	-0.00	0.04	-0.09	0.09
Spatial distance	-0.09	0.02	-0.13	-0.05
Sampling interval	-0.49	0.01	-0.52	-0.47
Sex category: male-male	0.12	0.11	-0.10	0.33
Sex category: female-male	0.06	0.06	-0.05	0.17
Social Network	0.10	0.13	-0.15	0.35
Social Network: Sex category, male-male	0.28	0.14	0.01	0.56
Social Network: Sex category, male-female	0.30	0.13	0.04	0.56

Table S2.13: Results of MCMCglmm models predicting microbiota diversity with covariates. Results from simplified MCMCglmm models predicting either asymptotic microbial Shannon diversity or asymptotic richness as a function of temporal, individual host and methodological factors. Models were simplified to remove the non-significant ($p>0.05$) variables age and sex. Significant ($p<0.05$) terms are shown in bold.

Asymptotic Shannon Diversity					
	post.mean	**l-95% CI**	**u-95% CI**	**eff.samp**	**MCMCp**
(Intercept)	83.55	48.71000	117.94433	1000.0	<0.001
month2	15.19	-2.16278	34.97057	1269.9	0.108
month3	10.98	-8.55660	25.70075	1000.0	0.188
month4	**18.48**	**0.75664**	**34.82477**	**888.5**	**0.024**
month5	15.51	-5.66490	35.37272	1000.0	0.162
month6	2.08	-14.00984	17.46371	911.8	0.810
month7	2.37	-24.16373	33.97004	1000.0	0.856
month8	**24.96**	**8.70720**	**43.91518**	**902.6**	**0.006**
month9	7.01	-14.94552	27.57888	809.6	0.514
month10	13.90	-1.87155	29.43618	1169.5	0.102
month11	0.88	-10.47376	12.65884	1000.0	0.900
month12	10.81	-7.44916	27.69678	1000.0	0.232

pcr_plate2	**18.37**	**9.29907**	**27.59661**	**1000.0**	**<0.001**
pcr_plate3	6.13	-2.55688	15.49759	1000.0	0.186
pcr_plate5	5.06	-12.85787	23.24887	1000.0	0.596
Habitat_Mixed	**-58.88**	**-92.06864**	**-25.08360**	**1144.8**	**0.002**
Habitat_Open_woodland	**-57.60**	**-88.02739**	**-26.77238**	**1146.2**	**0.002**
Habitat_Rhododendron	**-51.57**	**-82.94973**	**-18.69522**	**1184.4**	**0.002**
Region2	-7.88	-21.26818	6.96602	1000.0	0.304
Region3	14.64	-2.69574	32.01329	1000.0	0.090
Region4	-6.85	-24.49686	8.85641	927.1	0.444
Region5	1.75	-15.79396	19.06128	460.8	0.874
Region6	-0.07	-17.90074	15.07547	797.1	0.976
Region7	12.69	-4.78729	29.14240	886.3	0.136
Region8	-1.42	-26.49774	25.18387	1132.5	0.906
Region9	3.05	-10.21841	19.17771	1000.0	0.698
Asymptotic richness					
	post.mean	**l-95% CI**	**u-95% CI**	**eff.samp**	**MCMCp**
(Intercept)	0.018	1.089e+02	2.713e+02	1000	<0.001
Read Count	**0.003**	**1.676e-03**	**4.016e-03**	**1000**	**<0.001**
month2	**0.466**	**8.656e+00**	**8.777e+01**	**1000**	**0.030**
month3	**0.398**	**7.099e+00**	**7.081e+01**	**1000**	**0.016**
month4	**0.419**	**7.615e+00**	**8.403e+01**	**1173**	**0.030**
month5	0.237	-2.304e+01	6.739e+01	1000	0.308
month6	-0.175	-5.028e+01	1.863e+01	1000	0.342
month7	0.130	-4.848e+01	7.605e+01	1000	0.712
month8	0.297	-1.185e+01	6.396e+01	1000	0.148
month9	-0.246	-7.079e+01	1.649e+01	1124	0.268
month10	-0.142	-4.616e+01	1.831e+01	1000	0.392
month11	1.465	-2.417e+01	2.552e+01	1000	0.896
month12	**0.378**	**8.248e-01**	**7.357e+01**	**1000**	**0.046**
pcr_plate2	**0.324**	**1.141e+01**	**5.155e+01**	**1222**	**0.006**
pcr_plate3	6.562	-1.347e+01	2.638e+01	1000	0.518
pcr_plate5	-0.207	-6.065e+01	1.664e+01	1000	0.292
Habitat_mixed	-0.599	-1.387e+02	2.744e+00	1075	0.084
Habitat_open_woodland	-0.558	-1.171e+02	2.261e+00	1000	0.076
Habitat_Rhododendron	-0.514	-1.137e+02	1.160e+01	1000	0.130
Region2	-0.971	-3.852e+01	2.235e+01	1000	0.526
Region3	**0.387**	**3.255e+00**	**7.444e+01**	**1000**	**0.036**
Region4	-0.584	-3.967e+01	2.973e+01	1000	0.724

Region5	-0.134	-4.988e+01	2.543e+01	1000	0.508
Region6	-1.631	-3.515e+01	3.077e+01	1000	0.948
Region7	7.049	-2.751e+01	3.996e+01	1000	0.674
Region8	7.802	-4.797e+01	5.899e+01	1000	0.750
Region9	0.142	-1.771e+01	4.365e+01	1000	0.374

Table S2.14: Effects of social centrality metrics and non-social factors on gut microbiota Shannon diversity

Summary of social centrality metric effects on microbiota Shannon diversity, in MCMCglmm models including a single social centrality measure and controlling for all significant covariates identified in Table S2.13. Significance is inferred with MCMCp, a Bayesian p-value describing the extent to which credible intervals of posterior samples overlap zero.

	12h network		2min network	
	Posterior mean (95% credible interval)	**MCMCp**	**Posterior mean (95% credible interval)**	**MCMCp**
Intercept	6.4		4.7	
Degree	0.017 (CI -0.011-0.047)	0.272	0.102 (CI -0.032-0.227)	0.150
Weighted degree	-0.014 (CI -0.165-0.115)	0.864	0.032 (CI -0.127-0.172)	0.670
Eigenvector centrality	0.017 (CI -0.135-0.159	0.830	0.004 (CI -0.162-0.162)	0.999
Betweenness	0.040 (CI -0.071-0.138)	0.472	0.074 (CI -0.042-0.175)	0.188
Information Centrality	0.070 (CI- 0.062-0.180)	0.266	0.090 (CI -0.021-0.224)	0.144
Bridge Propensity	0.073 (CI -0.160-0.309)	0.546	0.060 (CI -0.037-0.158)	0.240

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3

Fine-scale variation in environmental exposure has weak effects on the gut microbiota of wild mice

List of contributions

- *Aura Raulo* designed the study, helped develop the new RFID tracking technology, designed the behavioural assay, collected the data from Wytham, completed all laboratory analyses on gut microbiota profiling prior to sequencing, developed analytical methods, analysed the data and wrote the manuscript
- *Genevieve Finerty* helped with home range analysis and the analysis of microhabitat variation and provided feedback on the manuscript
- *Maude Quicray* collected data from Wytham and helped develop the behavioural assay and plan the analyses of behavioural data
- *Holly English* helped collect field data using RFID loggers and provided feedback on home range analyses
- *Emma Dale* helped collect field data using RFID loggers
- *Curt Lamberth* led development of RFID tracking devices and helped collect field data
- *Josh Firth* supervised the research project and provided feedback on the analyses and the manuscript
- *Tim Coulson* supervised the research project and provided feedback on the analyses and the manuscript
- *Sarah Knowles* supervised the research project, helped develop the tracking technology and design the study, collected data from Wytham, planned and supervised laboratory methods, developed analytical methods and provided feedback on analyses and the manuscript.

Abstract

The diversity and composition of an individual host's microbiota is thought to affect the beneficial services it can provide, yet what determines microbiota variation within natural populations remains poorly understood. Recent research has emphasized the role of microbial transmission from the external environment to the gut as a potentially important force shaping variation in the gut microbiota. Individuals can vary in their exposure to different environments both by living in different environments and due to varying space-utilizing behaviours within the same environment. Here, we investigate the effects of environmental exposure on gut microbiota variation within a wild population of mice (*Apodemus sylvaticus*) at a very fine spatial scale. We trapped, sampled and compared the microbiota of mice living in different environments within a single woodland in the UK. In one location, we then used a new RFID-based tracking system to intensively monitor ranging behaviour of mice over a 10-month period to estimate activity behaviour, home range size and microhabitat diversity for 104 individuals. For a subset of individuals, we also sampled and analysed soil microbiota from the location they were trapped. Mice were found to have a localized gut microbiota, whereby geographic location among nearby trapping sites predicted 2-4% of total variation in gut microbiota composition and mice shared more microbial taxa with their local soil than with other soil in the same woodland. Fine-scale behavioural variation did not predict gut microbiota composition, but individuals with larger home ranges had significantly higher microbiota diversity. Further, counter to expectation, vegetation diversity within a mouse's home range negatively predicted gut microbial diversity. Overall these effects suggest that at this scale individual variation in environmental exposure has relatively weak effects on gut microbiota composition and diversity, and that fine-scale behavioural variation is not a major influential force shaping individual variation in the gut microbiota of wild wood mice.

3.1 Introduction

The mammalian gut microbiota plays an important part in the development, physiological pathways and even behaviour of their host. For instance, microbes of the gut are well known to aid digestion by breaking down poisonous and indigestible compounds (Kohl et al., 2014, 2016; Monachese et al., 2012; Turnbaugh et al., 2006) as well as regulate the metabolism and temperature homeostasis of their host (Chevalier et al., 2015; Fontaine et al., 2021; Visconti et al., 2019). The gut microbiota is also crucial for immune function, by defending the host against pathogenic infections (Giannetti & Staiano, 2015; Hooper et al., 2012; Zhang & He, 2015), guiding immune maturation in early life (Hooper et al., 2012) and tuning the host's immune system to recognise microbial enemies from allies (Honda et al., 2016).

Importantly, unlike other influential systems of the body, the gut microbiota is in a constant state of flux as microbes are exchanged between the host and its external environment. Consequently, while host animals control their microbiota composition to an extent, for example through immune mechanisms or selective feeding (Coyte et al., 2015), environmental influences still play a massive role in shaping these communities. Recent research has emphasized the role of transmission of microbes from the external living environment to the gut as an important force shaping individual variation in gut microbiota in humans, laboratory animals as well as wild populations (Moeller et al., 2018; Robinson et al., 2019; Rothschild et al., 2018). For example, human microbiota composition was shown to be strongly shaped by living environment, with humans sharing microbes with the buildings they live in (Dunn et al., 2013). A recent study even showed that the gut microbiota of students sharing the same room becomes increasingly similar to not only roommate's microbiota but also to the microbiota of the room itself (Sharma et al., 2019).

The tendency of microbiota to influence host physiology and at the same time be shaped by environmental transmission creates a unique biological pathway through which an individual's contact patterns with their environment can drive variation in their physiology and homeostasis. For example, exposure to environmental microbiota (soil) was shown to lead to higher gut microbial alpha diversity

and more realistic immune function in a mouse model of asthma (Ottman et al., 2019) and soil microbiota exposure was also shown to regulate mouse microbiota subsequently down-regulating anxiety-like behaviours (Liddicoat et al., 2020). An emergent line of research building on these environment-microbiota-host physiology links, focuses on the so called "Biodiversity hypothesis", that states that environmental biodiversity and human immune function are interrelated through microbial transmission processes (Hanski et al., 2012). Here, a sequence of studies comparing Finnish, Russian and Estonian children has emphasized the role of an individual's local environment in shaping their gut and skin microbial diversity. Specifically, children growing up in more rural environments, surrounded by higher local plant diversity or with more direct contact with natural soil, have been shown to host more environmental microbes (microbes present in the soil) on their skin and in their gut, and harbour overall more diverse microbiotas compared to urban children living in microbially poor environments (Lehtimäki et al., 2017; Ruokolainen et al., 2015, 2017, 2020). Importantly, this enhanced alpha diversity and prevalence of environmentally acquired microbes are suggested to be beneficial for host immune function. For example, both children and laboratory mice with more diverse, environmentally acquired microbiota developed a more well-regulated immune function, less prone to allergies and autoimmune diseases (Ottman et al., 2019; Ruokolainen et al., 2015, 2017)

The effects of contact with environmental microbes on the gut microbiota have been studied only in humans and laboratory rodents to date, and the role of environmental contact in shaping wild animal microbiota remains unclear. Most existing studies exploring fine-scale environmental differences in the gut microbiota focus on spatial variation alone, which will only partially reflect environmental transmission processes and can also be affected by variation in diet and host factors that vary spatially (e.g. host genetics). On a broad geographic scale, abundant evidence suggests that environmental variation shapes the microbiota in wild populations. For example, comparing 24 wild rabbit populations sampled across Spain, 12% of variation in gut microbiota composition was explained by their geographic location (Funosas et al., 2021) and location explained up to 16% of variation in gut microbiota among wild mice sampled across eight locations over 100km apart in western Europe (Linnenbrink et al., 2013). However, we know far less about to what extent environmental differences shape the gut microbiota within populations, though preliminary evidence suggests such fine-scale environmental variation may

not be unimportant. Variation between eleven trapping locations explained up to 15% of variation in gut microbiota within a freely interbreeding population of house mice inhabiting a small island of only 57-hectare area (Goertz et al., 2019). Similarly, gut microbiota similarity within an interconnected population of wild horses decreased linearly with geographic distance along the 50 km long Sable island (Stothart et al., 2021) implying that local environment had a significant effect on horse microbiota. Despite these findings on location-specificity of gut microbiota, firm evidence for transmission of microbes from the environment to the gut of wild animals is currently lacking, but some suggestive evidence for these processes do exist. In a study of baboons across multiple spatially overlapping populations, gut microbiota similarity was not only predicted by geographic distance but also strongly predicted by local soil properties, specifically the amount of sodium in the soil (Grieneisen et al., 2019). While these baboon populations belonged to more or less distantly related subspecies, the effect of local soil type on their gut microbiota was independent of, and stronger than effects of genetic or geographic distance. While this result could reflect indirect effects of soil on the microbiota through diet, it could also reflect a more direct influence of bacterial transmission from the environment (soil) on the baboon microbiota.

Importantly, individual variation in environmental microbial exposures can arise not only from spatial environmental heterogeneity, but also from how individuals behave within their environment. Animal individuals exhibit considerable repeatable variation in their behavioural profiles, called "animal personalities", "behavioural syndromes" or "coping styles" (Carere et al., 2010; Koolhaas, 2008), which can lead them to utilize the same available space in very different ways. For example, grey squirrels with more explorative and bold personalities were more likely to be exposed to and subsequently infected by a gastro-intestinal parasitic worm *Strongyloides robustus* (Santicchia et al., 2019) and similarly, tadpoles with more active personality accumulated higher parasite loads (Koprivnikar et al., 2012) Behavioural differences clearly drive variation in fine-scale environmental contacts within animal populations, capable of inducing variation in environmental microbial transmission patterns and consequently microbiota composition.

Notably, while research on animal personalities driving infection risk is abundant, effects of behavioural variation on gut microbiota composition are largely absent outside the context of social behaviour. Similarly, to our knowledge, studies on how environmental transmission might affect the microbiota at a fine (within-population) spatial scale are currently lacking. Specifically, a key open question concerns whether naturally existing variation in environmental exposures among members of a population may shape variation in the composition or diversity of their microbiota. If contact with the environment serves as a microbial transmission route that can shape overall microbiota composition, one can hypothesise that microbiota alpha diversity will be predicted by individuals' diversity of environmental exposures, as reported in humans (Hanski et al., 2012; Lehtimäki et al., 2017). While the roles of environmentally acquired microbes and general microbiota alpha diversity in shaping host health and fitness remain ambiguous (Reese & Dunn, 2018), having a richer microbiota and hosting environmental microbes could have potential benefits for the host, from increased caloric uptake (Suzuki et al., 2020; Turnbaugh et al., 2006) to a better regulated and resilient immune system (Round & Mazmanian, 2009; Zhang & He, 2015). For example, more diverse microbiotas may protect their host against pathogen invasions (by outcompeting invaders; Tuddenham & Sears, 2015) as well as autoimmune reactions, by tuning the acquired immune system to recognise enemies from allies (Honda et al., 2016.). The latter point was emphasized by a study showing that transplanting a natural microbiota (with more even diversity of different bacterial taxa) from wild mice into laboratory mice enhanced their immune function against both infections and autoimmune disease and ultimately increased their survival (Rosshart et al., 2017). Thus, uncovering the impact of environmental transmission on animal microbiotas is an important goal, if we are to understand the microbiota's role in the interplay between the environment and animal ecology and evolution.

Here we set out to study both broad-scale effects of a shared environment and the fine-scale effects of variation in environmental contacts within a shared environment on the gut microbiota of wild wood mice (*Apodemus sylvaticus*) in Wytham Woods, Oxford. We trapped mice, collected vegetation data and soil microbiota samples across 5 different nearby sites within the same woodland to estimate to what extent nearby geographic location and local soil microbiota affect overall gut microbiota composition. We then used a novel RFID-based tracking system to intensively follow a set of 104 mice within one of

the study sites, a 2.3-hectare woodland plot, and used this tracking data together with behavioural assays performed in short-term captivity to estimate how individuals vary in three aspects of behaviour relevant to environmental exposure: 1) home range size, 2) activity behaviour and 3) microhabitat diversity. Finally, we tested whether these proxies of environmental exposure predicted both composition and alpha diversity of the gut microbiota. We set out to test four main hypotheses:

1) If there is fine-scale geographic variation in the gut microbiota, community composition should be more similar among individuals from the same site than among individuals from different sites within the same woodland
2) If mice acquire some gut microbes from soil, their gut microbiota should share more microbes with soil samples from their own site than with soil samples from other sites
3) Within a site, individual variation in environmental exposure (home range size, activity behaviour or microhabitat diversity) should predict variation in gut microbiota composition (beta diversity), as different exposure patterns may lead to transmission of different microbes
4) Mice with more diverse environmental exposure (larger home ranges, more active behaviour or more diverse microhabitat) should have higher gut microbial alpha diversity

3.2 Methods

Mouse sampling and tracking

In the first part of this study, wood mice were trapped across 5 different study sites within Wytham Woods (Figure S3.1A) during June 2018- June 2019, using 60-120 Sherman traps per fortnightly trapping. These sites were all within the same 150-hectare area, but still far enough apart that regular movement of animals between sites is expected to be rare. Previous research (Godsall et al., 2014) suggests wood mouse home ranges do not generally exceed 8000m^2 (diameter of approx. 100 m, in a circular range), while the shortest distance between study sites was 180 m (Figure S3.1). Consistent with this, no individual mouse was ever captured in two different sites. Trapping sites were 1 ha rectangles, except for one which was 2.3ha, and sites varied in habitat type (meadow, sedge-dominated, coniferous woodland, 2 x deciduous woodland). Sites were not trapped evenly throughout the year (Figure S3.1B),

making site-effect on microbiota samples slightly confounded with seasonal variation. For this reason, we used sampling month as a covariate in all models with "site" as a predictor.

Upon trapping, mice were injected with a passive tracking tag (PIT-tag) for permanent identification, demographic data on mouse individuals were recorded (sex based on anogenital distance and age, based on weight and pelage colour) and faecal samples for gut microbiota analysis were collected from traps into sterile 2ml tubes using sterilized tweezers, and frozen at -80 C within the same day. Traps showing any sign of rodent contact were carefully washed and sterilised using bleach between trapping sessions. For a subset of individuals from all sites, we also collected a paired representative local soil sample. This soil sample was collected from the mouse's trapping location by picturing a meter-wide triangle around the trapping location and collecting a spoonful of soil (~200 mg) from 3 cm underground in each of the three corners into a sampling tube. Traps were set at dusk, collected at dawn and captured mice were released at their exact trapping location within 8 hours of trap collection.

In the second part of the study we performed in-depth monitoring of mouse movements and microhabitat use over a 10-month period on one of these study sites, our main trapping grid of 2.56 ha ("Holly Hill"). Here, we tagged and tracked movements of resident mice using a set of 60 custom-built motion-activated PIT-tag loggers, devices that record the time-stamped presence of any tagged rodent within the ~1m^2 range of their detection coil (Figure 1). Loggers were not baited with any kind of lure, and were evenly distributed across the grid and rotated fortnightly to ensure spatially even monitoring. This was done by giving each logger a 400m^2 "territory" of 4 contiguous 10m x 10m grid cells around which they were rotated fortnightly in a systematic design, so that each logger covered its entire territory over a 2-month period. In this way, each 10m x 10m grid cell of the study area was covered by a logger 25% of the time (one fortnight every two months). With this design, the maximum distance to nearest logger from any point was 11.2 m. For the home range analysis, which is a relatively data-hungry method, we additionally included logger data collected during the fall (July-November) with a set of 60 extra loggers, positioned at known mouse burrow entrances, approximately evenly across the grid, which were not rotated (Figure S3.2). Details on how mouse burrows for logging were identified and chosen can be found in Supplementary Material, Appendix S3.1. To ensure that the subsequent higher logger density in the fall did not bias our home range estimates, we used the subset of individuals with the most logger data to compare home ranges derived with or without this extra logger data (Supplementary Material,

Appendix S3.2). Microbiota samples from tracked individuals were collected fortnightly from 1.5 months prior (Dec 2018) until the end of the logging period (Nov 2019).

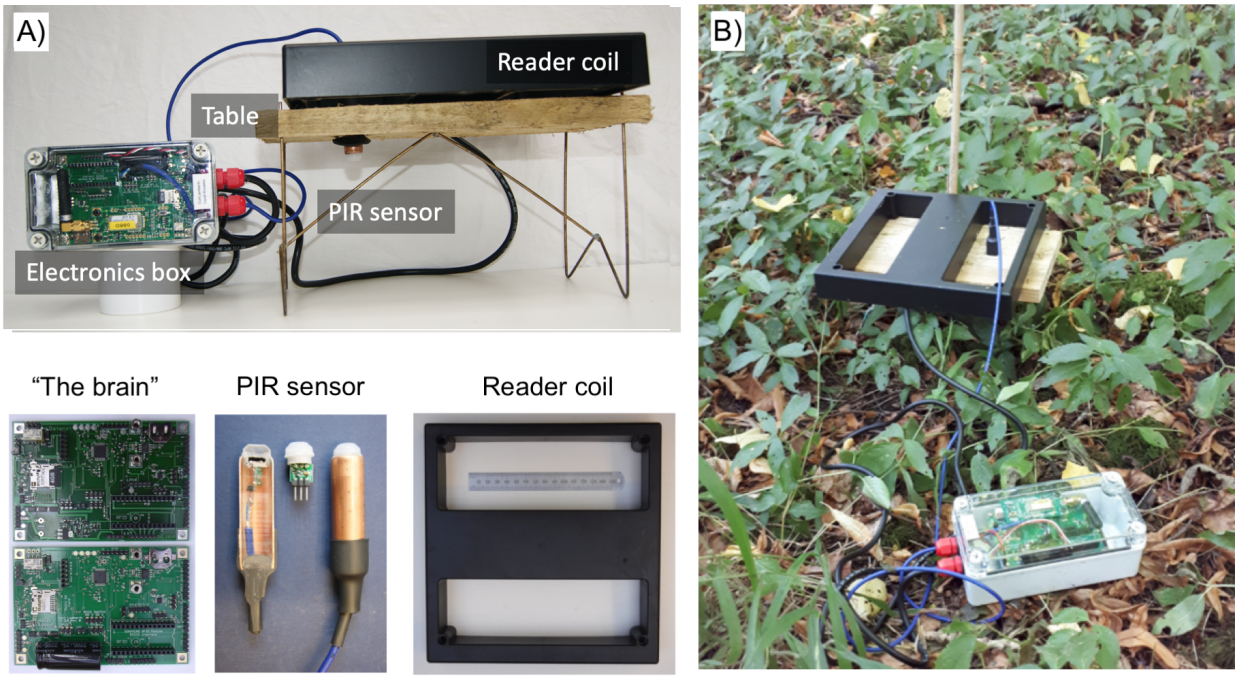


Figure 1. **The custom-made RFID-logger.** A) Logger consisted of a motion sensor ("PIR sensor"), that woke up the reader coil whenever a warm object, such as a rodent, came nearby. The coil, sitting on a wooden trestle (which shields the PIR sensor from sunlight and allows rodents to pass under as well as around it), creates a ~1 m^2 oscillating detection field that would interfere – and subsequently log- any 124 kHz RFID tag within this field. All captured wild rodents were tagged with a subcutaneous passive integrated transponder (PIT-) tags containing a 10-digit HEX-code identification number readable in this oscillation frequency. When a rodent tag was read by the coil, this identification number was received by the chip in the electronics box ("the brain") which controlled the device and its data. The tag number, together with a time stamp and technical information about the logger (e.g. temperature and battery level) were then saved by the brain on an SD-card and additionally transmitted to an interactive internet database through an internal antenna (in the electronics box) connected to the broadband network covering Wytham Woods (Wytham Data Net). The logger was programmed to wake up automatically every 4 hours to send an "I'm alive" signal to the data base, enabling researchers to notice when a logger had or was about to run out of battery, and charge it. Battery life of the logger was 1-4 months depending on the seasonally shifting level of PIR triggers from rodent activity and sunlight on low-level vegetation.

Behavioural assays

To compliment field-based measures of home ranges and movement behaviour from loggers, we also subjected a subset of trapped individuals on Holly Hill (n=50) to a behavioural assay shortly after capture, the Open Field Test (OFT). The OFT is a simple behavioural assay commonly used to measure movement behaviour in captive as well as wild-caught animals (Dingemanse et al., 2002; Gould et al., 2009), see for example (Dingemanse et al., 2002). The OFT setup is pictured in Figure 2. Prior to handling, a mouse was released from its trap to a cloth bag and from here released through a small hatch into the OFT arena. The box was positioned in a separate small room with covered windows to create low light conditions. When the mouse had entered the box, the hatch door was closed, lights were turned off and the individual was left alone in the box (with researchers leaving the room). An overhead mounted night-vision camera recorded mouse movement for 5 minutes after which the mouse was retrieved by opening the hatch door and letting it run back into a trap. The OFT arena was thoroughly cleaned between assays, aided by a removable plastic floor and acryl-spray-painted inner sides of the plywood walls. Videos were used to derive estimates of activity level and boldness. We used the video-processing software *AnimApp* (Rao et al., 2019) to record the location coordinates of a mouse within the box during each frame of the video (25/second). Unreliable coordinates were filtered out using the app's detection reliability measure, the size of the circle it draws around the moving target, as well as our own filtering code, which looked for realistically impossible jumps between locations (e.g. when one frame reported the target further than one grid square away while the next frame reported the mouse in the original location). The resulting coordinates were then used to generate a movement path and calculate the total distance moved in 5 minutes (a proxy for general locomotor activity/movement behaviour, hereafter called "assay activity") as well as the propensity to enter the centre region of the box (a proxy of bold behaviour, (Gould et al., 2009), hereafter called "assay boldness").

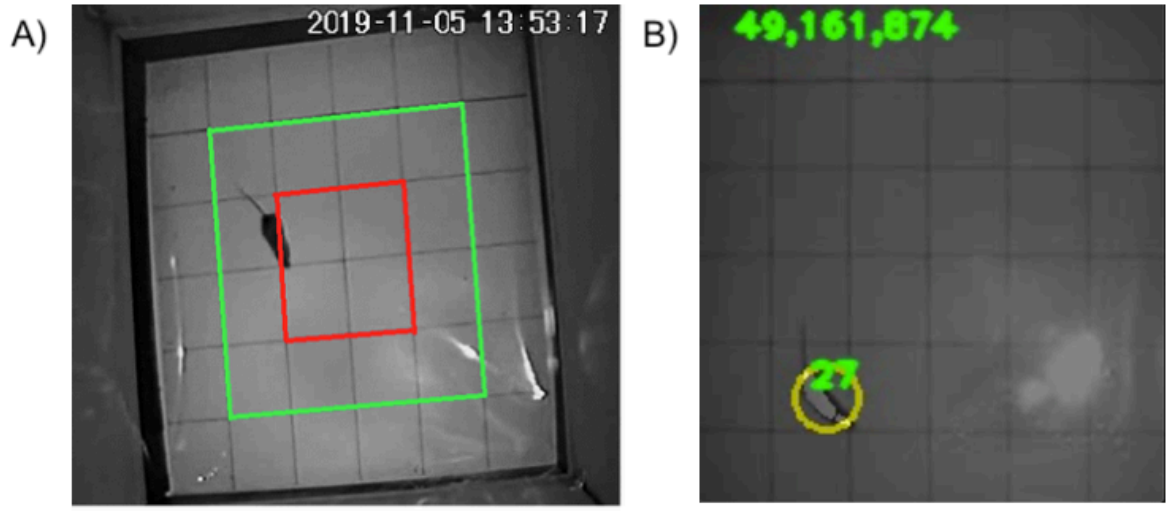


Figure 2. Open field Test setup. A) Picture of a mouse in the 1m^2 assay box (with 1m high plywood sides) and gridded markings on the base. Squares outside the green lines constitute the periphery (sides) and squares inside the red lines constitute the centre area. B) Screen shot of assay video being processed by the AnimApp-software, which draws a circle around the moving object (here a mouse) and tracks its location in the arena. Numbers in the top indicate the coordinates and angle of the mouse and number on the mouse indicates the size of the detection circle, which is a measure of confidence/resolution of the tracking.

Home range and activity estimates from loggers

Logger data, which included the time-stamped record of tagged individuals in a specific location was aggregated within ten-minute periods, such that all records of an individual at the same location within each ten minute-period were considered a single unique "logger visit". Using these nightly logger records, we derived three measures to describe individuals' natural movement patterns: **1) Nightly activity**, the mean number of unique consecutive "logger visiting bouts" per night, across nights when an individual was detected (Figure 3A). A unique logger visiting bout is defined as any period of time (or number of visits) spent at a single logger between which an individual is not detected in any other location. Thus, every time a mouse was detected in a logger that was not the same as where its last detection was, this was counted as a unique visiting bout. For example, if a mouse's logger record in time order was for example A-A-A-B-A (Where A and B are different loggers), this would equal to three unique logger visiting bouts. **2) Nightly ranging distance**, the maximum distance between unique logger

locations on a given night, averaged across nights when an individual was detected (Figure 3B). **3) Home range size**, an estimate of the area across which a mouse was detected during the entire 10-month period (Figure 3E). An animals' home range can be defined as "the area, usually around a home site, over which the animal normally travels in search of food" (Burt, 1943) and is commonly presented as a utilization distribution describing the probability of space use with respect to time (Powell & Mitchell, 2012). We quantified home ranges from logger data using an autocorrelated kernel density estimator ("AKDE"; (Fleming et al., 2015) implemented using the *ctmm* package (Calabrese et al., 2016). Home range boundaries were delineated at the 75% level to provide an estimate of the core home range, the smallest area you could expect to find an individual in with 75% probability. Home ranges were calculated only for individuals satisfying our criteria for a complete and stable observation record, based on variograms estimating temporal autocorrelation in spatial records (Supplementary Material, Appendix S3.3, Figure S3.3). Under these criteria, home ranges could be estimated for 104 of the 157 mice recorded on loggers. Overall, therefore, we derived five measures of mouse space use and movement across field-based and captive settings, which capture alternate aspects of spatial and movement behaviour (Figure 3A-E).

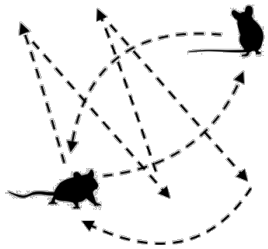
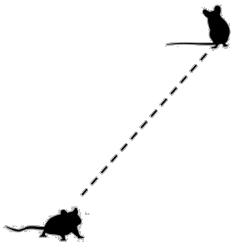
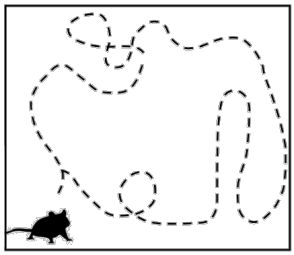
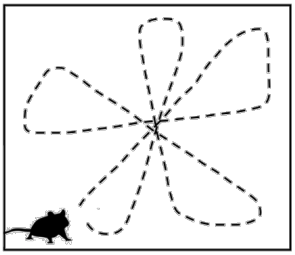
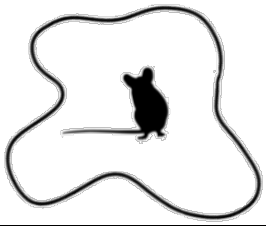
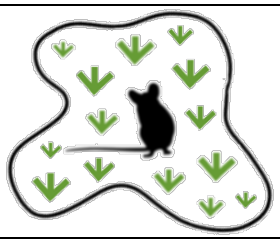
		
Behavioural variable	**A) Nightly activity**	**B) Nightly ranging distance**
Description	Mean number of unique logger visits per night	Average within-night maximum distance between detection locations, across all active nights
Implications for environmental exposure	High value implies more intensive exposure to the environment	High value implies broader exposure to the environment
		
Behavioural variable	**C) Assay activity**	**D) Assay boldness**
Description	Metric distance (mm) travelled in 5 minutes in the open field test	Number of occasions on which the mouse entered the centre area of the assay arena
Implications for environmental exposure	High value implies a tendency for more intensive exposure to environments	High value implies lower tendency to avoid open spaces
		
Behavioural variable	**E) Home range size**	**F) Microhabitat diversity**
Description	Area (m^2) of the 75% core of the kernel utilization distribution of logger visits.	Shannon diversity of main ground cover-types within the home range
Implications for environmental exposure	High value implies exposure to larger area of environment	High value implies exposure to a more diverse micro-environment

Figure 3. Descriptions of different measures of environmental exposure variables and their implications for environmental contact patterns potentially relevant for microbial transmission from the environment to the gut.

Vegetation survey and habitat diversity estimation

To enable characterisation of plant community composition and diversity in each mouse's home range, we performed a vegetation survey of the main Holly Hill plot in May 2020, when flowering plants are easiest to identify. For each 10 x 10 m grid cell we recorded percentage cover for eight main types of ground cover, as well as presence-absence of all other identifiable herb and tree species. The main ground cover types were defined as open ground or any plant species that, during a pilot data collection round, had been observed to grow in continuous patches covering more than 1 m^2 of area. Main ground cover types were: 1) open ground (OG), 2) dog's mercury (DM; *Mercurialis perennis*), 2) blue bell (BB; *Hyacinthoides non-scripta*), 3) bramble (BR; *Rubus fruticosus*),4) grass (G; species in the family *Poaceae*), 5) sedge (S, *Carex pendula*), 6) Enchanter's night shade (EN; *Circaea lutetiana*), 7) wild garlic (WG; *Allium ursinum*) and 8) Currant (RI, *Ribes rubrum*) (Figure S3.4). We also recorded the percentage of open canopy and amount of dead wood (with a score ranging 0= no, 1=little, 2=medium amount or 3= a lot of dead wood) in each grid cell. Lastly, on a coarser scale we recorded the presence/absence of tree species (with trunk diameter > approx. 10 cm) and other, less abundant herb species for each 10m x 10m grid cell. All identified plant species and their prevalence across the grid are summarized in Supplementary Table S3.1. To summarise vegetation community composition, we ran a Principal Components Analysis (PCA) first using all habitat variables (Figure 4) and then only main ground cover habitat types (Figure S3.5). Both showed a similar pattern, where the first axis of variation roughly represented the amount of light (with amount of open ground and open canopy at the extreme ends) and the second axis was more related to vegetation type ranging from relatively open woodland with dog's mercury and bluebells to more bushy vegetation including bramble, sedge or grass (Figure 4). Since the variation patterns were similar in both PCAs, we chose to focus on results from the PCA using only percentage cover variables. The percentage cover of each main vegetation type within each mouse's home range was extrapolated by overlaying the boundary of the 75% home range (as described above) on the grid map, and taking the mean of abundance scores for all vegetation types across grid cells that fell within, or intersected with, this boundary.

These values were then used as measures of abundance to calculate a vegetation habitat Shannon diversity index (ref) for each mouse. We also calculated another habitat Shannon diversity index for all plant species (including rarer herbs and trees), using species incidence (proportion of grid cells within a home range a species was observed) as the abundance measure, as described in (Chao et al., 2014).

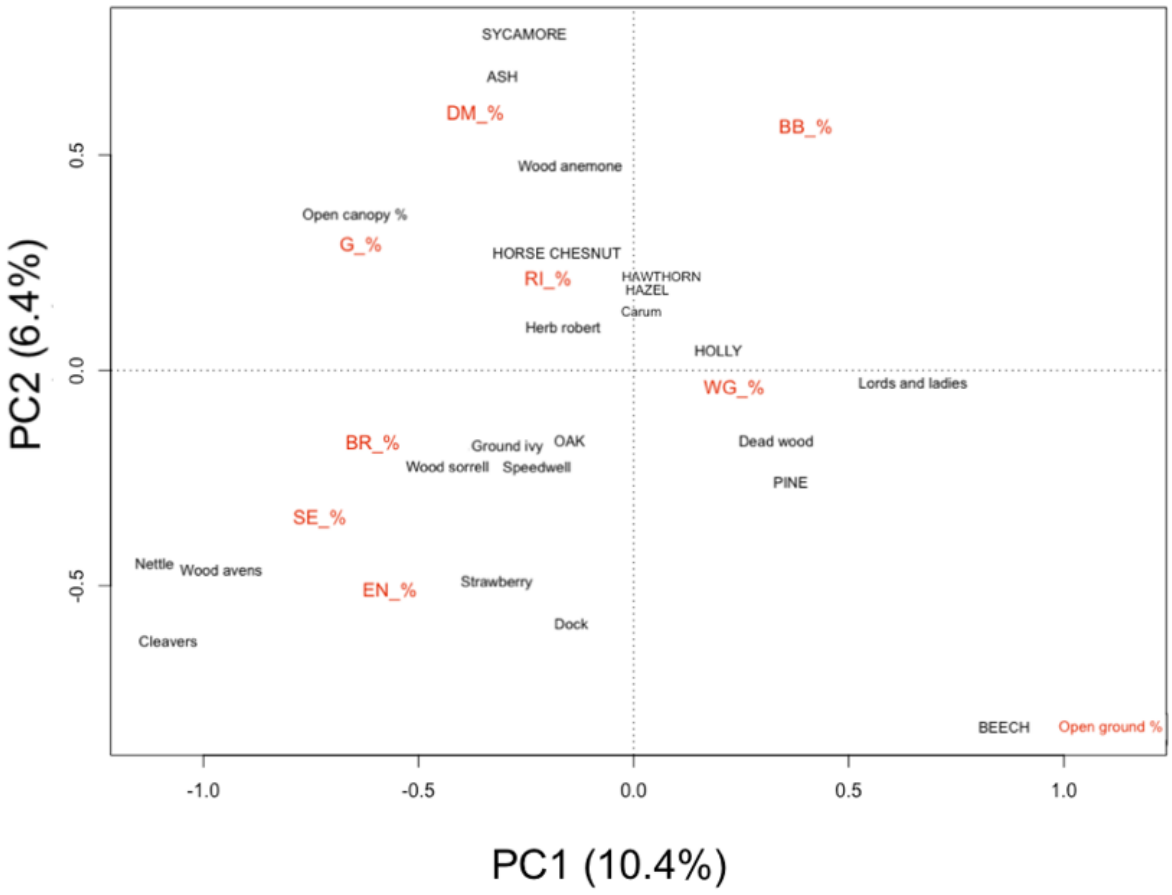


Figure 4. Principal Components Analysis illustrating relative position of each habitat variable on the first two PC axes across all collected habitat variables. Tree species are written in capitals, herbs and other habitat variables in lower case. The main ground cover variables are shown in red.

Microbiota characterization

The gut microbiota was characterised from faecal samples using 16S rRNA amplicon sequencing, with 401 samples from 193 individual wood mice profiled in total. Soil microbial communities were profiled from 24 soil samples across three sites (See Table 1).

Table 1: Sample size and site information across study locations

Study Site	Area	Habitat	Mouse microbiota samples	Soil microbiota samples	Mouse individuals sampled
Site 1 (Old Main)	1 ha	Deciduous woodland	6	4	6
Site 2 (Meadow)	1 ha	Open meadow	4		4
Site 3 (Conifer)	1 ha	Coniferous woodland	22	5	22
Site 4 (Sedge)	1 ha	Sedge-dominated wetland	2		2
Site 5 (Holly Hill)	2.56 ha	Deciduous woodland	367	15	192

DNA was extracted from samples using the Zymo Fecal/Soil 96-well kits, with soil and gut samples randomized into DNA extraction batches and sequenced and analysed together. Primers 515F and 926R (Walters et al., 2016) were used to amplified a section of the V4-V5 region of the 16S rRNA gene. Sequencing was performed on an Illumina MiSeq at the Centre for Genomic Research, Liverpool. 16 extraction (water) controls (8 extraction controls and 8 pcr controls) and 6 mock communities were prepared and sequenced alongside 634 microbiota samples. Full details on library preparation and sequencing and an evaluation of sequencing success can be found in Supplementary Material, Appendix S3.4 and Figure S3.6. All subsequent data processing, bioinformatics and statistical analyses were performed in R 4.0.3 (R Core Team, 2020). Sequence data were processed through the DADA2 pipeline (Callahan et al., 2016) to infer amplicon sequence variants (ASVs) and taxonomy was assigned using the Silva Database (Quast et al., 2013). The resulting data had observable levels of contamination (some bacterial DNA in the negative control samples as well as a spatial autocorrelation signal in microbial community similarity across samples on some extraction plates), which we dealt with by removing taxa identified as contaminants using the *decontam* algorithm (Davis et al., 2018), and by having the extraction- and PCR-plate of each sample as technical covariates in all models. Full description of the contamination issue and mitigation can be found in Supplementary Material, Appendix S3.5. Decontaminating and subsequent pre-processing was done separately for soil and mouse gut microbiota samples, in package *phyloseq* (McMurdie & Holmes, 2013). This included using sample completeness and rarefaction curves made using package iNEXT (Hsieh et al., 2016) to decide a read depth filtering threshold (5000 reads for mouse samples and 1000 reads for soil samples) below which samples were dropped from the dataset (Figure S3.7) and filtering out taxa belonging to Cyanobacteria from both gut and soil samples, as these ASVs may represent plant chloroplasts. Finally, we normalized ASV counts

to proportional abundance within each sample for beta diversity analyses (McKnight et al., 2019). We estimated gut microbial alpha diversity by calculating asymptotic values of ASV richness and Shannon diversity in package iNEXT. To estimate similarity in microbiota composition among sample (beta diversity) we used both a presence/absence-based metric (the Jaccard Index of similarity) as well as the abundance-weighted Bray-Curtis index of dissimilarity.

Statistical analyses

To test whether microbiota composition (Jaccard Index, the proportion of ASVs in a pair that are shared) was predicted by sampling site, we used a Wilcoxon rank-Sum test. We tested whether the microbiota of mouse-mouse, soil-soil or mouse-soil sample pairs was more similar within sites than between sites. Since the response variable is a set of non-independent pairwise comparisons, a permutation test was used to assess significance, in which we compared the observed test statistic to the distribution of test statistics generated when using one of 1000 random permutations of Jaccard Index values across sample pairs.

Permutational analysis of variance (marginal PERMANOVAs) on Jaccard distances and Bray-Curtis dissimilarity matrices, were used to assess environmental and host-level predictors of microbiota composition across samples. Models were run using the adonis2 function in package *vegan* (Oksanen et al., 2008). In a first model including all soil samples and one randomly selected faecal sample per mouse, we tested the effects of sample type (soil/mouse), season (month) and sampling site on microbiota composition, after which separate PERMANOVAS were performed for faecal and soil sample sets, to examine temporal (month) and spatial (site) variation. For mouse samples we then ran a model with all samples (repeated samples per individual), to assess the variance explained by individual identity. After this, we used a subset of data including one randomly selected sample per individual to estimate the effects of individual level variables (age, sex, body mass, home range size, logger-derived behaviour variables, microhabitat diversity) alongside sample-level variables (sampling month, read depth, extraction plate, PCR plate) on microbiota composition. We also ran an alternative model with the same variables except instead of microhabitat diversity, first two principal component axes of habitat variation

were used (habitat PC1 and habitat PC2). The effects of assay activity and assay boldness on microbiota composition were tested in a PERMANOVA on the subset of samples for which this data was available.

Lastly, to test whether our various measures of environmental exposure predicted gut microbial alpha diversity in mice, we used Bayesian mixed models in package *MCMCglmm* (Hadfield, 2010). Two separate (gaussian regression) models were performed with either gut microbial richness or microbiota Shannon diversity as the response, and our four environmental exposure variables as predictors: home range size, microhabitat diversity, nightly activity and nightly ranging distance. The following fixed effects were also included: sampling month (10-level factor), host sex, age (Adult, Juvenile), body mass and sample read depth, as well as random factors for mouse ID, extraction plate and PCR plate. All numeric variables were scaled between 0-1 to enable comparison of posterior means. A separate MCMCglmm model was performed on the subset of samples from mice that undertook the OFT test (the same day as the sample was collected) to examine effects of assay activity and assay boldness, alongside the same set of covariates listed above.

3.3 Results

Microbiota similarity among wood mice and between mice and their local soil

The wood mouse gut microbiota contained 1760 unique bacterial ASVs overall, and was dominated by microbes belonging to two bacterial families Muribaculaceae and Lactobacillaceae (Figure 5). Local soil microbiota was richer in microbial taxa (ASVs as well as families) but more homogenous in composition than mouse microbiota, with 4006 bacterial ASVs dominated by microbes in the families Xanthobacteriaceae and Chitinophagaceae (Figure 5). In this dataset, mouse gut and local soil microbiota shared in total 63 bacterial ASVs belonging mostly to the families Xanthobacteriaceae and Methyloligellaceae, though the precise numbers may have been affected by contamination in the data (See Supplementary Material, Appendix S3.5). 39 of these shared ASVs were more abundant in mice, while 20 were more abundant in soil, and thus more likely to represent microbes living in the soil.

Reads belonging to these potential soil-inhabiting microbes comprised on average 0.8% reads (range 0-0.9%) in a mouse gut microbiota sample.

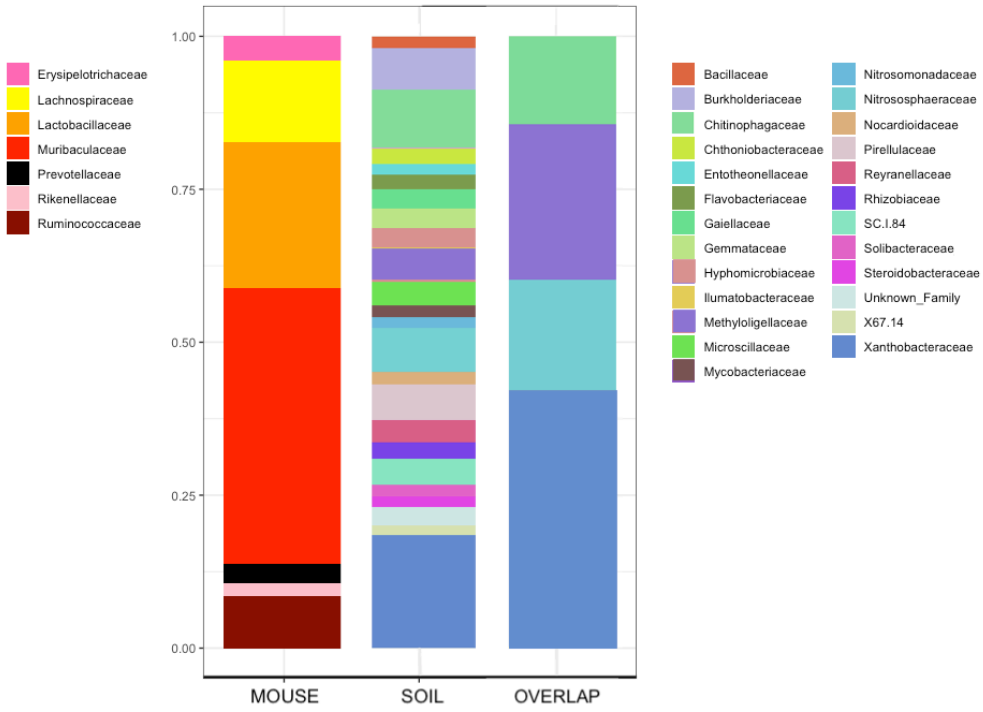


Figure 5. Composition of the soil and wood mouse gut microbiota at the family level. Plot includes all microbial families that were present at >1% abundance within each sample type. The most abundant bacterial families shared between mouse gut and soil microbiota (right panel) are Chitionphagaceae (mint green), Methyloligellaceae (purple), Nitrososphaeraceae (turquoise) and Xanthobacteriaceae (blue)

The gut microbiota of wood mice from the same site shared significantly more microbial taxa (ASVs) than that of mice caught at different sites within Wytham (permuted Wilcoxon rank sum test on Jaccard Index, $p<0.01$, Figure 6B). Soil samples from the same site also shared more taxa than those from different sites (permuted Wilcoxon rank sum test, $p<0.01$, Figure 6B), though site differences in soil microbiota composition were less striking. When comparing mice between sites, their average Jaccard Index was 0.08, while for mice from the same site it was 0.12, meaning that they shared 12% of all ASVs they had. Soil microbiota was much more similar across sites, with samples sharing on average 38% and 46% of their ASVs between sites and within site respectively (Figure 6B). While wood mice shared few taxa overall with soil (on average <1% of their gut microbial ASVs shared with a soil sample), the

proportion of shared taxa was approximately twice as high for pairs of mice and soil from the same site as compared with mice and soil from a different site (0.9% shared ASVs vs 0.4% shared ASVs, Figure 6).

Compared to soil microbiota, mouse gut microbiota was more variable (Figure 6), largely due to individual repeatability and temporal variation in composition. 59-60% variation was explained by host individual identity (marginal PERMANOVA on Jaccard similarity: R^2=0.59, p<0.01; Bray-Curtis dissimilarity: R2=0.60, p<0.01) while 10-13% of variation across all mouse samples was explained by month (marginal PERMANOVA on Jaccard similarity: R^2=0.10, p<0.01; Bray-Curtis dissimilarity: R^2=0.13, p<0.01). When using only one random sample per individual, month explained 12-16% of variation in microbiota composition, while study site explained only 2-3% (marginal PERMANOVA on Jaccard similarity: R^2=0.02 p<0.01; Bray-Curtis dissimilarity: R^2=0.03, p<0.01).

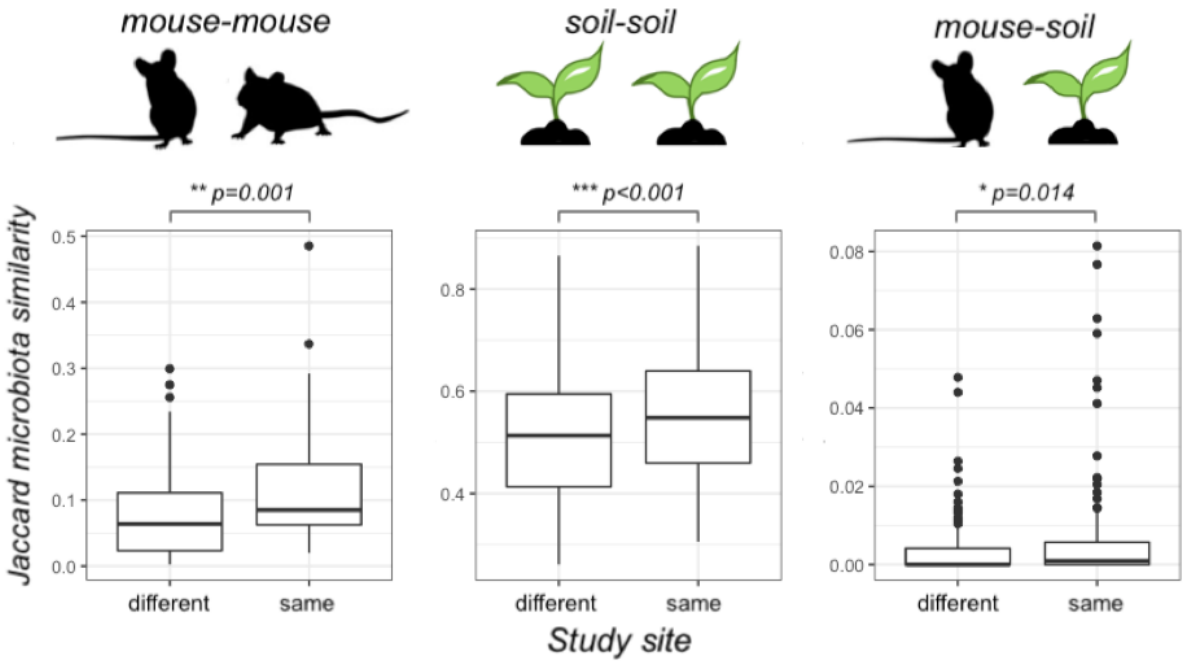


Figure 6. Microbiota similarity patterns among soil and mouse gut sample pairs within and across study sites in Wytham Woods. Boxplots depict Jaccard similarity values of mouse-mouse, soil-soil and mouse-soil sample pairs taken from the same or different sites. p-values are based on a permuted Wilcoxon rank sum test predicting microbiota Jaccard Index with study site similarity (same vs different).

Variation in wood mouse behaviour and patterns of environmental exposure

Over the 10-month logging period on the main grid, 86% of (167 out of 192) mice tagged on the plot since 1.5 months before the logging started were recorded on loggers, with a mean of 23 (sd 25.3) nights of active data per individual (Figure S3.8). Wood mice showed considerable and individual variation in their behavioural profiles, and some measures of activity across field and captive contexts were correlated. Mice that scored high for activity in the captive open field test (Assay activity) tended to also range slightly further within a night (Nightly activity) and mice who ranged further within a night had significantly larger home ranges (Figure 7). Males had higher average values of all measures of environmental exposure but assay boldness and home range size were the only variables where this sex difference was significant (two-tailed t-test, $p=0.038$ and $p=0.030$ respectively). Microhabitat diversity and composition varied considerably across the study site and accordingly, home ranges in different parts of the study site encapsulated varying microhabitats (Figure 8).

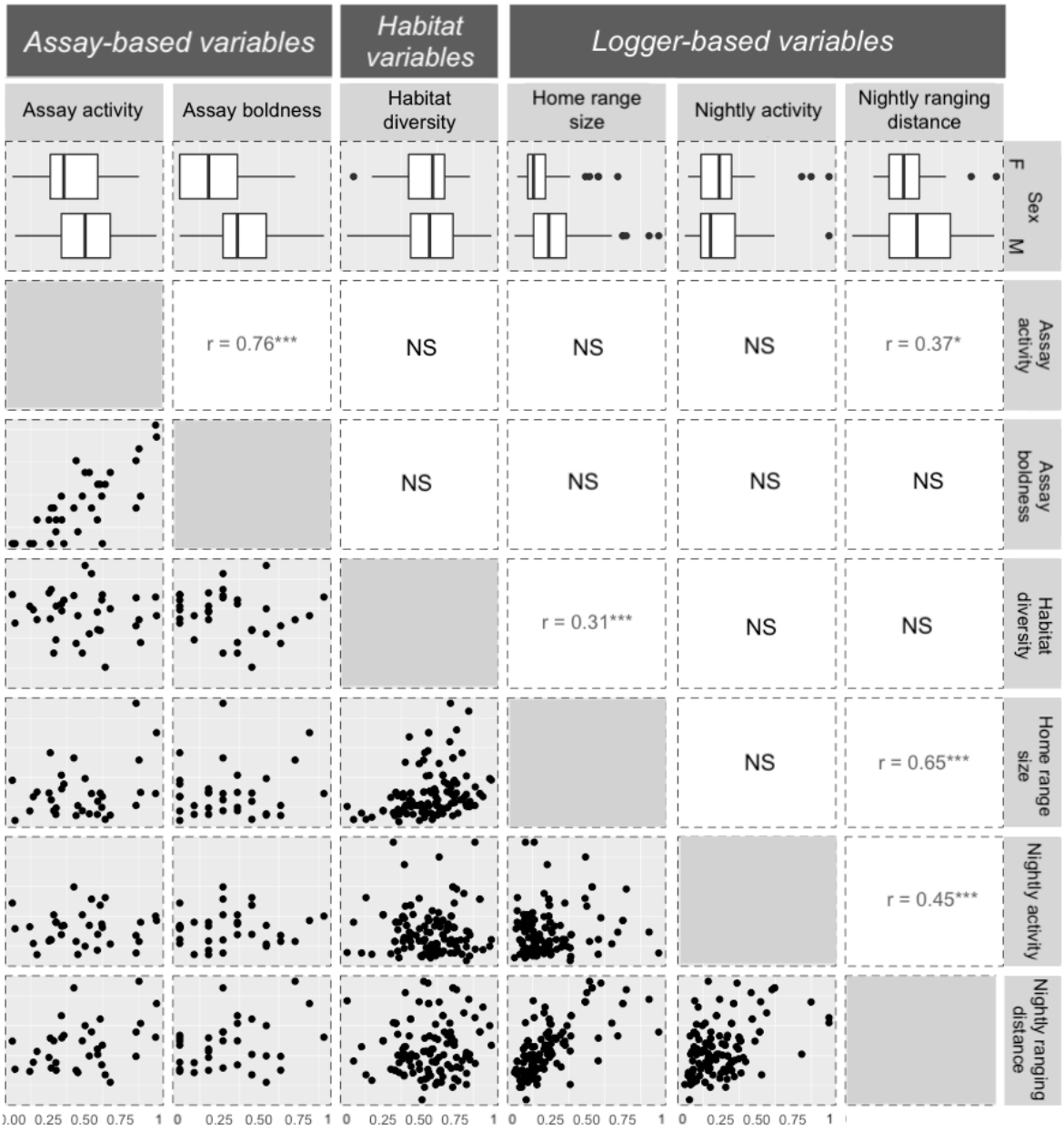


Figure 7. **Pairplot of correlations among the behavioural measures describing different aspects of environmental exposure**. Top row summarizes variation between sexes in each variable. Lower triangle visualizes correlation between intersecting variables and upper triangle reports significant pairwise correlations (Pearson's correlation coefficient).

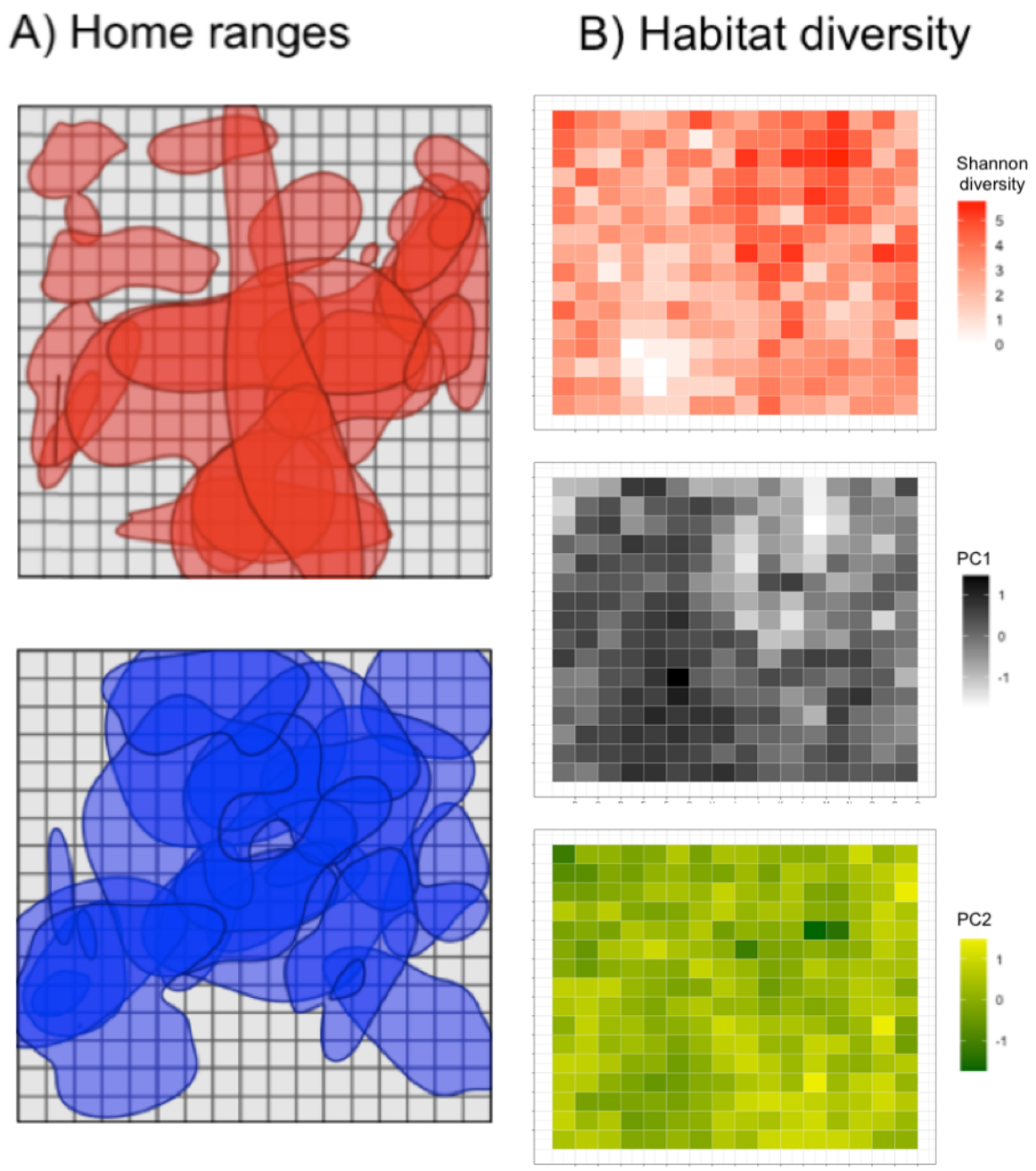


Figure 8. **Distribution of mouse home ranges and habitat variation across the Holly Hill study site**. A) Spatial location of the home ranges of 20 randomly chosen example female (red) and male (blue) mice. B) Spatial distribution of habitat variables (from top to bottom: habitat Shannon diversity, PC1 and PC2 of habitat composition) across the 10 x 10 metre grid cells of the 2.4 ha study site. In all subplots, x-axis represents direction approx. from west to east and y-axis from south to north.

Effect of environmental contact variables on microbiota composition

No measures of host movement behaviour or home range size from loggers, microhabitat diversity or microhabitat composition predicted gut microbiota composition among mice on the main study plot (Table S3.2), though the effect of Habitat PC1 on microbiota composition was near significant (marginal PERMANOVA R^2=0.01, p=0.07 Tables S3.2B, S3.2E). In models examining mice that performed the OFT, assay activity significantly predicted microbiota composition, explaining 3% of variation in microbiota composition across mice (marginal PERMANOVA R^2=0.03, p=0.01, Tables S3.2C, S3.2F), while assay boldness did not predict microbiota composition (Tables S3.2C, S3.2F).

Effect of environmental contact variables on microbiota alpha diversity

Mouse home range size positively predicted both measures of gut microbial alpha diversity (Richness: Posterior mean= 0.15, CI= 0.03 to 0.28, CIs; Shannon diversity: Posterior mean= 0.23, CI= 0.03 to 0.41, Tables S3.3A, S3.3B). Microhabitat diversity negatively predicted alpha diversity in the same models (Richness: Posterior mean= -0.16, CI= -025 to -0.05, CIs; Shannon diversity: Posterior mean= -0.21, CI= -0.35 to -0.06, Tables S3.3A, S3.3B). These effects were of similar effect size (Figure 9) and independent of each other as well as covariates (mouse sex, age and body mass, sample read depth and sampling month). However, when either home range or microhabitat diversity were added as predictors separately from each other (but including other covariates), only microhabitat diversity remained a significant negative predictor of gut microbial diversity, while the positive effect of home range size was no longer significant. To assess whether any single habitat variable was driving this negative trend of habitat diversity on microbiota diversity, we ran an additional model predicting microbiota diversity with separate habitat variables as well as habitat diversity estimates calculated while dropping each habitat variable from the data. Coverage of habitat types was associated with levels of general habitat diversity (Figure S3.9C) and some habitat types had independent effects on microbiota diversity, especially the amount of wild garlic within a home range had a strong positive influence on gut microbiota richness (Figure S3.9A). However, no single habitat variable drove the correlation between habitat diversity and microbiota diversity (Figure S3.9B). For mice that performed the OFT, neither measure of assay movement behaviour predicted gut microbiota alpha diversity (Table S3.3C, S3.3D).

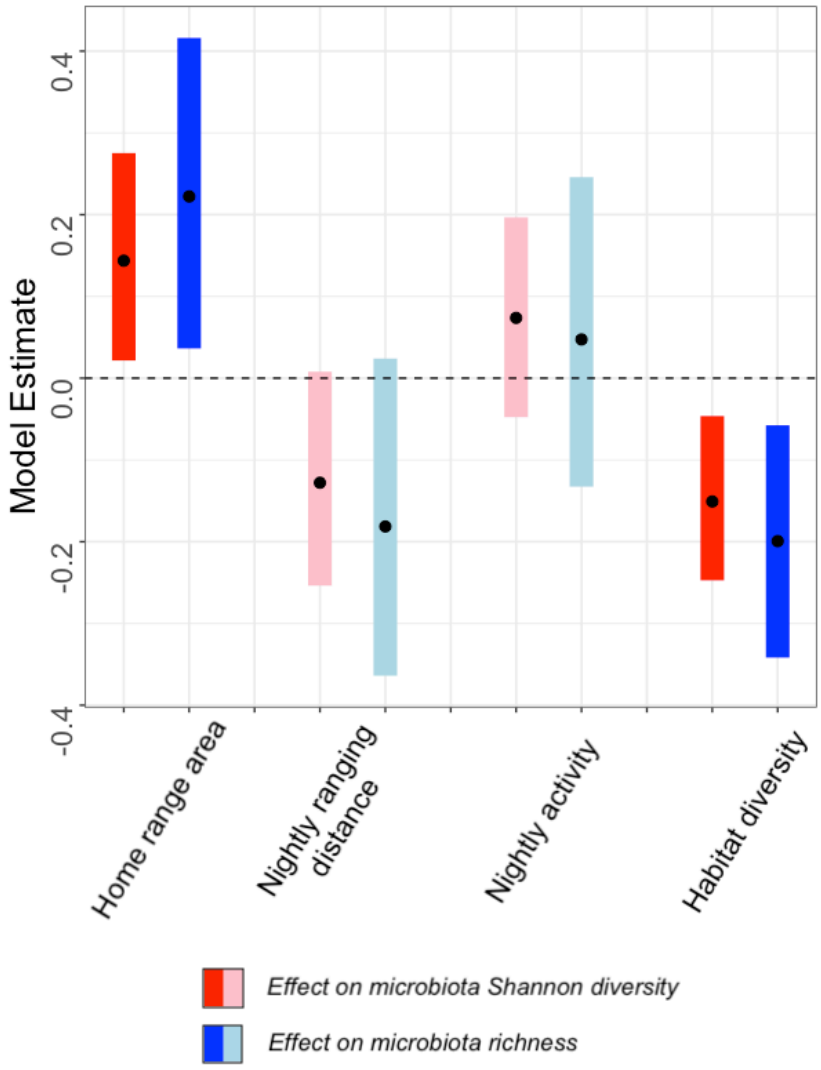


Figure 9. Effects of environmental contact measures on gut microbial alpha diversity of mice. Effect size estimates (points along y-axis) and their 95% credible intervals (lines along y-axis) of environmental contact variables (x axis), plotted from Bayesian regression models with gut microbiota Shannon diversity (red colour) or richness (blue colour) as the response. Brighter colours mark significant effects, where credible intervals do not overlap zero.

3.4 Discussion

We set out to study how the wood mouse microbiota is influenced by variation in environmental exposures across individuals, arising from fine-scale habitat variation as well as differences in space use behaviour among individuals. We found evidence that across different sites within the same 150-hectare area, mice harboured a level of location-specificity in their microbiota. This is in line with earlier research in wild house mice, showing that gut microbiota profiles of wild house mice were predicted by spatial distances between mice within an island as small as 57 hectares (Goertz et al., 2019). The effect of spatial distance on microbiota composition is likely present at larger spatial scales as well. For instance, between multiple mammalian species separated by distances ranging up to thousands of kilometres across the western hemisphere, the compositional similarity of gut microbiota declined exponentially with distance (Moeller et al., 2017). The observed location-specificity of gut microbiota among wood mice is likely to some extent affected by variable diets in different habitat. A recent study on these same Wytham wood mice reported parallel effects of habitat type on gut microbiota composition and diet, as measured through metabarcoding of dietary items from mouse faeces (Marsh, 2020). In addition to dietary variation among habitats, our results suggest that part of this location specificity may arise from microbial transmission from soil to gut, as implied by our finding that mice tended to share more microbes with their local soil than other more distant soils in the same woodland. However, both the effect of location and soil on mouse gut microbiota were relatively weak compared to other factors predicting gut microbiota variation. For instance, while individual identity explained over half the gut microbiota's compositional variation among repeatedly sampled mice, less than 5 % of this individual variation was explained by their capture site. Further, while mice shared twice as many microbes with their local soil than other soils, overall, no more than 1% of the taxa in their gut overlapped with microbes found in any soil sample. With such weak effects, it is also possible that they are influenced by the observed contamination in the data. For instance, while the fact that mice did share more microbes with their local soil compared to other soil is unlikely to be affected by contamination in the data as the contamination effect was random across samples, the increased noise in the data can mean that we may be under-estimating the actual sharing of microbes between mice and their local soil compared to other soils.

However, as the mouse and soil microbiota profiles were remarkably distinct, the level of any contamination homogenising microbiota profiles across sample types is likely very low.

Overall, the small effect sizes of this result appears to run counter to conclusions from an earlier study in wild baboons, in which local soil properties were the single most powerful predictor of gut microbiota variation across 14 populations of two interbreeding sub-species, explaining variation in microbiota composition more than host species or genetic relatedness among individuals (Grieneisen et al., 2019). However, this study related baboon gut microbiota not with soil microbiota but with general geological soil properties, such as amount of exchangeable sodium, which likely also predict local plant community and therefore baboon diet. Our study, which directly compares microbes present in the gut and local soil of mice, specifically explores the transmission of microbes between the external environment and the gut, and excludes selective effects the soil can have on the gut microbiota through diet. Mice in different sites lived or foraged in somewhat different habitats, such as a meadow or a woodland, yet site-differences in gut microbiota were small.

Due to a lack of replication, we could not compare the effect of habitat (e.g. deciduous of conifer woodland) with that of spatial distance on microbiota composition at this scale. The relative importance of spatial distance and habitat variation on gut microbiota is a topic calling for more research, especially since the effects of space and environment on microbiota have been demonstrated separately in multiple studies of wild animals. For example, the gut microbiota of Howler monkeys differed significantly between more or less human-managed forest habitats (Amato et al., 2013) while American moose gut microbiota was found to be predicted by their spatial distances across a relatively homogenous landscape (Fountain-Jones et al., 2019). The effects of space and environmental variation on gut microbiota composition have only been directly compared in a study of multiple mammalian species across the western hemisphere, which showed that more distant populations of mammalian species harbour more different microbiotas independent of variation in their diet (Moeller et al., 2017). Attempts to disentangle the effects of spatial ecology from more qualitative aspects of the environment have perhaps been more thorough in the study of free-living environmental microbiota. For instance, a study of biogeography of British soil microbiota found that microbial communities in soils are most strongly structured by soil pH

but independent of this environmental effect, soil communities were also significantly spatially structured (Griffiths et al., 2011). Such patterns of soil microbiota variation could directly influence the gut microbiota of animals living in contact with soils. However, regardless of ecology of free-living microbiota, similar interplay between spatial ecology and environmental selective effects could be also shaping the composition of gut microbial communities as such. For instance, the composition of any give microbiota is likely influenced by both selective forces induced by variable diets (environmental effect) as well as dispersal-limitations of microbes induced by sheer space between hosts (spatial effect).

While mice had location-specific microbiotas within a single woodland, finer scale variation in environmental exposures due to different microhabitats and space use behaviours had minimal detectable effects on overall microbiota composition. Of all the measured aspects of environmental contact, the only significant effect was between assay activity and microbiota composition. This meant that active mice who moved further in the 5-minute open field test had significantly different microbiota composition from less active mice, and this measure of activity explained up to 3 % of variation in microbiota composition across the 50 assayed mice. To our knowledge, this is the first evidence of covariation between activity behaviour and microbiota in a wild animal. This correlation could reflect effects of individual behaviour on environmental exposure and subsequent microbial transmission, but as none of the other measures of behaviour or environmental contacts had any effect on microbiota composition, this seems unlikely. Rather, this behaviour-microbiota correlation, if reflecting a true causal relationship, may more likely reflect the effects of gut microbes on the behaviour of their host or the simultaneous effects of host physiology (e.g. hormonal regulatory pathways) on behaviour and microbiota. Hormonal state of the host, especially the level of glucocorticoid "stress hormones" of the Hypothalamic Pituitary Adrenal (HPA)-axis is known to affect both behaviour (Carere et al., 2010) and the gut microbiota (Bailey et al., 2011; Bharwani et al., 2016). In line with this, individual variation and temporal fluctuations in HPA-axis activity were associated with gut microbiota composition in wild red-squirrels (Stothart et al., 2016). Further, HPA-microbiota effects are bidirectional: mammalian gut microbiota is also known to influence multiple aspects of host behaviour through their effects on the HPA-axis (Wu et al., 2021). For example, the germ-free state in mice was shown to induce more active movement patterns (Heijtza et al., 2011) and experimental administration of *Lactobacillus* bacteria was

shown to lower HPA-axis activity and consequently improve anxiety and depressive-like behaviours in conventional laboratory mice with intact microbiotas (Bravo et al., 2011). A recent study also found that microbiota changes specifically induced by exposure to natural soils modified the anxiety-behaviour of laboratory mice (Liddicoat et al., 2020). In wood mice, gut microbiota composition could potentially function as a nutritional cue for the host, influencing their behaviour to adapt to their nutritional needs by searching for specific kinds of diets. For instance, a highly productive microbiota with high digestive capacity may enable feeding on a broader range of diets and require a much less active foraging style, as reflected by the more passive foraging styles of ruminant ungulates compared to non-ruminating herbivores such as horses. Detecting correlations between behavioural phenotypes and microbiota compositions in natural populations is the necessary first step in disentangling to what extent natural variation in microbiota influences behaviour and to what extent specific behavioural patterns shape microbiota (Davidson et al., 2020). This kind of findings are then amendable to experimentation using naturally occurring microbiota variation, with methods such as microbiota transplantations from wild donor individuals to germ-free recipients in the lab. These kind of research would tell us more about how much of the behavioural phenotype is caused by gut microbiota and vice versa.

While overall microbiota composition was largely unrelated to individual differences in space use and microhabitat, we found that microbiota alpha diversity was associated with individual behaviour and environmental contact patterns even on a fine scale. Supporting our hypothesis that a broader range of environmental exposures would enrich the gut microbiota, we found that home range size positively predicted microbiota alpha diversity. Similar findings come from a study of mountain goats with a significantly positive correlation between core home range size and gut microbiota richness (Wolf et al., 2021). However, against our predictions and in contrast to evidence from human children, habitat diversity was significantly negatively associated with microbiota diversity. Comparison with environmental variation experienced by human children living in urban or rural habitats (Lehtimäki et al., 2017; Ruokolainen et al., 2017) may be unrealistic as our study subjects experienced an overall much more homogenous habitat. Within this singular woodland plot, however, mice with a more diverse set of plant species and ground-cover types had significantly less diverse gut microbial communities. This might reflect cryptic influences of stressors or dietary resources associated with microhabitat diversity.

For example, some dietary items might have strong positive influence on gut microbiota diversity, and only exist in low-diversity habitats. Indeed, one plant species, wild garlic (*Allium ursinum*), stood out as having disproportionately strong positive influence on mouse gut microbiota diversity while growing in extreme low-diverse areas in general. However, our observed correlation between microhabitat and gut microbial diversity was not fully driven by any single plant species or ground cover type, leaving this as an anecdotal note. An alternative explanation for the observed trend is that mice occupying home ranges with low microhabitat diversity may experience more stress due to having to move across more open spaces, as low diversity areas were generally enriched in open ground with no vegetation cover. Chronic stress on the other hand is known to lower microbiota alpha diversity in rodents (Bharwani et al., 2016; Stothart et al., 2016). Overall, these effects of environmental contact on individual alpha diversity are not convincingly consistent with the idea of individuals with broader environmental exposure having more diverse microbiotas. Future research should look into the effects of environmental variation on gut microbiota alongside variation in hormonal status of the host, especially the HPA-axis, as the glucocorticoids of this physiological pathways are strongly reactive to variation in the environment and are known to influence as well as be influenced by gut microbiota (Foster et al., 2017).

Overall, these results suggest that spatial variation in the wild wood mouse microbiota exists on relatively fine spatial scales, but is only minimally influenced by individual-level variation in microhabitat and space use behaviour. Rather, gut microbiota variation seems to be related to population-level variation between living environments. Some of these location differences likely arise due to exposure to varying environmental pools of microbes, but based on the low levels of microbial sharing between mice and their local soil, this may not be the main cause for the rise of these ”local microbiotas”. Rather, homogeneous microbiotas within a local population may arise through social contact patterns between mice, and the subsequent metacommunity dynamics these networks create for the gut microbes transmitting from individual to individual. Indeed, our previous research has demonstrated an effect of social contact on the microbiota of this same species (Chapter 2), that is much stronger that the effects of environmental contacts reported here. The next natural step would be to assess the relative importance of social vs environmental transmission on the gut microbiota of wild ranging animals, and to distinguish socially transmittable microbes from environmentally transmittable ones. This will make it easier to

answer questions about what kind of contact landscapes, both social and environmental, may be enriching for the gut microbiota or most risky in terms of pathogen transmission.

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Supplementary Material S3 for

Chapter 3

Fine-scale variation in environmental exposure has weak effects on gut microbiota of wild mice

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Supplementary Appendices

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Appendix S3.1. Identifying mouse burrows

Mouse burrow entrances were identified during a 6-month period (Dec 2018-Jul 2019), by following captured mice to their burrow following release. At the point of release, mice tended to run to a nearby familiar burrow for cover, and we made use of this by following each released mouse to locate and mark the burrow entrance it ran into. Burrow entrances were catalogued in a map of mouse burrows across the study site. Over time, the number of newly found burrows plateaued (with constant effort in mouse following, at every trapping session) implying we had identified most of the active mouse burrows in the area by Jul 2019. Identified burrows were given a confidence score of certain, semi-certain or uncertain, based on how clearly the researcher had seen the mouse entering it and whether it had other identifying features of a mouse burrow (e.g. signs of digging or mouse faeces around the entrance). Burrow loggers were not rotated but left at a fixed location, chosen from the burrow catalogue such that only burrows labelled "certain" were considered and that burrow loggers were as evenly distributed across the study site as possible (See Figure S3.2).

Appendix S3.2. Validation of using logger data with varying density for home range analysis.

For ease, figures mentioned in this appendix are included within this appendix and referred to alphabetically.

As movement-model based home range analysis requires an extensive number of relocation data points for an individual, we included observations from all logger data for home range analysis to maximize the number of individuals for whom home range could be reliably estimated. This induced a sampling inbalance in our data, as we had more loggers out covering the same area during Fall (n= 60 AG-loggers, July-November) than in Spring (n= 60 AG-loggers and 60 B-loggers, February-June). To ensure this was not biasing our home range estimates between seasons, we chose a subset of well-tracked mice from the fall period and created a comparable pair of home ranges for each individual

using 1) the full logger data (dense data home ranges) and 2) subset of logger data from the 60 above ground loggers only (sparse data home ranges). Across example mice, these two home range estimates were largely overlapping (Figure A below) and had area estimates that were highly correlated ($r=0.97$, $p<0.001$; Figure B below). This implies that our initial criteria for data density acceptable for reliable home range estimation were realistic, estimates were asymptotic and thus including any additional observation data had negligible influence on the estimates. Based on this, we decided to use all available logger data to infer home ranges for as many mice as possible.

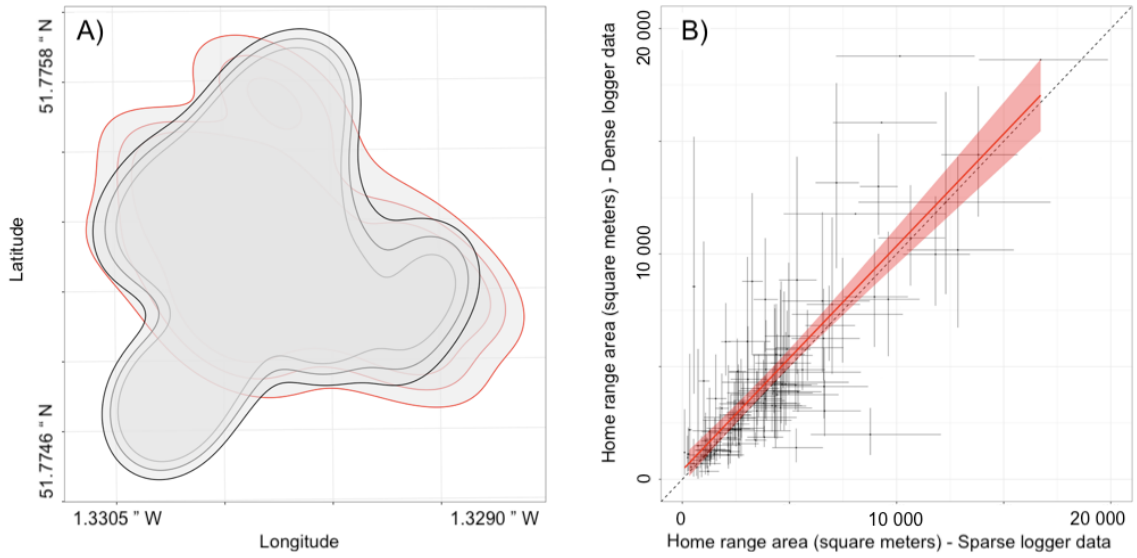

Figure A) Example of overlap between the home range of a single mouse constructed from sparser (red) vs denser (grey) logger data. **B)** Correlation between home range area estimates based on sparser (x-axis) and denser (y-axis) logger data, and the regression line (red line with confidence interval) from a linear model predicting one with the other. The area estimate combinations (points) have both horizontal and vertical confidence intervals (black lines).

Appendix S3.3. Details on home range estimation from logger data.

To ensure that data quality was suitable for home range analysis we first filtered out all individuals that did not have at least five unique "logger visits" in at least three unique locations. We then inspected the remaining data using empirical variograms, a method visualising the autocorrelation structure in locational timeseries by presenting the semi-variance (proportional to home range size) as a function of increasing time lags, with clear asymptotes diagnostic of range-residency (Fleming et al., 2015). Here, a lack of asymptote indicates either the data is too incomplete to reveal the home range extent, or that the animal is not exhibiting site fidelity (Calabrese et al., 2016). Based on this, we excluded individuals who's variogram showed a positive linear relationship between estimated distance and time lag between logger observations, as these were considered signs of either logger histories that were too incomplete for reliable home range estimation (e.g. due to small amount of data or edge effects on the study grid) or spatially shifting home range location (unstable home range). Under these criteria, home ranges could be estimated for 104 of the 157 mice recorded on loggers. To estimate home range, for each individual we first fit a series of range-resident movement models using model selection to identify the best model fit, given the data (Fleming et al., 2015) following the workflow described by (Calabrese et al., 2016). We applied the AKDE to these movement models using an error-informed model (with error set to be 1 meter, approximately the detection diameter of loggers). The method accounts for error by first Kriging data to reduce location error before placing the kernels (Fleming et al., 2018; Fleming & Calabrese, 2017). Although the observation model of fixed station data is different to tracking data that the ADKE was developed for, the scales of movement observed for mice was larger than the grid resolution. Accordingly, visual inspection of the kernels against the grid setup and tracking data showed little evidence of excess density at the stations.

Appendix S3.4. Microbiota analysis with 16S rRNA marker gene

Sample analysis order

The microbiota samples were analysed in two batches, each including i) DNA extraction, ii) library preparation with two-step PCR and purification and iii) sequencing on Illumina MiSeq platform. Both batches included soil samples as well as faecal samples of two rodent species (Wood mouse, *Apodemus sylvaticus;* Yellow-necked mouse*, Apodemus flacvicollis*). The first batch had additionally faecal samples from bank voles (*Myodes glaerolus*). The distribution and number of different samples across 96-well extraction plates in both sequencing batches are summarised in a table below. All samples were randomised across 96-well plates for DNA extraction and re-ordered for 96-well plates for library preparation to enable detection of extraction and PCR-related technical effects on the final data. The latter re-ordering was not entirely random: Rather samples 1-25 from all Extraction plates within a sequencing batch (extraction plates 1-4) went on the same PCR-plate (PCR-plate 1), samples 25-40 across all extraction plates on the next PCR-plate etc. This way samples from all extraction plates ended up on all PCR-plates. Because samples were analysed partially overlapping with sample collection period, sequencing batches had a slight temporal difference: First batch contained only samples from November 2018-July 2019, while the second batch contained samples from November 2018-November 2019. However, sequencing batch in fact explained minimal amounts of variation across all sequenced microbiota samples (marginal PERMANOVA across all (435) wood mouse samples: R^2=0.01, F=6.5, p=0.001)

Sequencing batch	**Extraction plate**	**Apodemus sylvaticus *(faecal)***	**Apodemus flavicollis *(faecal)***	**Myodes glaerolus *(faecal)***	**Soil**
HH1	HH1-EX2	40	0	2	6
	HH1-EX3	62	5	11	3
	HH1-EX4	72	3	6	0
	HH1-EX5	58	4	6	2
HH2	HH2-EX1	87	1	0	0
	HH2-EX2	82	2	0	0
	HH2-EX3	58	8	0	20
	HH2-EX4	51	9	0	6

DNA extraction

DNA was extracted from samples with a 96-well plate format Zymo Quick-DNA™ Fecal/Soil Microbe kits (Zymo) according to manufacturer's instructions, using a Qiagen TissueLyser plate-format bead beater for DNA homogenization. Each DNA extraction plate contained one mock community (ZYMOBiomics Microbial Community Standard cat. No.D6300) to evaluate extraction, PCR and sequencing accuracy. After extraction, DNA concentrations in samples and controls were quantified with a Qubit 3.0 Fluorometer to verify extraction success. Control samples were additionally amplified for bacterial DNA with a 40-cycle PCR (see below) and product run on gel to make sure no bacterial DNA amplified from negative controls. No bands were seen on these gels.

Library preparation and sequencing

Extracted DNA samples were re-ordered for library preparation plates and amplified in a two-step PCR with primers 515F and 926R targeting an approximately 370 bp sequence in the V4-V5 region of the bacterial 16S rRNA gene (Walters et al., 2016). Each plate contained 95 samples and one negative PCR-control. First round PCR had 20µl reaction volume, consisting of: 10µl KAPA 2x Mastermix (KAPA Biosystems), 0.25µl each primer at 10µM, 4.5µl ultra pure water and 5µl extracted DNA. Cycling conditions were as follows: denaturation at 98°C for 2min, 10 cycles of 95°C for 20s, 65°C for 15s, 70°C for 45s, followed by a final extension at 72°C for 5min and a 4°C hold. To reduce PCR-induced variation, this first round PCR was done in duplicates after which the products pooled (by mixing 10µl +10µl of each product on a clean plate) and purified on AMPure magnetic bead purification plate (according to manufacturer's instructions). After purification, the second round PCR was prepared by mixing 9 µl of purified product with a 11 µl of master mix containing 10 µl KAPA Mastermix and 0.5+0.5 µl of forward and reverse Illumina Nextera barcoded indexing primers, added in unique combinations per each sample. The second PCR included 15 cycles with the same thermocycle conditions and was followed by the same AMPure purification step as the first PCR. Finally, amplified, indexed and purified 16S libraries were quantified for concentration (with Qubit), diluted to even concentration of 3-5 ng/ul and pooled for sequencing. Of each library preparation plate, the negative PCR control and 7 real samples (in diagonal order) were run on agrose gel in the end to ensure

amplification success and lack of PCR-contamination. Extraction controls were not re-run on gels after amplification. Pooled libraries (x2) were sent off to the Centre for Genomic Research in Liverpool, where each pool was size-selected using a Pippin Prep, by excluding fragments outside the expected range. Libraries were then sequenced on two separate sequencing runs using 2x250bp paired-end sequencing on an Illumina MiSeq.

Bioinformatics and preprocessing of microbiota sequence data

Raw sequence data was demultiplexed into their original samples and then processed through the DADA2 pipeline (version 1.14)(Callahan et al., 2016) . Here, *cutadapt* (Martin, 2011) was first used to determine optimal trimming length to remove primer and adapter sequences. Based on this, 24 base pairs from the beginning of each read were trimmed away using the trimLeft argument in FilterAndTrim function in DADA2. As part of this function, and following visual inspection of sequence quality, low-quality tails were also trimmed, leaving 290 bp for forward and 230 bp for reverse reads. Next, sequences were dereplicated (identical sequences combined and their quality scores merged), amplicon sequence variants (ASVs) were inferred using the DADA2 algorithm and reverse paired-end reads were merged. Reads that could not be merged or were of abnormal length (<365 bp or > 371 bp) were removed from the dataset.

After this step, data from the two sequencing batches were combined and processed together, using the R package *phyloseq* (McMurdie & Holmes, 2013). First, chimeric sequences were removed from the combined data and taxonomy was assigned to ASVs against the Silva Database (Quast et al., 2013; version 128). Following this, we used iNext package in R (Hsieh et al., 2016) to inspect rarefaction and sample completeness curves across different sample types. Unsurprisingly, soil and rodent gut microbiota samples differed markedly in their diversity and completeness (Figure S3.6). Based on this, as well as the attempt to control for any potential cross-contamination between these sample categories, data was divided into soil vs rodent sample data and the remaining pre-processing steps were completed separately for these sample types: This included

1) identifying potential contaminant ASVs with *decontam* algorithm (Davis et al., 2018) and filtering these away from the data sets (See Appendix S3.5 for full description of contamination mitigation),

2) Removing singletons and doubletons, i.e. very rare taxa only present in abundance of <3 reads within sample,

3) Based on iNEXT completeness and rarefaction curves deciding thresholds for read depth (6000 for mouse samples and 1000 for soil samples) below which samples were dropped out of the data as incomplete and unrepresentative. For soil samples, to keep as many of them in the data as possible, we chose a threshold a little lower than optimal completeness threshold (1000 instead of 1500).

4) Filtering away all ASVs assigned to Phylum "Cyanobacteria" because due to shared ancestry, these ASVs may in fact be chloroplasts from plants rather than real bacteria and

5) normalizing the read count data per sample as proportions.

The original number of unique sequence variants (ASVs) inferred by DADA2 algorithm was 15996 (6174 in wood mouse samples and 7530 in soil), but after decontaminating the data, removing singletons and doubletons and all ASVs assigned to Cyanobacteria, wood mouse samples had 1760 ASVs and soil samples had 4006 ASVs left.

Visualization of mock community composition revealed that our laboratory- and bioinformatics pipeline successfully captured the microbial diversity present in mock samples, though relative abundances varied somewhat from their theoretically true levels (Fig S6).

Appendix S3.5. Contamination in the microbiota data

For ease, tables and figures mentioned in this appendix are included within this appendix and referred to alphabetically.

Short summary of the contamination issue:

1. Evidence for cross-contaimination between samples on DNA extraction plates was detected through a) low to medium levels of DNA in some of the sequenced extraction controls and b) in some plates, a small but significant extraction-plate-wise spatial autocorrelation signal on microbiota profiles (samples extracted closer to each other shared more microbial taxa)
2. This kind of cross contamination is a known and common issue with plate-format DNA-extraction kits, such as ours (Minich et al., 2019).
3. Since our samples were randomised across extraction plates (Figure S3.5A), contamination has a random effect on microbiota profiles and as such is unlikely to create any biases in the observed biological signals on microbiota composition. However, contamination does increase the noise in the data and can thus reduce our ability to detect weak biological effects on microbiota composition.
4. We used a probabilistic de-contamination strategy utilizing the *decontam* algorithm (Davis et al., 2018) to minimize the contamination signal in our data.
5. We show that the effect of contamination on microbiota is unrelated to and independent of real biological effects in our data. Adding plate-effects in models on microbiota alpha- or beta diversity only explains residual variation unexplained by our measured biological predictors. Despite this, we decided to add the plate effects as covariates in all our models predicting microbiota variation.

Deatiled description of the contamination issue:

Alongside 634 soil and gut microbiota samples (belonging to four rodent species), we sequenced 6 mock communities (See Appendix S3.3), 8 extraction negative controls and 8 PCR negative controls. Prior to sequencing, we quantified DNA concentration in our control samples and ran all control

samples on agarose gel to detect any signs of amplified bacterial DNA. These checks suggested no contamination (no detectable levels of bacterial DNA in control samples), but despite this, considerable levels of amplified bacterial DNA was found in some control samples after sequencing (Table A).

Contamination was much stronger in extraction controls, even ones that went through a clean (uncontaminated) PCR, implying that our DNA extraction protocol was vulnerable to contamination. This is a common issue with plate-format extraction kits, such as the ZYMO 96-well plate format faecal/soil DNA extraction kit we used (Minich et al., 2019). This kind of contamination likely happens through spillage between samples during the lysis step, and is prone to increase "technical noise" in the microbiota data. While this noise is random across biological groups in our data (as our samples were randomised across extraction plates, see figure S3.5A), it can potentially diminish or mask some real biological differences between samples.

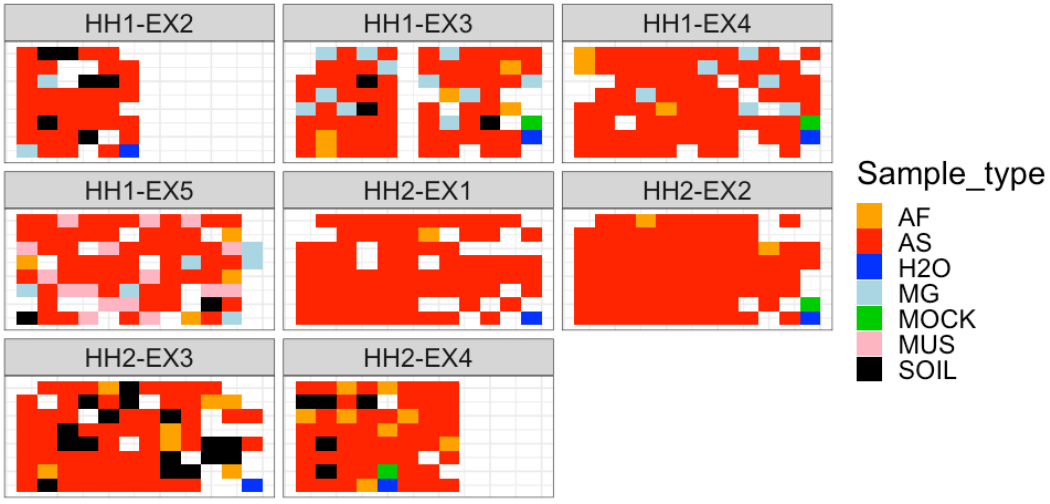

Figure S3.5A. Distribution of different sample types across extraction plates. Sample types extracted together: AF= Yellow-necked mouse faeces, AS=wood mouse faeces, MG= Bank vole faeces, MUS= house mouse faeces, SOIL= soil samples, H2O= water control, MOCK=ZYMO microbial Mock community standard

Based on the assumption that contamination happened through spillage during extraction step, we set out to explore the contamination with probabilistic means, attempting to describe, extract and control for the signal of contamination in the data as well as we could, with the following steps:

1. First, we tested for spatial autocorrelation in the contamination signal, wherein samples that were closer to each other on the plate had more similar microbiota composition (Jaccard similarity). *We found this contamination signal on most plates (Table S3.5A, Figure S3.5B).*

Extraction plate	post.mean	l.CI	u.CI	Control sample read depth	Mean microbiota sample read depth
HH1-EX2	0.0013	-0.0016	0.0039	1	23601.74
HH1-EX3	0.0004	-0.0003	0.0011	18693	32170.72
HH1-EX4	-0.0004	-0.0012	0.0004	3658	40454.12
HH1-EX5	0.0037	**0.0029**	**0.0045**	353	52596.60
HH2-EX1	0.0051	**0.0042**	**0.0060**	9554	38158.83
HH2-EX2	0.0072	**0.0064**	**0.0080**	157	47817.52
HH2-EX3	0.0022	**0.0012**	**0.0030**	44	42698.56
HH2-EX4	0.0058	**0.0044**	**0.0074**	749	40259.57

Table S3.5A. Contamination evidence per extraction plate, based on each plate's control sample read depth (last column) and statistically modelled contamination signal among samples (middle columns). Latter is described by posterior means and credible intervals from dyadic MCMCglmm models (one per plate) predicting microbiota Jaccard dissimilarity with spatial distance between samples on extraction plates. When credible intervals do not overlap zero, samples closer to each other had significantly more similar microbiota composition.

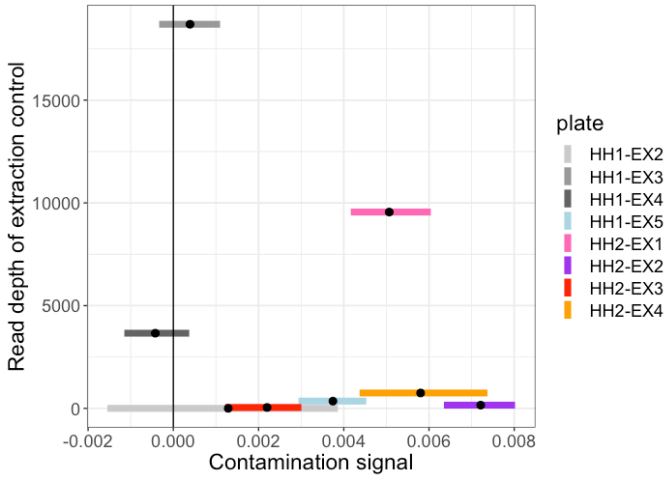

Figure S3.5B. Levels of contamination across extraction plates (colour) based on bacterial DNA reads in plate-specific extraction negative control samples (y axis) and above-described statistically modelled spatial contamination signal (x-axis). When credible intervals (lines) of the effect estimates (points) do not overlap zero (non-grey lines), samples closer to each other had significantly more similar microbiota composition. The two measures of contamination are clearly uncorrelated.

2. We then tested whether this contamination signal was stronger on plates with contaminated control samples, and *found that control sample contamination levels were unrelated to within-plate spatial contamination signal strength (Pearson's correlation test, cor= -0.3, p=0.4), implying that control samples were a poor proxy for contamination occurring within plates.* In line with this, a recent study on contamination issues in 16S data showed that negative controls are a poor measure of contamination and that removing sequences or ASVs as "contaminants" based on DNA observed in the negative controls erroneously removed many real ASVs (Karstens et al., 2019).

3. Next, we explored on what kind of within-sample abundance range the contamination signal was strongest and *found that contamination signal is strongest when keeping only the low-abundance ASVs in each sample compared to when keeping more abundant ASVs, implying that contaminants were mostly rare within a sample (Figure S3.5C).*

4. Based on step 3 above, we tested the effect of different abundance-based filtering strategies (varying thresholds for minimum abundance within sample) on the strength of the contamination signal and two benchmarking biological signals (1- difference between host species and 2- temporal distance (sampling interval) between samples – both biological differences that are expected to exist in the data based on earlier literature (Knowles et al., 2019; Raulo et al., 2021). *We found that no abundance-based filtering strategy diminished the contamination signal without diminishing the biological signals, implying that abundance-based filtering was not an optimal de-noising strategy.*

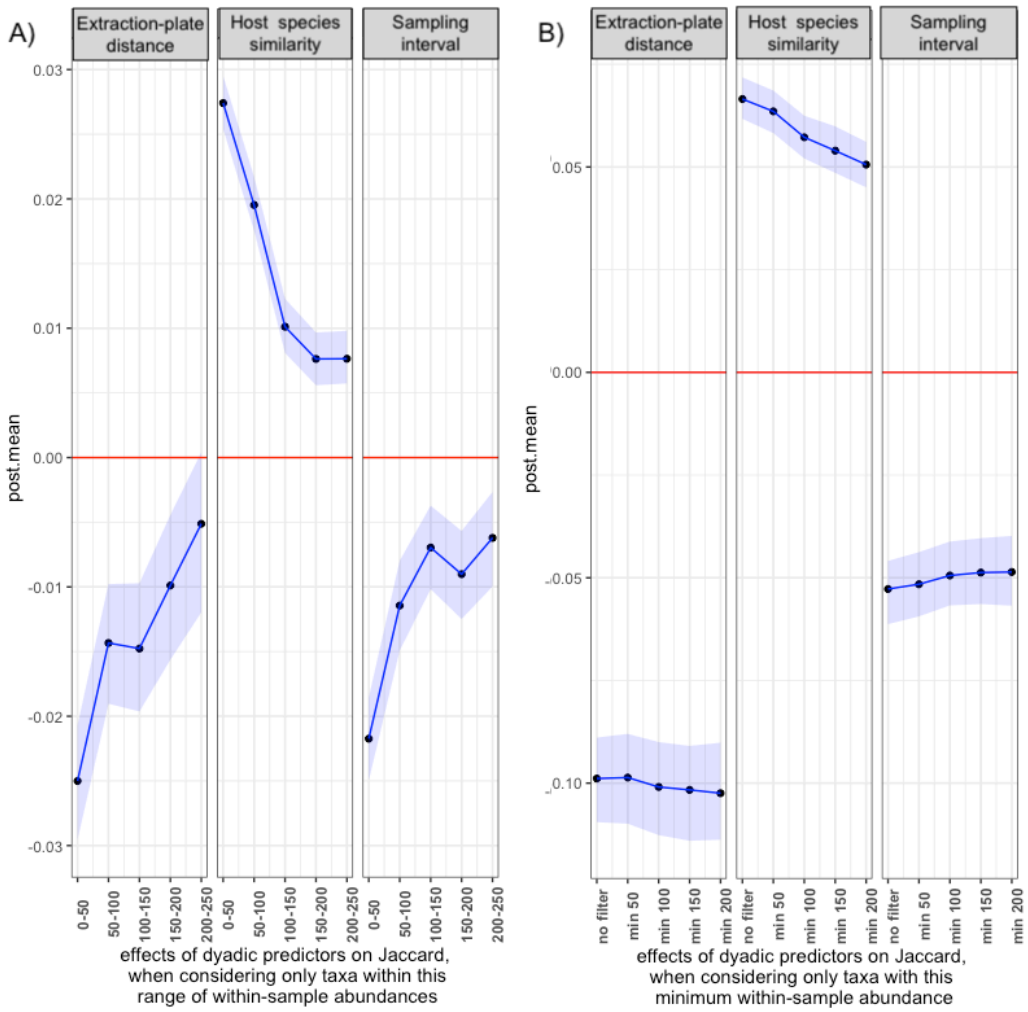

Figure S3.5C. Posterior means (points along y axis) and credible intervals (blue ribbon) of contamination signal and biological signals (panels) in microbiota composition (Jaccard similarity) in data with different within-sample abundance (read count) filtering criteria for ASVs (x axis). Where credible intervals touch zero (red line), the effect is not significant. A) Effect of predictors on microbiota composition in different low abundance ranges of taxa within sample. All effects are strongest (furthest away from zero) in data including only rare taxa with 0-50 counts within sample. B) Effect of predictors on microbiota composition in data filtered with varying minimum-counts-per-sample abundance filters (x axis). No abundance-based filtering threshold decreases the contamination signal in the data, while biological signals diminish with increasing abundance filter threshold (with increasingly abundant taxa filtered away per sample).

After this, we tested an alternative, ASV-based filtering strategy using the decontam-algorithm (*decontam* package in R; (Davis et al., 2018)). This algorithm was originally developed for removal of external laboratory contaminants, and not cross-contamination (Minich et al., 2019), but even to date it is commonly used without specifications over where contamination might have come from. Here, we tested its power specifically as a cross-contamination filter, by decontaminating different sample types (e.g. soil vs. wood mouse) separately of each other. In this method the prevalence (presence/absence across samples) of each ASV in real samples of a given sample type is compared to the prevalence of the same ASV in negative controls to identify contaminants. As this is done through a probabilistic model, the algorithm priduces a probability value for each ASV over how likely they are to be a contaminant in that sample type. These probability values can then be used to create a threshold for filtering away likely contaminants. We tested the effect of filtering the data based on different probability thresholds of what counts as a (cross) contaminating ASV, on the same contamination and biological signals as above. *We found that processing the data with decontam-algorithm with probability threshold 0.70 minimizes the contamination signal and strengthens the benchmarking biological signals, implying that it reduces noise in the data caused by contamination (Figure S3.5D).*

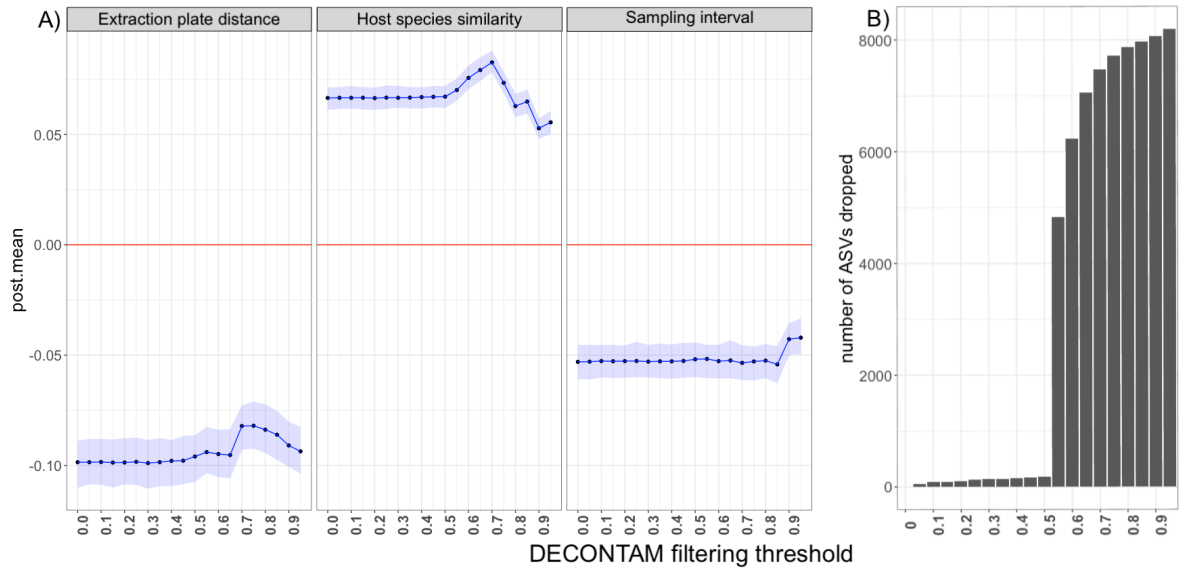

Figure S3.5D. A) Posterior means (points along y axis) and credible intervals (blue ribbon) of contamination signal and biological signals (panels) in microbiota composition (Jaccard similarity) in data with varying decontam probability threshold (x axis). At probability threshold 0.7, contamination signal diminishes (approaches zero) while the two biological signals remain as strong or strengthen. B) number of ASVs dropped from the data with each decontam threshold. Here using the whole data, mouse and soil samples together (15996 ASVs in the non-filtered data set)

5. Finally, we tested whether the contamination signal had any effect in the observed biological trends on microbiota in this data. We did this by adding the contamination signal (extraction distance between samples) as a control variable in a model predicting microbiota similarity with the above-mentioned biological predictors, and examined whether this control variable affected the uncertainty (credible intervals) or effect size (posterior mean) of biological predictors. *We found that even though contamination signal was a significant predictor of a small amount of variation in microbiota similarity across samples, this effect was completely independent of/ unrelated to other effects in the data. Thus adding the contamination signal as a covariate had no effect on the explanatory power of biological predictors over microbiota composition (Figure S3.5E). This is as expected, under the assumption that contamination had a non-biased, normally distributed effect on samples with biological differences.*

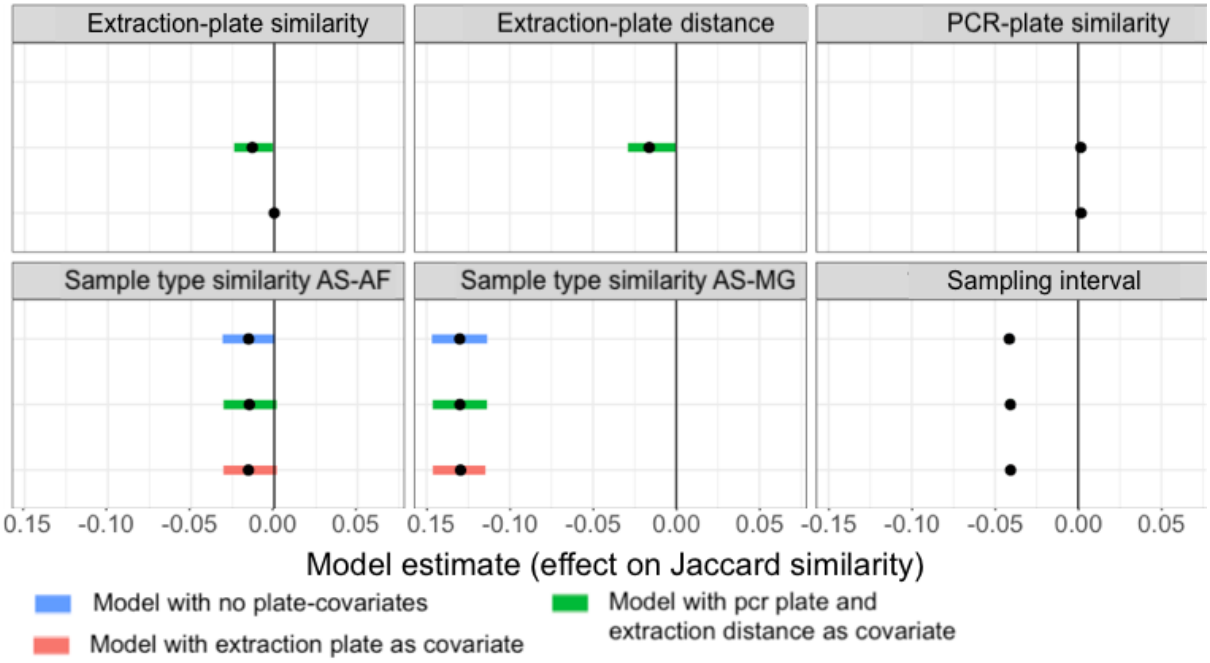

Figure S3.5E. A) Posterior means (points along x axis) and credible intervals (coloured lines) of contamination signal (upper panel) as well as biological signals (lower panel) in microbiota composition. Estimates are based on three dyadic MCMCglmm models (colour) predicting microbiota Jaccard dissimilarity with biological predictors only (blue), biological predictors and extraction plate similarity (same vs different; red) or biological predictors and extraction distance as covariates. All models yield similar estimates with similar uncertainty. PCR-plate similarity (same vs different) was also added as a covariate to models with extraction plate effects, but had no effect on microbiota composition.

Based on these insights, we decided to implement a general contamination mitigation strategy for our data, which included the following steps:

- Prior to other pre-processing of the microbiota data, samples were filtered with decontam-algorithm, using the optimised probability threshold 0.70. To maximize detection of cross-contaminants between sample types, filtering was done separately for soil microbiota samples and samples belonging to different rodent species.
- Very rare taxa within sample (<3 reads) were filtered out from each sample

- Extraction- and PCR plate were added as random factors in all models predicting microbiota sample-wise metrics (e.g. alpha diversity)
- In models predicting pairwise similarity between microbiota samples (e.g. Jaccard similarity), extraction distance (scaled between 0-1) as well as PCR-plate similarity (same/different) were added as technical covariates.

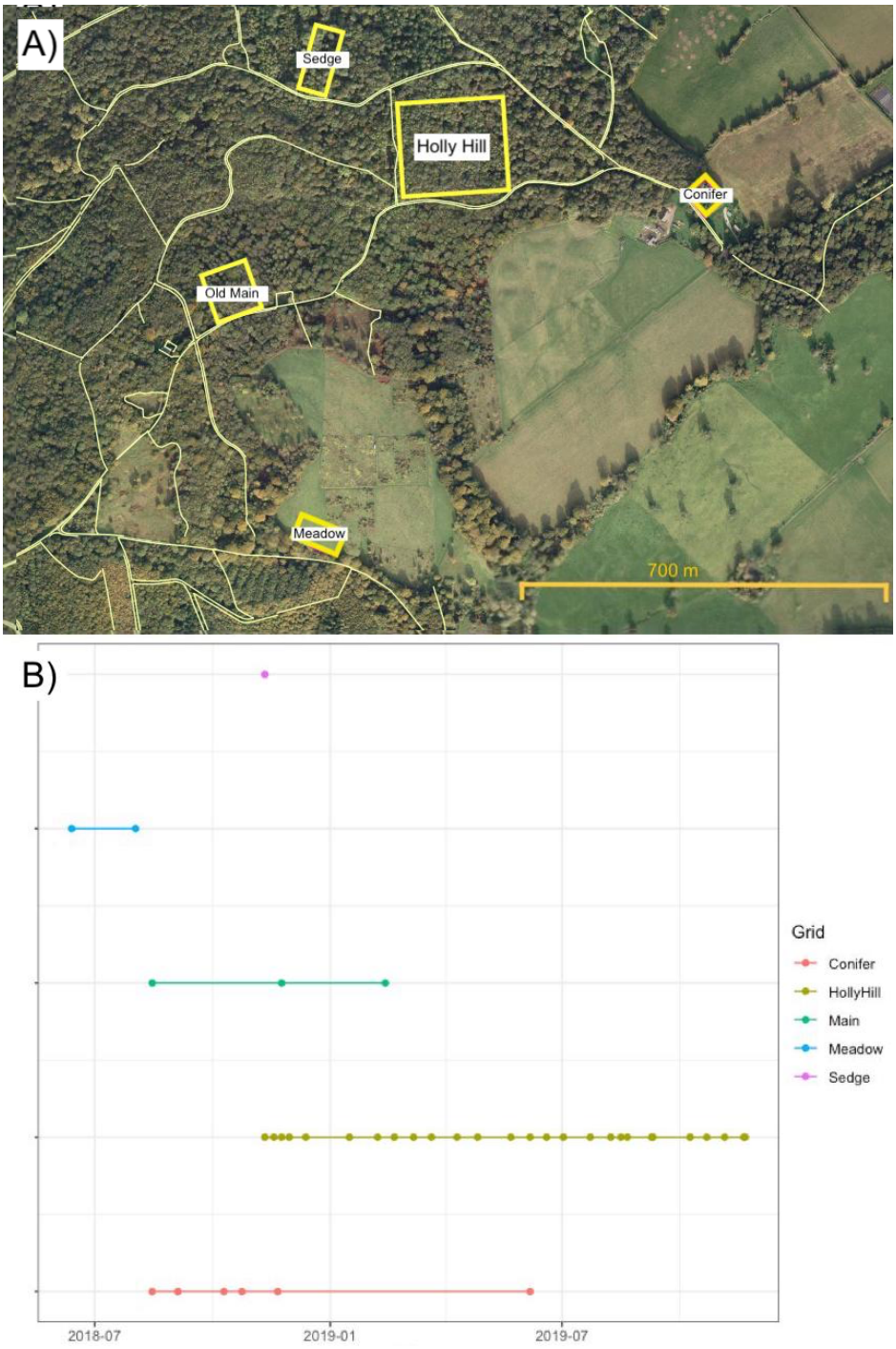

Figure S3.1. Spatial and temporal distribution of sampling of different sites across Wytham. A) Map of the study grids in Wytham Woods. B) distribution of trapping occasions on the five different sites across the year

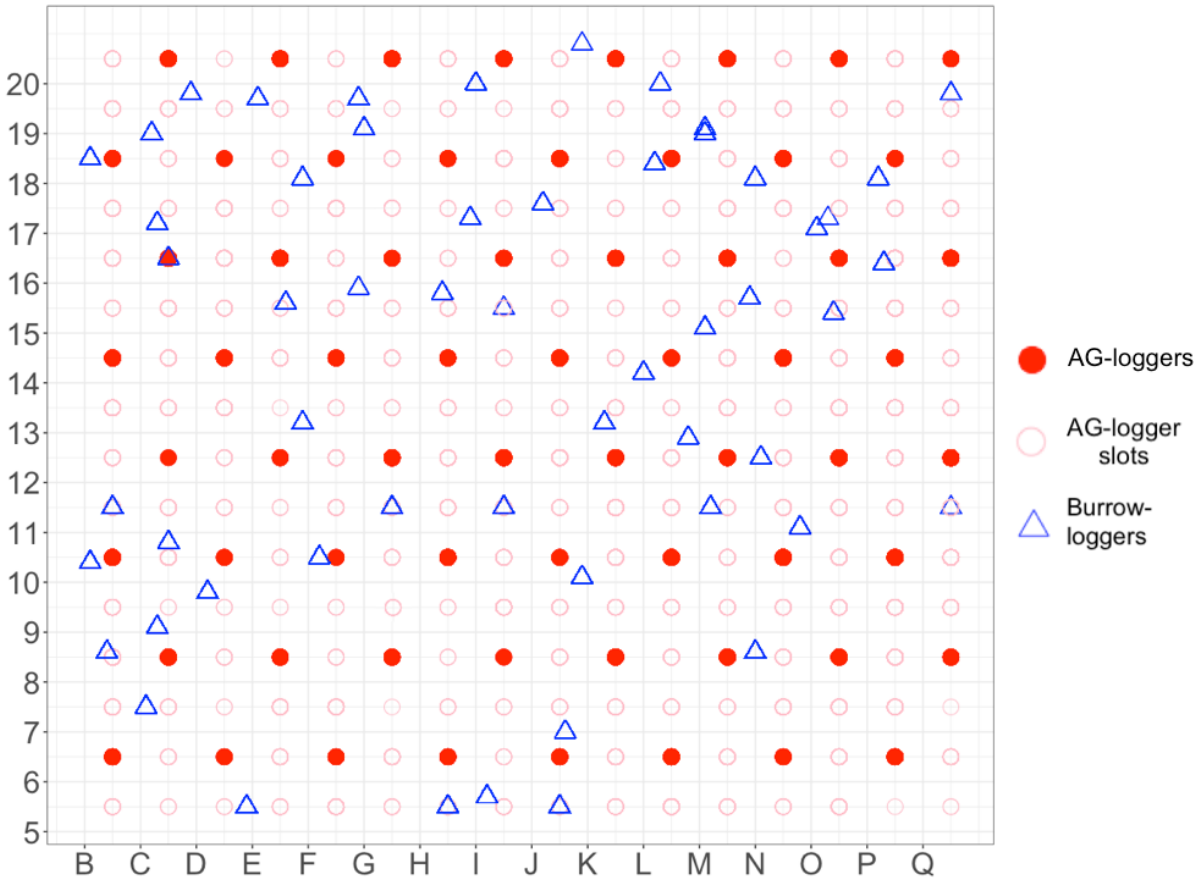

Figure S3.2. Logger positions on the study grid. Above-ground (AG-) loggers were positioned in an even chequerboard design, with each logger having a square territory of four 10 x 10m grid cells. Loggers were placed in the middle of a 10 x 10 m grid cell (solid red dots) for two weeks, and then fortnightly rotated from February to November by moving each logger to an empty grid cell (hollow pink dots), one cell (=10 m) to the north (increasing numbers in y axis). Throughout the rotation, loggers stayed in their latitudinal transect (letter, x-axis) and moved across their longitudinal transects (numbers, y-axis) and when a logger reached the north-limit of the grid (20-transect), the next rotation it was moved back down to the south limit (5-transect). This way each grid cell contained a logger for two weeks of every two months. Burrow-(B-)loggers (blue triangles) were positioned at a set of known mouse burrow entrances across the grid from July until November, and were not rotated. B-logger data was used to complement AG-logger data in home-range estimation only, and not in any other logger-derived measures.

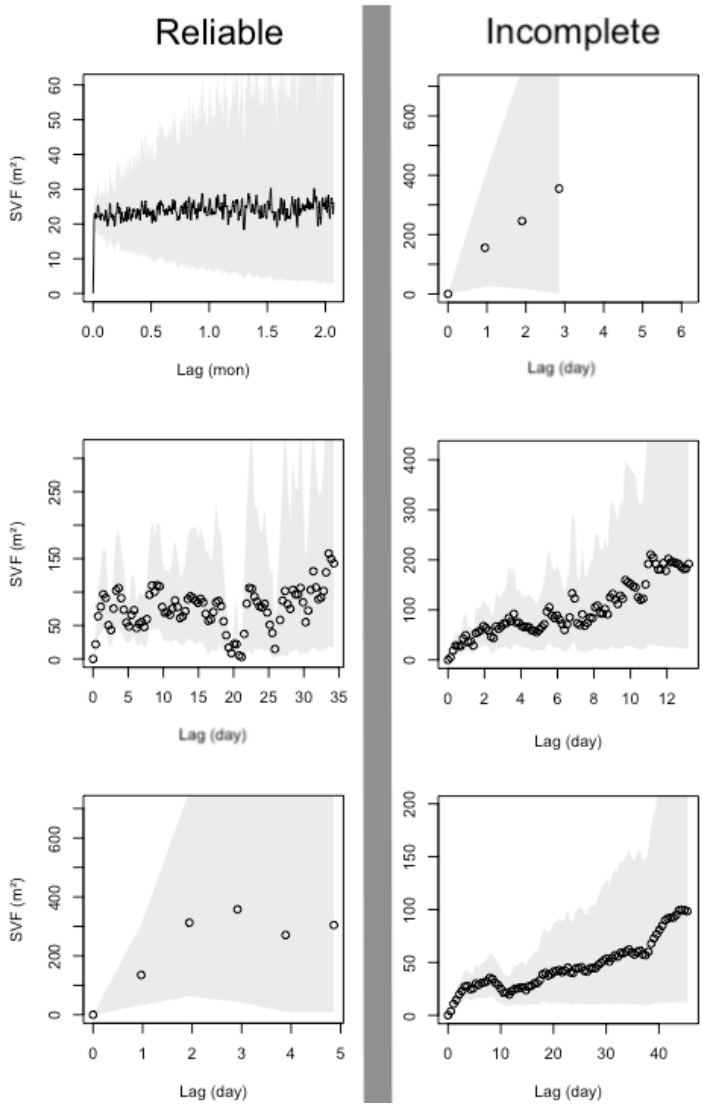

Figure S3.3. Examples of variograms used as an inclusion criterion for home range estimation. Six example variograms describing the completeness and stability of location-observation record of six individuals. Variograms were used to filter out individuals with insufficient logger data for reliable home range estimation. Here, each box is the observation record of one individual mouse, where x-axis describes time lag in days/months between logger observations and y-axis (SVF) describes the expected distance between observations. Where the distance estimate grows linearly with time-lag between observations (right-hand panel), individual's home range is unstable (shifting) or observation data is not complete enough to capture the true home range and thus estimates for these individuals are not reliable.

Figure S3.4: **Main ground cover habitat types in Holly Hill study site.**

Photos are taken from Wytham Woods by myself and others, except the "currant"-landscape which is from a similar woodland in Sussex, England (Photo credit Paul Kirtley).

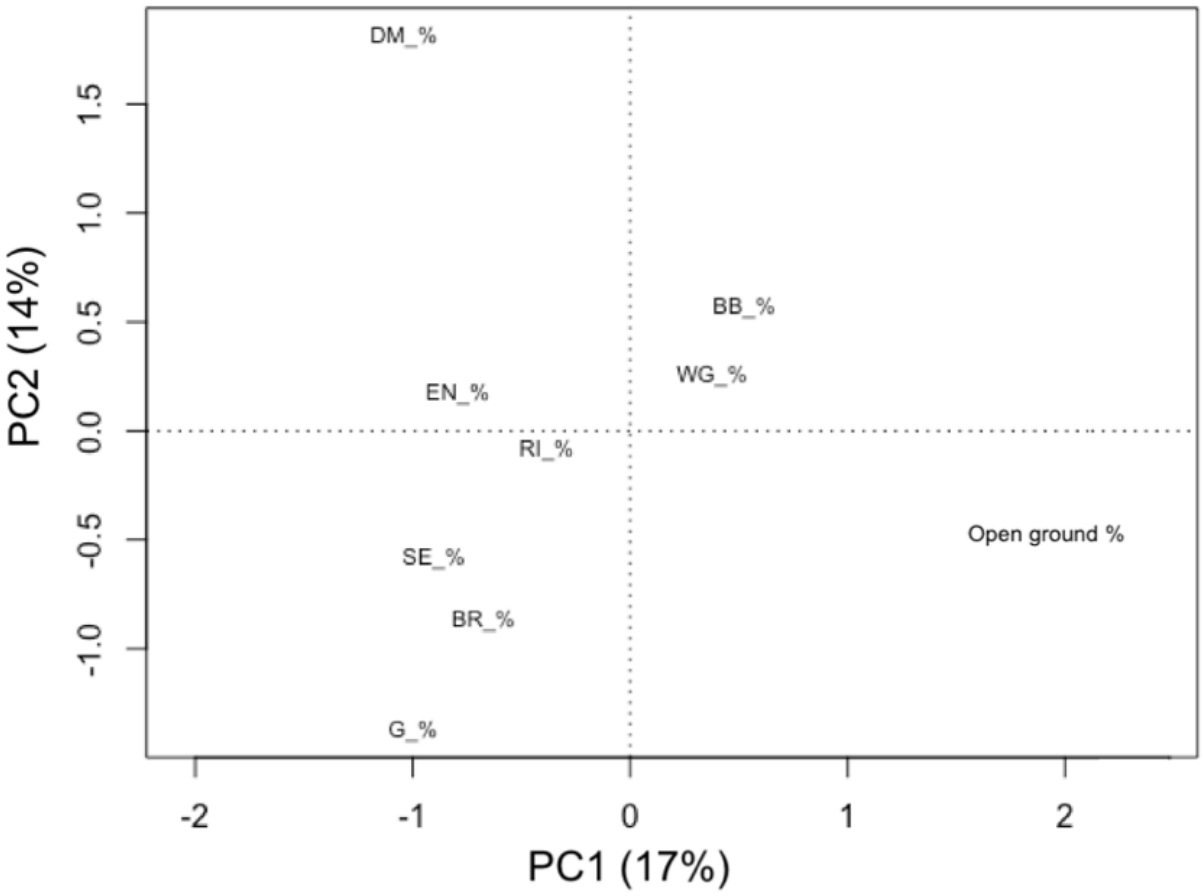

Figure S3.5. Principal Components Analysis including only main ground cover variables. Position of main ground cover variables between two main axes of variation across their distribution across the grid largely follows the same patterns as with full habitat data (Figure 4 in the main text).

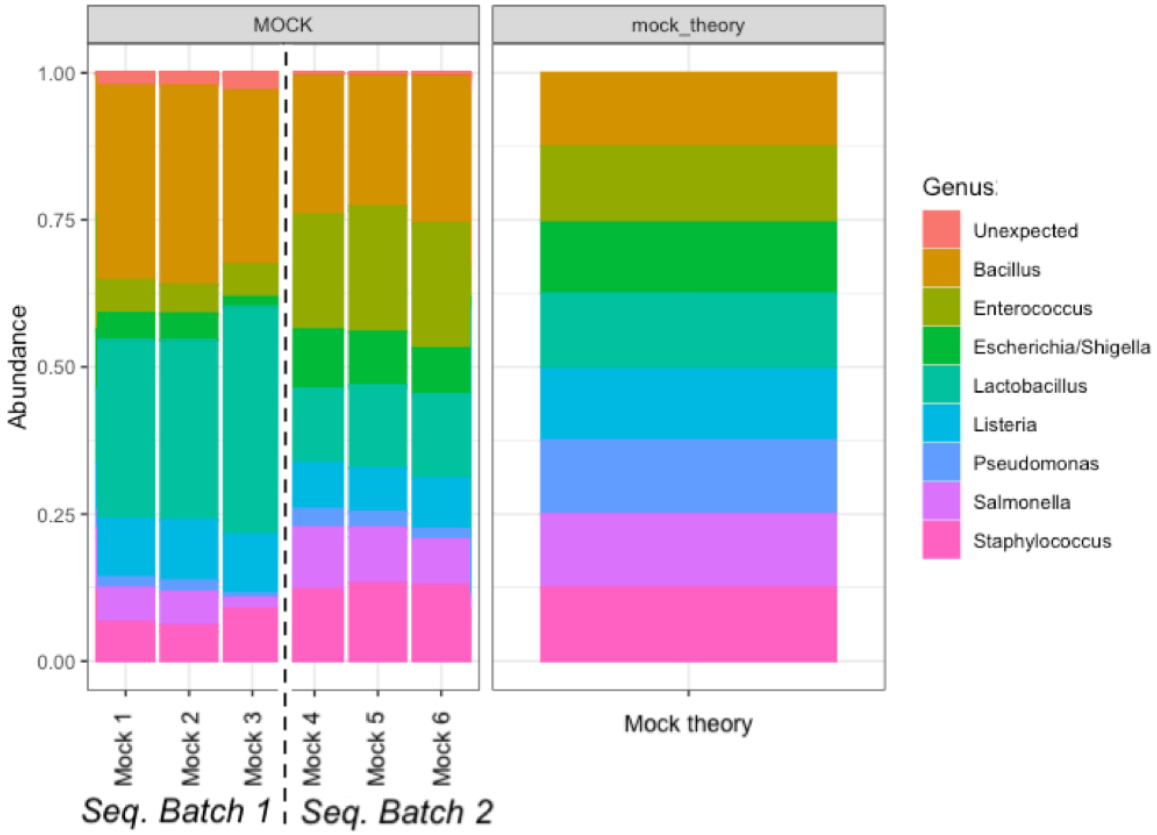

Figure S3.6. Microbial standard (mock) community profiles.

Community composition in sequenced mock community samples compared to the expected composition (Mock theory).

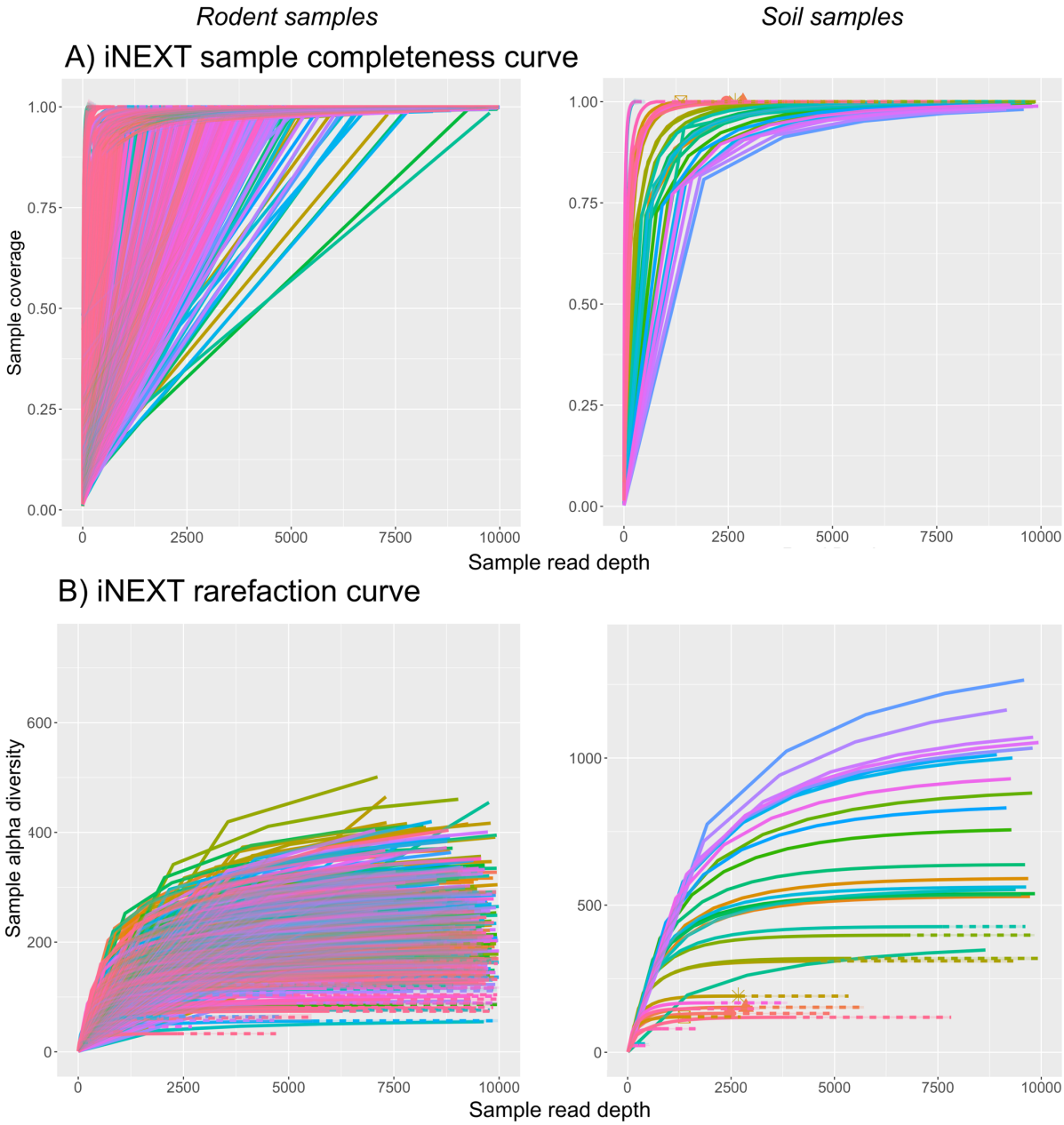

Figure S3.7: iNEXT analysis results

A) Sample completeness curve, showing completeness plateaus above read counts of approximately 6000 for rodent samples and 1500 for soil samples. B) Rarefaction curve, showing diversity estimates stabilize at read counts above approximately 2500 for rodent samples and 1500 for soil samples.

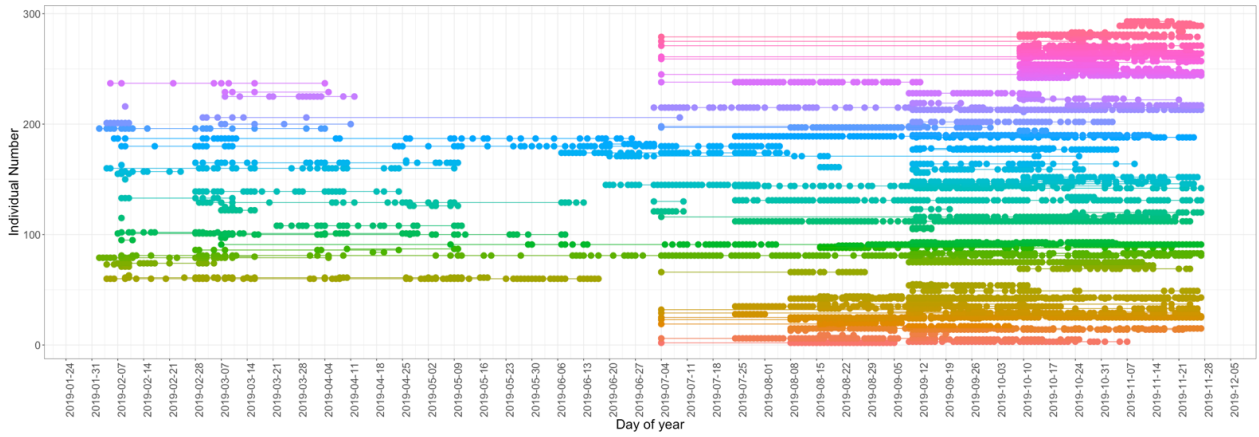

Figure S3.8. Logger detections per individual across time in Holly Hill study site 2019.

Nights of logger detection (dots) across the calendar year (x axis) are shown for each of the 167 individual wood mouse in the logger data (colour and rows of y-axis).

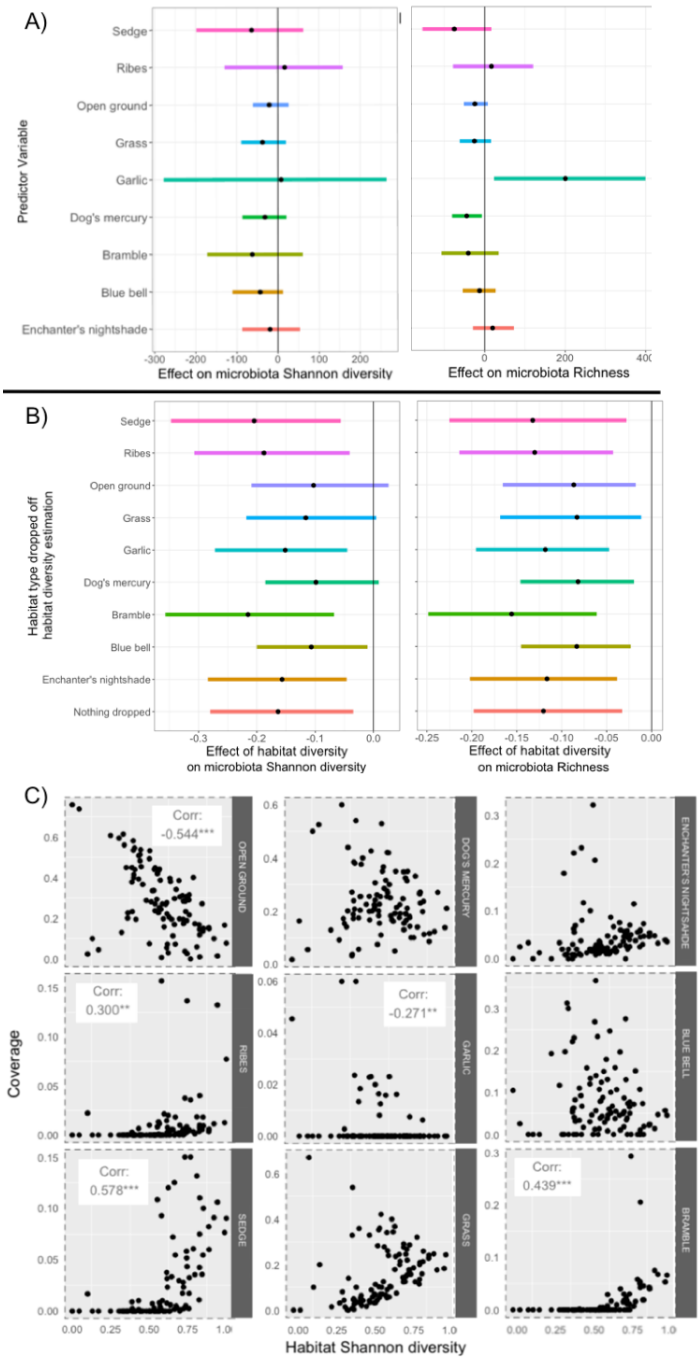

Figure S3.9. Effect of lower-level habitat variables on habitat diversity estimates and gut microbiota of mice. A) The influence of dropping each of the main ground cover types away from the data on model estimates (points) and credible intervals (coloured lines) of the effect of overall habitat diversity on gut microbial diversity. B) The effect of each main ground cover variable on gut microbial diversity in a multivariate model (with all other habitat variables as separate predictors) C) Covariation among coverage (y axis) and overall habitat Shannon diversity (x axis) of each ground cover type (facets).

Table S3.1. Prevalence of tree species and most common herb species and their prevalence across the Holly Hill site.

Main ground coverage herb species were species that grew in patches covering >1m^2 of area. Other herbs were plants growing in smaller patches. In this data we only included herb species that were found in more than three 10 x 10 m grid squares.

Species	**Overall prevalence (percentage of 10 x 10 m gird squares where present)**
Tree species	
Beech (*Fagus sylvatica*)	54
Sycamore (*Acer pseudoplatanus*)	71
Ash (*Fraxinus Excelsior*)	57
Oak (*Quercus robur*)	15
Hawthorn (*Crataegus monogyna)*	27
Field maple (*Acer campestre)*	<1
Holly (*Ilex aquifolium*)	3
Pine *(Pinus sylvestris)*	1
Hazel (*Corylus avellane)*	7
Horse Chesnut (*Aesculus hippocastanum)*	<1
Main ground coverage herbs	
Dog's mercury (*Mercurialis perennis)*	48
Blue bell (*Hyacinthoides non-scripta)*	26
Enchanter's nightshade (*Circaea lutetiana)*	46
Currant (*Ribes spicatum)*	11
Grass (family *Poaceae)*	46
Bramble (*Rubus fruticosus)*	5
Wild Garlic (*Allium ursinum)*	1
Other herbs	
Lords and ladies (*Arum maculatum)*	47
Nettle (*Urtica dioica)*	23
Bracken (*Genus Pteridium*)	41
Cleavers (*Galium asparine*)	22
Herb Robert (*Geranium robertianum*)	1
Lesser Celandine (*Ficaria verna*)	11
Wood Avens (*Geum urbanum*)	34
Ground Ivy (*Glechoma hederacea)*	3
Wood Anemone (*Anemonoides nemorosa)*	2
Wood sorrel (*Oxalis acetosella*)	2
Speedwell (*Genus Veronica)*	<1
Dock (*Genus Rumex*)	1
Honey suckle (*Genus Lonicera*)	<1
Genus *Carum*	1
Wild Strawberry (*Fragaria vesca*)	3

Table S3.2: The effect of environmental exposure variables on gut microbiota composition. Results are from a marginal PERMANOVA predicting microbiota Jaccard distance (1-Jaccard similarity) matrix with individual-level environmental contact variables alongside technical and demographic covariates (sample read depth, extraction and PCR plate, month, sex, age of host). A) Model using logger-based environmental contact variables and habitat diversity, B) Model using logger-based environmental contact variables and first two principal component axes of habitat variation, C) Model using assay-based environmental contact variables. D-F repeat the same models (as A-C) with an abundance weighted Bray-Curtis dissimilarity metric as a measure of microbiota variation instead of Jaccard. All models use a dataset including one randomly selected sample per individual, with as the response. Only one sample per ID was included so that the variance explained by individual-level factors could be directly compared without pseudoreplication. Significant terms ($p<0.05$) are shown in bold.

Table S3.2. A) Marginal PERMANOVA predicting Jaccard microbiota dissimilarity with logger-based environmental exposure variables and habitat diversity (n=242)					
	Df	SumOfSqs	R2	F	p
Sample read depth	1	0.306	0.011	1.185	0.2
Extraction plate	7	1.69	0.06	0.936	0.749
PCR plate	8	1.984	0.071	0.961	0.641
Sex	1	0.268	0.01	1.037	0.391
Age	1	0.336	0.012	1.302	0.103
Month	**11**	**3.358**	**0.12**	**1.183**	**0.017**
Home range area	1	0.186	0.007	0.72	0.905
Habitat Shannon diversity	1	0.266	0.009	1.03	0.383
Nightly ranging distance	1	0.29	0.01	1.123	0.25
Nightly activity	1	0.27	0.01	1.044	0.37
Residual	70	18.067	0.645		
Total	103	28.019	1		
Table S3.2. B) Marginal PERMANOVA predicting Jaccard microbiota dissimilarity with logger-based environmental contact variables and habitat composition (n=242)					
	Df	SumOfSqs	R2	F	Pr(>F)
Sample read depth	1	0.415	0.011	1.212	0.09
Extraction plate	7	2.25	0.061	0.938	0.863
PCR-plate	8	2.684	0.073	0.979	0.635
Sex	1	0.37	0.01	1.081	0.251
Age	1	0.393	0.011	1.148	0.179

Month	**11**	**4.238**	**0.116**	**1.125**	**0.013**
Habitat PC1	1	0.429	0.012	1.253	0.07
Habitat PC2	1	0.371	0.01	1.082	0.268
Home range area	1	0.303	0.008	0.885	0.757
Nightly ranging distance	1	0.371	0.01	1.084	0.237
Nightly activity	1	0.364	0.01	1.062	0.286
Residual	69	23.641	0.645		
Total	103	36.677	1		

Table S3.2. C) Marginal PERMANOVA predicting Jaccard microbiota dissimilarity with assay-based environmental contact variables (n=50)

	Df	SumOfSqs	R2	F	p
Sample read depth	1	0.340	0.019	0.999	0.435
Extraction plate	3	1.020	0.058	0.999	0.474
PCR plate	4	1.424	0.081	1.046	0.280
Sex	1	0.330	0.019	0.968	0.501
Age	1	0.404	0.023	1.187	0.142
Month	**3**	**1.299**	**0.074**	**1.272**	**0.024**
Assay activity	**1**	**0.477**	**0.027**	**1.402**	**0.034**
Assay boldness	1	0.369	0.021	1.084	0.261
Residual	34	11.572	0.656		
Total	49	17.628	1		

Table S3.2. D) Marginal PERMANOVA predicting Bray-Curtis microbiota dissimilarity with logger-based environmental contact variables and habitat diversity (n=242)

	Df	SumOfSqs	R2	F	Pr(>F)
Sample read depth	1	0.285	0.01	1.131	0.222
Extraction plate	7	1.856	0.067	1.052	0.27
PCR-plate	8	2.179	0.078	1.081	0.163
Sex	1	0.25	0.009	0.991	0.429
Age	1	0.373	0.013	1.48	0.036
Month	**9**	**2.711**	**0.097**	**1.195**	**0.016**
Habitat Shannon diversity	1	0.232	0.008	0.921	0.593
Home range area	1	0.164	0.006	0.653	0.97
Nightly ranging distance	1	0.195	0.007	0.772	0.847
Nightly activity	1	0.253	0.009	1.005	0.458
Residual	72	18.143	0.651		
Total	103	27.867	1		

Table S3.2. E) Marginal PERMANOVA predicting Bray-Curtis microbiota dissimilarity with logger-based environmental contact variables and habitat composition (n=242)

	Df	SumOfSqs	R2	F	Pr(>F)
Sample read depth	1	0.354	0.013	1.379	0.085
Extraction plate	7	1.639	0.059	0.913	0.818
PCR-plate	8	2.011	0.072	0.98	0.559
Sex	1	0.279	0.01	1.086	0.272
Age	1	0.319	0.011	1.245	0.138
Month	**9**	**3.375**	**0.12**	**1.197**	**0.013**
Habitat PC1	1	0.361	0.013	1.409	0.064
Habitat PC2	1	0.295	0.011	1.152	0.24
Home range area	1	0.213	0.008	0.83	0.74
Nightly ranging distance	1	0.286	0.01	1.116	0.256
Nightly activity	1	0.28	0.01	1.093	0.305
Residual	71	17.695	0.632		
Total	103	28.019	1		

Table S3.2. F) Marginal PERMANOVA predicting Bray-Curtis microbiota dissimilarity
with assay-based environmental contact variables (n=50)

	Df	SumOfSqs	R2	F	p
Sample read depth	1	0.232	0.017	0.913	0.578
Extraction plate	3	0.766	0.056	1.005	0.457
PCR plate	4	1.122	0.082	1.103	0.211
Sex	1	0.236	0.017	0.927	0.543
Age	1	0.328	0.024	1.291	0.121
Month	**3**	**1.088**	**0.08**	**1.428**	**0.027**
Assay activity	**1**	**0.422**	**0.031**	**1.659**	**0.037**
Assay boldness	1	0.287	0.021	1.128	0.267
Residual	34	8.640	0.632		
Total	49	13.665	1		

Table S3.3: The effect of environmental exposure variables on gut microbiota alpha diversity. Results from MCMCglmm models predicting either asymptotic microbial Shannon diversity or asymptotic richness with individual-level environmental contact variables alongside technical and demographic covariates (Sample read depth, month, sex, age of host) and random intercept across samples coming from the same individual, pcr-plate or extraction plate. A-B) Models predicting alpha diversity with logger-based environmental contact variables, C-D) Models predicting microbiota alpha diversity with assay-based environmental contact variables. Significant ($p<0.05$) terms are shown in bold.

Table S3.3 A) Predicting gut microbial Shannon diversity with logger-based environmental exposure variables and habitat diversity (n=242)					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	**0.655**	**0.325**	**0.957**	**1000**	**<0.001**
Sample read depth	-0.161	-0.625	0.250	269.5	0.458
Month: 2018-12	-0.106	-0.454	0.294	1097.5	0.586
Month: 2019-01	0.023	-0.350	0.415	1000	0.904
Month: 2019-02	0.049	-0.237	0.323	1007	0.724
Month: 2019-03	-0.008	-0.303	0.273	984	0.958
Month: 2019-04	0.014	-0.273	0.335	896.1	0.928
Month: 2019-05	0.159	-0.253	0.519	1000	0.408
Month: 2019-06	0.144	-0.168	0.442	970.6	0.344
Month: 2019-07	0.091	-0.171	0.388	1000	0.546
Month: 2019-08	0.072	-0.197	0.348	1000	0.618
Month: 2019-09	0.074	-0.216	0.350	1000	0.628
Month: 2019-10	-0.077	-0.340	0.200	1000	0.610
Month: 2019-11	-0.028	-0.320	0.243	1000	0.862
Sex: Male	0.006	-0.047	0.065	1000	0.832
Age: Juvenile	0.009	-0.178	0.168	1000	0.940
Body mass	-0.004	-0.012	0.003	1000	0.312
Habitat Shannon diversity	**-0.205**	**-0.348**	**-0.060**	**1000**	**0.006**
Home range area	**0.226**	**0.034**	**0.409**	**1000**	**0.024**
Nightly ranging distance	-0.184	-0.381	-0.001	1000	0.064
Nightly activity	0.054	-0.134	0.240	1000	0.584
Table S3.3 B) Predicting gut microbial richness with logger-based environmental exposure variables and habitat diversity (n=242)					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	**0.397**	**0.189**	**0.619**	**1097.75**	**0.002**
Sample read depth	**1.237**	**0.856**	**1.634**	**32.03**	**<0.001**
Month: 2018-12	-0.024	-0.303	0.237	1024.59	0.870

Month: 2019-01	0.011	-0.239	0.254	1000	0.930
Month: 2019-02	0.083	-0.106	0.285	1000	0.412
Month: 2019-03	0.044	-0.166	0.241	1000	0.658
Month: 2019-04	0.026	-0.183	0.227	1000	0.796
Month: 2019-05	0.228	-0.012	0.491	1000	0.090
Month: 2019-06	0.09	-0.105	0.310	1187.87	0.376
Month: 2019-07	0.120	-0.066	0.322	1097.03	0.248
Month: 2019-08	0.088	-0.095	0.275	1000	0.346
Month: 2019-09	0.103	-0.088	0.289	1071	0.286
Month: 2019-10	0.025	-0.177	0.197	1000	0.774
Month: 2019-11	0.049	-0.124	0.271	1000	0.626
Sex: Male	0.005	-0.033	0.041	1000	0.760
Age: Juvenile	-0.047	-0.163	0.060	1000	0.432
Body mass	-0.001	-0.006	0.004	1000	0.816
Habitat Shannon diversity	**-0.155**	**-0.245**	**-0.051**	**1000**	**<0.001**
Home range area	**0.153**	**0.033**	**0.276**	**1000**	**0.020**
Nightly ranging distance	-0.135	-0.274	-0.009	1000	0.050
Nightly activity	0.077	-0.058	0.195	1000	0.232
Table S3.3 C) Predicting gut microbial Shannon diversity with assay-based environmental contact variables (n=50)					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.522	-0.117	1.069	1000	0.100
Sample read depth	0.764	-0.661	2.128	689.8	0.312
Month: 2019-09	-0.021	-0.259	0.201	1000	0.854
Month: 2019-10	**-0.220**	**-0.407**	**-0.003**	**1000**	**0.030**
Month: 2019-11	-0.195	-0.454	0.065	513.3	0.142
Sex: Male	0.029	-0.126	0.171	1000	0.702
Age: Juvenile	-0.181	-0.672	0.232	1000	0.412
Body mass	-0.007	-0.03	0.023	1000	0.568
Assay activity	0.162	-0.26	0.550	1095.9	0.432
Assay boldness	-0.102	-0.558	0.301	1000	0.640
Table S3.3 D) Predicting gut microbial richness with assay-based environmental contact variables (n=50)					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	**0.554**	**0.229**	**0.959**	**1000**	**0.002**
Sample read depth	**2.00**	**1.152**	**2.752**	**1000**	**<0.001**
Month: 2019-09	-0.033	-0.162	0.119	1000	0.650
Month: 2019-10	**-0.133**	**-0.254**	**-0.007**	**988.3**	**0.032**
Month: 2019-11	-0.124	-0.277	0.018	1000	0.098
Sex: Male	0.026	-0.065	0.108	1000	0.588
Age: Juvenile	-0.179	-0.466	0.091	1000	0.176
Body mass	-0.009	-0.025	0.008	1000	0.212
Assay activity	-0.14	-0.382	0.099	1000	0.250
Assay boldness	0.114	-0.135	0.374	1000	0.422

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4

Social and environmental transmission spread distinct sets of gut microbes in wild mice

List of contributions

- *Aura Raulo* designed the study, helped develop the new RFID tracking technology, collected the data from Wytham, completed all laboratory analyses on gut microbiota profiling prior to sequencing, developed analytical methods, analysed the data and wrote the manuscript
- *Emma Dale* helped collect field data using RFID loggers
- *Holly English* helped collect field data using RFID loggers and provided feedback on home range analyses
- *Genevieve Finerty* helped with home range analysis and the analysis of microhabitat variation and provided feedback on the manuscript
- *Curt Lamberth* led development of RFID tracking devices and helped collect field data from Wytham
- *Josh Firth* supervised the research project, developed social network analysis methods and provided feedback on the analyses and the manuscript
- *Tim Coulson* supervised the research project and provided feedback on the analyses and the manuscript
- *Sarah Knowles* supervised the research project, helped develop the tracking technology and design the study, collected data from Silwood and Wytham, planned and supervised laboratory methods, developed analytical methods and provided feedback on analyses and the manuscript.

Abstract

Gut microbiota composition is an influential part of the host animal, varying greatly between individual hosts and holding consequences for their metabolism, immunity and behaviour and a range of other traits. Recent research has emphasized the role of microbial transmission from outside the host as a key force shaping these communities. Separate lines of research have shown that gut microbes are transmitted through social contact behaviours between hosts as well as physical contact with their environment, but studies distinguishing social and environmental transmission effects on the microbiota are lacking. Here we used a novel RFID-based tracking system to collect high resolution data on social relationships and space use patterns of wild wood mice (*Apodemus sylvaticus*) over a 10-month period, alongside parallel profiling of their gut microbiota from faecal samples. We use a dyadic Bayesian regression framework to disentangle the relative contributions of transmission via social contacts (social networks representing spatiotemporal co-occurrence), shared space use (home range overlap) and habitat similarity among mice. We find positive and independent effects of social association, spatial overlap and habitat similarity on the proportion of gut microbial taxa shared by mice. However, social effects on the microbiota were far stronger than those of spatial overlap or habitat similarity. Additionally, we find evidence that distinct subsets of microbes transmit through social contacts and contact with the natural environment. While social association predicted the sharing of both aerobic and anaerobic (and especially anaerobic) taxa, overlapping space use predicted the sharing of different, mostly aerobic and spore-forming taxa. These findings provide the first evidence for parallel, independent social and environmental transmission of microbiota, involving biologically distinct subsets of the mammalian gut microbiota. These results suggest the importance of social microbiota transmission may be currently underestimated, and consideration should be given to the detrimental consequences of social isolation on natural microbiota transmission and consequent health in mammals including humans.

4.1 Introduction

Host-associated microbiotas, especially the diverse communities inhabiting the vertebrate gut, are increasingly recognised as an influential part of their host's biology, influencing the development (Bates et al., 2006; Gloria Dominguez-Bello et al., 2019; Goldszmid & Trinchieri, 2012), physiology (Foster et al., 2017a; Round & Mazmanian, 2009), behaviour (Davidson et al., 2020; Montiel-Castro et al., 2013; Wu et al., 2021) and ultimately ecology, and evolution of their host (Alberdi et al., 2016; Foster et al., 2017b; Gilbert, 2020; Theis et al., 2016). Many biological effects of the microbiota depend on community composition, which can show vast multidimensional variation among host individuals, populations and species, and strong temporal dynamics within individual hosts.

Even though the gut microbiota may be one of the most variable traits affecting animal fitness, we know little about the forces that shape this variation in natural systems. Importantly, since the microbiota is an ecological community, the forces underlying its compositional variation are expected to be governed by ecological processes (Costello et al., 2012; Coyte et al., 2015; Karkman et al., 2017) such as competitive and dependent associations among microbial species ("intra-community dynamics") or interactions between the host and its microbiota as a whole ("host-microbiota interactions"). Importantly, however, host-associated microbes are living in an inherently patchy landscape of hosts, like islands in a sea of less suitable habitat. This means that host-associated microbiotas can be conceptualised as metacommunities, in which the composition of any individual hosts' microbiota is likely to be influenced by its connectedness to others (Miller et al., 2018). Because of this, while processes happening inside the host can greatly influence these communities, the transmission of microbes from outside the host remains a fundamental force shaping microbiota composition (Robinson et al., 2019).

Transmission of microbes into a host can happen through various forms of contact with its external environment. In mammals, this starts in birth by contact with microbes in their mother's birth canal (Dominguez-Bello et al., 2010) and continues throughout life as microbes spread through other types of physical contact with conspecifics as well as the wider ecosystem. For example, sharing a living space was found to be an influential force shaping the gut microbiota of humans, even more so than genetic relatedness (Rothschild et al., 2018). Further, the gut and skin microbiota of human children was

shown to be strongly influenced by variable physical contact with local biodiversity and natural soils (Lehtimäki et al., 2017; Ruokolainen et al., 2017, 2020). This spread of microbes seems to happen both through shared contact with the natural environment (e.g. soil) as well as direct contact with others, and recent research has specifically emphasized the importance of social behavior in the spread of gut microbes among interacting animals. For example, intimate social relationships appear to homogenize the gut microbiotas of humans; (Dill-McFarland et al., 2019) found that friends and spouses share more gut microbes than strangers, and this effect was strongest among spouses who reported having a physically close relationship. Similarly, social group membership has been shown to predict composition of the microbiota in multiple species of primates (Bennett et al., 2016; Gogarten et al., 2018; Moeller et al., 2016; Perofsky et al., 2017; Raulo et al., 2018; Tung et al., 2015; Wikberg et al., 2020) as well as some other social mammals (Antwis et al., 2018; Leclaire et al., 2014) and further, stronger pairwise social relationships predicted more homogenous microbiota between interacting pairs of baboons (Tung et al., 2015), lemurs (Perofsky et al., 2017; Raulo et al., 2018) and wild mice (Raulo et al., 2021).

The host social environment is gaining increasing attention as a source of microbiota spread. Consequently, the social network of hosts has been framed as an important microbial transmission landscape, "a social archipelago of host islands" (Sarkar et al., 2020), in which the population structure of microbes across host "islands" is defined by the host's social network. However, as microbes are also readily transmitted from the non-social environment (Lax et al., 2014; Liddicoat et al., 2020; Sharma et al., 2019), this island metaphor is not sufficient to capture transmission dynamics of the microbiota in full (Miller & Bohannan, 2019). Further, because microbes transmit from the same environment in which social contacts happen, it has been hard to separate the effects of transmission via social contact from convergent exposure to the same environmental pools of microbes. Social behavior happens in space (interacting individuals need to be in the same place at the same time), and thus any measure of social contact is prone to be confounded with shared patterns of environmental exposure (Albery et al., 2021). Importantly, both social contact and environmental exposure can function as transmission routes for microbes and may have independent effects on microbiota composition. For instance, we recently showed that spatial proximity and social association strength both contributed to predicting gut microbiota similarity between wild wood mice, but that the effect of social association was much stronger (Raulo et al., 2021). Further, we have shown in unpublished work (Chapter 3) that mice share more gut

microbes with other local mice as well as local soil compared to more distant mice and soil respectively. Importantly, social and environmental contacts may be expected to serve as transmission pathways for different sets of microbes, because microbial species vary in their ability to survive outside the host. Aerotolerant and spore-forming microbes, which can survive well outside a host organism, may be transmitted through social as well as environmental contact while anaerobic, non-spore forming bacteria, which cannot persist for long outside a host, may rely solely on intimate physical contact to spread from one host to another. Consistent with this, (Tung et al., 2015) found that the microbial taxa driving a correlation between grooming social networks and gut microbiota similarity in baboons were enriched for those with an anaerobic or non-spore forming lifestyle.

Despite accumulating evidence that both social and environmental transmission pathways can shape the gut microbiota, these effects have rarely been studied together and directly compared. Thus, we know little about their relative importance in shaping the gut microbiota or whether they transmit different types of microbes. This is an important question, because whether any biologically important variation in the gut microbiota arises through variation in exposure to environment or social relationships matters for our predictions about what kind of host lifestyles are harmful or beneficial in maintaining a heathy gut microbiota while avoiding pathogen spread. For example, microbes less transmissible through the environment (anaerobic, non-spore forming) gut microbes have been suggested to have a more mutualistic relationship with their host while aerobic microbes have been shown to be more easily horizontally transmitted and more likely pathogenic (Moeller et al., 2018). While research on human children suggests that environmental exposure (the amount of contact with natural soils and biodiverse local environments) is important for healthy microbiota development and subsequent immune function (Lehtimäki et al., 2017; Ruokolainen et al., 2015, 2017), the role of socially transmitted bacteria in the same processes remains unstudied. Similarly, the opposite bias prevails in the studies of social transmission: across studies of social microbiota transmission in primates, the effect of environmental transmission remains unexplored, since it cannot be easily separated from that of social associations happening in shared space, particularly in species that live in tight social groups.

To bridge this gap in our understanding, we set out to dissect the effects of environmental and social transmission on the gut microbiota composition of wild wood mice (*Apodemus sylvaticus*). Wood mice are a nocturnal woodland rodent species inhabiting small and stable home ranges and importantly

for our aims, they do not form tight-knit social groups but are considered a semi-social species, with a non-modular social organization (Raulo et al., 2021). Previously we showed that these mice have a social structure which is only partially dependent on spatial proximity; mice that live close to one another are not always those with the strongest social associations (often observed together). This makes wood mice a good model species for effectively disentangling the effects of shared space and social association on microbiota composition. Social microbiota transmission between individuals might happen through physical contact behaviours (e.g. mating, huddling, grooming, licking, fighting, see Chapter 1: Figure 4 A-D), whereas environmental transmission may happen through contact with microbes present in soil, food items and other natural surfaces (see Chapter 1: Figure 4 E-I). In addition to direct transmission between individuals and convergent exposure to the same environmental pools of microbes, there is a scenario that encompasses both, whereby microbes spread from one host to another through the environment. This type of transmission may happen through exposure to faeces in the environment, or possibly coprophagy, though the latter has not been observed in wood mice. We used a tracking system based on passive radio-frequency identification (RFID) technology to intensively monitor the home ranges, social networks and microhabitat use of 164 wild wood mouse individuals for a period of 10 months. Combining this data with gut microbial profiles characterised from faecal samples, we first explore the relative contributions of environmental and social transmission of gut microbes, by predicting microbiota similarity among pairs of mice as a function of their social association strength, overlap in space use, and similarity in habitat. Second, we dissect the environmental and social transmission signals detected, by modelling how each bacterial genus contributes to them. With these insights we examine whether environmental and social contacts transmit separate subsets of the mouse gut microbiota.

4.2 Methods

Field data collection

During July-November 2019, we collected faecal samples and tracked the movements of 164 wild wood mice living within a 2.56 ha (160m x 160m) square study area (Holly Hill) in Wytham Woods, Oxford.

This involved fortnightly trapping to tag mice and collect samples, alongside continuous passive tracking of tagged individuals using RFID technology. During trapping sessions, which were carried out in the area from November 2018 and throughout the study period, mice were aged and sexed, and newly captured individuals injected with a subcutaneous PIT-tag for permanent identification and tracking, after which all individuals were released at the exact location they were trapped. Faecal samples for microbiota analysis were collected from the traps of identified individuals into sterile sample tubes with sterile tweezers and frozen at -80^{o}C within 4 hours of collection. All traps with signs of rodent presence were carefully washed and sterilised in bleach solution before the next trapping session, to eliminate cross-contamination.

Mouse behaviour was monitored with a set of custom-built RFID-loggers distributed across the study site, recording the time-stamped presence of any mouse individual that came within its read range (~1m^2). A detailed description of the logger devices can be found in Chapter 3. Loggers were unbaited, and positioned across the grid according to two schemes: 1) our primary logging data came from 60 "above-ground" loggers (AG-loggers) which were spread evenly across the site and rotated fortnightly to ensure even spatial coverage of the area (see Chapter 3 for details on rotation protocol). 2) In addition to these, for some analyses we also used data from 60 additional "burrow loggers", (B-loggers) which were positioned at known mouse burrow entrances for the latter half of the study period (Jul-Nov 2019). Burrow loggers were approximately evenly distributed across the study site and were not rotated (see Supplementary Figure S3.2). Details on how mouse burrows for logging were identified and chosen can be found in Supplementary Appendix S3.1.

At the end of the study (Spring 2020), we completed a thorough survey of vegetation and microhabitat variation across the study site, in which the presence of common herbs and all tree species as well as the percentage cover by each of the eight main ground cover types in the area was recorded for each 10 x 10m grid cell of the plot. Details of the habitat survey can be found in Chapter 3.

Social network construction

Social networks were constructed from the full 10 months of spatially even above-ground logger data, for the whole study period (Full Social Network) and then separately for spring (Feb-Jul) and Fall (Jul-Nov) (seasonal networks). This division of the year into "seasons" was done based on cutting the whole study period in two equal halves, but it also approximately corresponded to the natural seasons of wood mouse breeding, as wood mice are reproductively inactive during late winter/early spring and become reproductively active during early summer and breed until late Fall. While reproductively active, wood mice are known to be generally less social and especially females also more territorial (Gurnell, 1978; Wolton, 1983, 1985). Networks were constructed and visualized using our custom social network inference and plotting functions in R (Supplementary Material, Appendix S4.1) with the help of R package *igraph* (Csardi, 2014). These functions took the logger data, consisting of time-stamped observations of tagged individuals in fixed locations, and calculated a pairwise association index for all mouse pairs based on the frequency they were observed in the same location within the same short time window. Specifically, the logger data was first filtered to include only the hours of normal activity time for this nocturnal species (16.00-08.00). Each 4pm-8am (16hr) period in the data was considered one "julian night", and the combination of each unique logging location and each julian night formed the primary units for social association inference. This means that within each night-location combination, each individual pair was labelled as associated or not, with 'associated' meaning that they were observed within 12 hours of each other at the same logger location during the same night, where night was defined as the 16-h period from 4pm to 8am. These instances of associations were then used to calculate an association index (described in detail in Chapter 2) defined as:

$$Adjusted\ SRI\ = \frac{X}{[X + y_{AB}\ + yA\ + yB]},$$

where X = the number of instances (number of night-location combinations) in which individuals A and B were observed associated (within 12 h of each other), y_{AB} is the number of instances in which A and B were *both* observed but not associated (observed in the same place during the same julian night but more than 12h apart), y_A and y_B are the number of instances in which both were known

to be alive but only A or B were observed respectively. Using an association criterion based on observation in the same location within 12h of each other during a 16h "night" allowed us to define association in a way comparable to earlier research on wood mouse social networks (Raulo et al., 2021) and yet separate nights clearly from one another while using all data from the times of the day when wood mice were seen to be most active.

By taking lifespan overlap into account, we are able to more accurately summarize the temporally fluctuating social structure of the mouse population in one static social network. Of note, the Adjusted SRI contains two fundamentally different types of zero - those arising because two individuals overlapped in lifespan, but never interacted during that time, and those arising because two individuals had non-overlapping lifespans. For the purpose of this study, we treated these zeros as equivalent, since a lack of interaction is expected to have the same effect on microbiota similarity regardless of whether individuals had opportunity to interact or not.

Because burrow loggers covered a spatially uneven and incomplete set of mouse burrows, and thus did not provide an unbiased sample of mouse co-occurrences, we could not readily use this data to derive proportional social association indices such as Adjusted SRI. However, for the purpose of comparing social networks at different types of location, we derived comparable above-ground and burrow social networks using a simpler method, and logger data limited to the Fall period (Jul-Nov) when both logger sets were deployed. For this, we used a matrix containing raw counts of instances (location-night combinations) that each pair of individuals was observed at the same location (burrow or above-ground location) within 12 hours of each other. While these numbers are not an optimal way of describing relative social relationships (as pairs of more active individuals will have more observations and score higher), they can provide insights on whether social associations based on above ground co-observations are representative of a potentially more intimate social association involving burrow sharing.

Estimating home range overlap

Home ranges were inferred from the logger data as described in Chapter 3. As reliable home range estimation requires a considerable amount of tracking data, home ranges were estimated using all available logger data (above-ground and burrow logger data combined), maximizing the number of individuals for which a home range could be estimated (104 mice out of 164 tracked). Details on the criteria used in home range estimation can be found in Chapter 3. To ensure that higher logger density in the fall (by including burrow loggers) did not bias estimates, we used a subset of well sampled mice from the Fall to show that home ranges calculated from the sparser AG-logger data were largely overlapping and comparable in size to home ranges based on all logger data pooled for the same individuals (see Supplementary Appendix S3.2).

Home ranges were primarily estimated as the (spatial) kernel utilization density distribution of each mouse's logger records (aggregated into unique location-mouse ten-minute-period combinations), calculated with the *ctmm* package in R (see Chapter 3). In other words, each individual mouse's home range was described as a three-dimensional probability distribution of space utilization, where the two base dimensions were actual space and the third dimension was utilization intensity, i.e. how frequently the mouse used a given region within its range. From these kernel density functions we calculated the overlap between each pair of the 104 home ranges with the "overlap" function in *ctmm*, using the Bhattacaryya coefficient of overlap between two probability distributions, defined as:

$$BC(h, g) = \int \sqrt{h(x)g(x)}\ dx$$

,where h and g are two probability distributions of value x respectively.

Estimating microhabitat similarity

Microhabitat similarity between mice was estimated using data on the percentage cover by each of the eight main ground cover types within each mouse's 75% core kernel density home range area (the smallest area in which a mouse had a 75% chance of being observed). The main ground cover types were

defined as: 1) open ground (OG; no plant coverage), 2) dog's mercury (DM; covered by *Mercurialis perennis*), 2) bluebell (BB; covered by *Hyacinthoides non-scripta*), 3) bramble (BR; covered by *Rubus fruticosus*),4) grass (G; covered by grass species in family *Poaceae*), 5) sedge (S, covered by *Carex pendula*), 6) Enchanter's night shade (EN; covered by *Circaea lutetiana*), 7) wild garlic (WG; covered in *Allium ursinum*) and 8) Currant (RI, covered by *Ribes spicatum*) (see Supplementary Appendix S3.4 & Supplementary Figure S3.4). The coverage (in m^2) of all main ground cover types were summed within each mouse's 75% core kernel home range, and normalized by dividing the total coverage of each habitat type by the area of the (75% core kernel) home range. Based on these values, we then used the *vegan* package in R to calculate a matrix of microhabitat Bray-Curtis dissimilarity values among mice, and used the complement of this (Bray-Curtis Index= 1-Bray Curtis dissimilarity) as a measure of *habitat similarity* between mice. This index of habitat similarity was defined as follows:

$$Habitat\ similarity = \frac{2C_{ab}}{S_a + S_b}$$

, where C_{ab} is the sum of lesser abundances of all ground cover type present within both home ranges *a* and *b* and S_a and S_b are the total number of ground cover types present within home range *a* and *b* respectively.

Gut microbiota profiling

We profiled gut microbial communities by extracting DNA from faecal samples and using primers 515F and 926R (Walters et al., 2016) to amplify and sequence the V4-V5 region of the 16S rRNA gene in bacteria/archaea. Full details of the laboratory work, library preparation and sequence data bioinformatics can be found in Chapter 3. In brief, we used the DADA2 algorithm to infer microbial sequence variants (ASV) from the sequence data and assigned taxonomy using the SILVA database (version 128) after which the data was decontaminated (Quast et al., 2013), filtered of non-gut-microbial taxa and samples with low read counts. Finally, abundance data was normalized to proportions of each ASV per sample.

Statistical analyses

Describing microbiota variation

To characterise variation in microbiota composition among individuals (beta diversity), we used the Jaccard Index, a pairwise measure of microbial community similarity that captures the proportion of microbial taxa (ASVs) detected across a pair of individuals, that are shared. This metric was selected because the proportion of shared taxa is an intuitive way to capture transmission signals (as transmission should affect the presence/absence, but not necessarily the relative abundance of taxa within a host, which should depend also on within-host replication). Previous work suggested that abundance-weighted measures of beta diversity (e.g. Bray-Curtis dissimilarity) show similarly transmission signals to the Jaccard Index (Raulo et al., 2021).

To estimate the amount of gut microbiota variation accounted for by stable differences between host individuals vs. temporal fluctuations within host individuals, we used Principal coordinates analysis (PCoA) and marginal PERMANOVAs (as implemented with the adonis2 function of package *vegan* (Oksanen et al., 2008) to predict the Jaccard Index across samples from repeatedly sampled individuals (n=255 samples from 82 individuals with a mean 3.1 samples per individual, range 2-10), using host ID and sampling month (as factor) as fixed effects.

Modelling the effect of different transmission pathways on microbiota

To test the effects of social and environmental transmission on microbiota composition we constructed a model that predicts microbiota sharing (Jaccard index) between pairs of mice simultaneously with their social association strength as well as spatial overlap and habitat similarity to infer transmission via social contacts and through indirect environmental pathways respectively. Here, the effect of social association controlled against the effects of spatial overlap and habitat similarity is meant to capture the effect of social contacts on microbiota transmission. Similarly, the effect of spatial overlap controlled against the other two main predictors is meant to capture the effect of indirect transmission of microbes through shared space, and the effect of habitat similarity controlled against the other two predictors is meant to capture the effect of convergent exposure to similar, if not the same, environmental pools of microbes.

To build this model we used a dyadic Bayesian mixed model framework implemented in package *brms* (Bürkner, 2017b), as validated and described in (Raulo et al., 2021). This model framework allows random effect structures that can account for the types of dependence inherent to pairwise comparisons as well as repeated sampling of the same individuals, such that it can be used to predict pairwise microbiota similarity (Jaccard index) with dyadic measures of social or spatial association (Bürkner, 2017a). Pairwise Jaccard indices for all sample pairs except those from the same individual mouse were modelled as a function of social association, spatial (home range) overlap and habitat similarity, as well as a set of technical and biological covariates: host age class similarity (same vs different), sex similarity, time interval in days between samples, sample extraction distance (the physical distance between two samples on plates during DNA-extraction, as described in Supplementary Appendix S3.5), read depth difference and PCR plate similarity). A beta regression (family=Beta) model was used as the response is a proportion (Jaccard index), and the model included a multi-membership random effect (random intercept) for the identity of samples (Sample A + Sample B) as well as individuals (Individual A + Individual B) involved in each pairwise comparison. In addition to this primary model, we ran two additional models exploring seasonal and sex-specific differences in the influence of social and environmental effects on the microbiota. Here, we modelled Jaccard Index as a function of the same set of predictors but for either a spring or fall subset of the social network, and included an interaction term between sex-similarity (same/different) and social association. The brms model uses a Markov chain Monte Carlo sampler to estimate posterior distributions (Bürkner, 2017a). We ran the model with 4 parallel chains (across 4 cores), each with 1000 warmup samples preceding 5000 actual iterations and used posterior checks to ensure reliable model performance.

Indicator taxa analysis

To identify indicator taxa that respond to different (environmental vs. social) transmission processes, we ran a series of models in which we dropped in turn each of the 186 identified bacterial genera from the microbiota data, recalculated the Jaccard Index and re-ran the above-described brms model. For each genus, we then derived an "importance score" for each effect of interest (social association, spatial overlap and habitat similarity), which reflected the extent to which dropping that genus from the analysis increased uncertainty in the effect estimate. The Importance of genus *G* for effect *E* was calculated as

the 95% credible interval width when G is excluded (CI_{excl}) minus the 95% credible interval width when G is included (CI_{incl}), divided by the number of ASVs (*n.ASV*) assigned to genus G:

$$Importance_G = \frac{CI_{excl} - CI_{incl}}{n.ASV}$$

To examine whether genera with high importance for different transmission signals varied in biological attributes relevant to transmission, we used Bergey's Manual of Systematics of Archaea and Bacteria and to classify the aerotolerance (aerotolerant or anaerobic) and spore-forming ability (spore forming or non-spore forming) of each bacterial genus. When this information was not found from Bergey's Manual (e.g. in case of some newly named taxa), we additionally searched for original research papers describing the genus or research papers specifically assessing the aerotolerance or spore-formation of bacterial genera. Full phenotypic trait data for each genus and further detail on how they were assigned are presented in Supplementary Table S4.1.

4.3 Results

Composition, structure and dynamics of the wood mouse gut microbiota

The wood mouse gut microbiota was dominated by bacteria belonging to the families Muribaculaceae, (formerly known as S24-7), Lactobacillaceae and Lachnospiraceae (Supplementary Figure S4.1A). 60% of ASVs could be assigned to a genus and of the remaining ASVs a great proportion (26% of all ASVs) belonged to unknown genera in the family Muribaculaceae. Accordingly, the most common genera detected were Lactobacillus, Lachnospiraceae_NK4A136_group and an unknown genus in the family Muribaculaceae. Mice had a strongly individualized microbiota composition with individual identity explaining 40% variation in community composition among samples from repeatedly sampled mice (marginal PERMANOVA on Jaccard Index, R^2=0.40, F=1.59, p=0.001). In addition to individual variation, microbiota composition also varied within individuals over time: 6% of variation among these

microbiota samples was explained by sampling month (marginal PERMANOVA, R^2= 0.06, F=1.63, p=0.001). This was reflected in a PCoA which showed that gut microbiota profiles distributed themselves along the first PC axis according to when they were collected (Supplementary Figure S4.1B). Mean interindividual microbiota similarity also showed seasonal variation, being higher during spring (February-June) than fall (July-November) (Wilcoxon rank sum test, p<0.001, Supplementary Figure S4.2).

Social and spatial population structure of wood mice

Wood mice had a non-modular social network, which did not cluster into social groups (Figure 1). Mice had on average 6.4 social connections (range 0-24) that varied considerably in association strength (mean non-zero Adjusted SRI=0.10, sd=0.15, range=0.005-1). Social associations, as measured by spatio-temporal co-occurrence, were of comparable strength across sex categories (female-female, female-male, male-male) and tended to be stronger in the spring compared to fall, especially in male-male pairs (Supplementary Figure S4.3A).

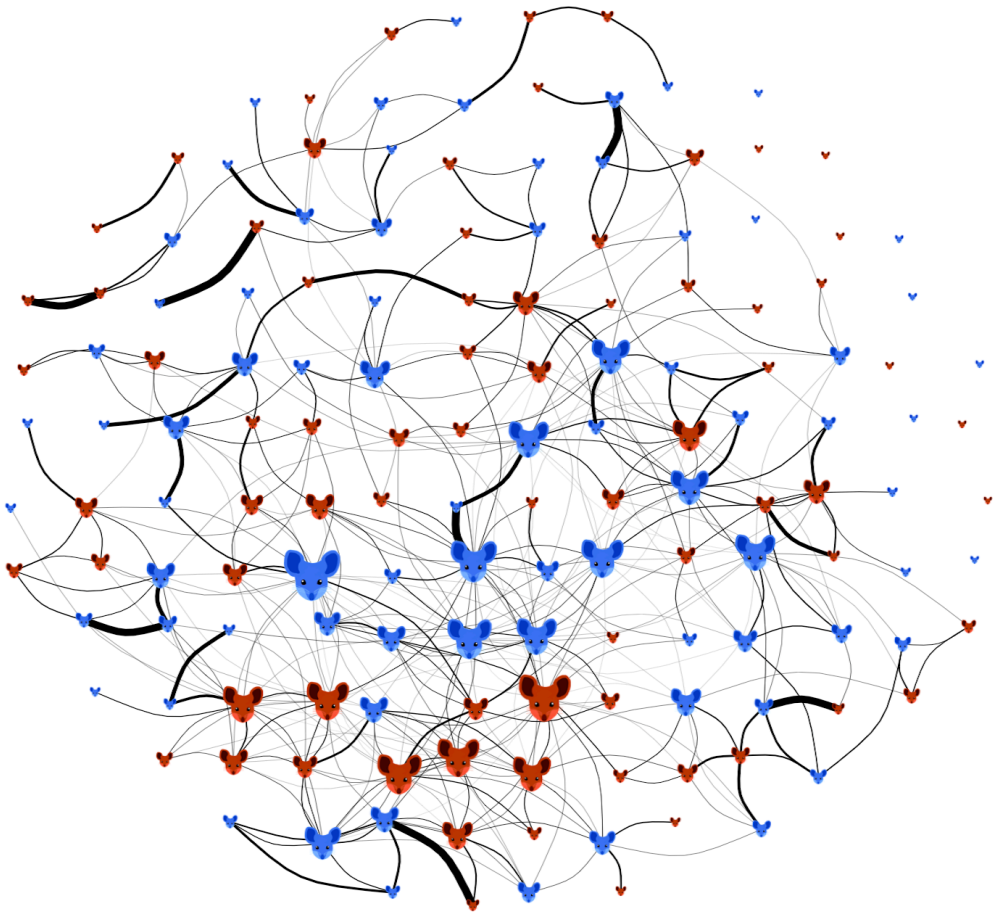

Figure 1. **Social network of wood mice**. Nodes are individual wood mice, either males (blue) or females (red). Edges are measures of social association (Adjusted SRI). Node size depicts the full degree of an individual, i.e. the number of social connections (larger = more connections). Line thickness denotes social association bond weight between individuals (thicker = higher Adjusted SRI value).

The home ranges of wood mice showed varying amounts of overlap (mean Bhattacaryya home range overlap=0.18, sd=0.24, range=0-0.98), the degree of which showed some variation according to season and sex. During spring, home ranges overlapped less among females than among males, with male-female pairs having intermediate levels of overlap (Supplementary Figure S4.3A). During Fall, all sex-categories had similar levels of home range overlap (Supplementary Figure S4.3A). Finally, habitat similarity varied across pairs of mice (mean Bray-Curtis habitat similarity=0.39, sd=0.16, range=0-0.98), but did not differ between sex categories or seasons. Social association strength, home range overlap and habitat similarity were all partially correlated with each other (Mantel test of matrix correlation: social

association-spatial overlap, r=0.26, spatial overlap-habitat similarity r=0.50, Social association-habitat similarity r=0.12, all p<0.001, Supplementary Figure S4.3B).

The logger data from burrows was much denser, containing twice as many logging minutes than that from the comparable set of fall above-ground loggers. Despite this, the social networks created from these different data sets were remarkably similar. Specifically, the raw count networks describing the number of times two mice were observed in the same above ground logger location (AG-network) or burrow logger location (B-network) had very similar distribution of edge values (Supplementary Figure S4.4). The two networks were also significantly correlated (Mantel test, r=0.52, p=0.001), suggesting either that mice who may have shared burrows were also more likely to be observed in the same above-ground locations during the same night, or that B-loggers also capture to some extent the same above-ground general activity as AG-loggers.

Effect of social association, spatial overlap and habitat similarity on gut microbiota similarity between mice

Social association, spatial overlap and habitat similarity all had significant positive effects on microbiota similarity between mice that were independent of each other and other covariates (Figure 2). The effect of social association on microbiota similarity was particularly strong, and over five times stronger than effects of spatial overlap or habitat similarity (Figure 2, Supplementary Table S4.2).

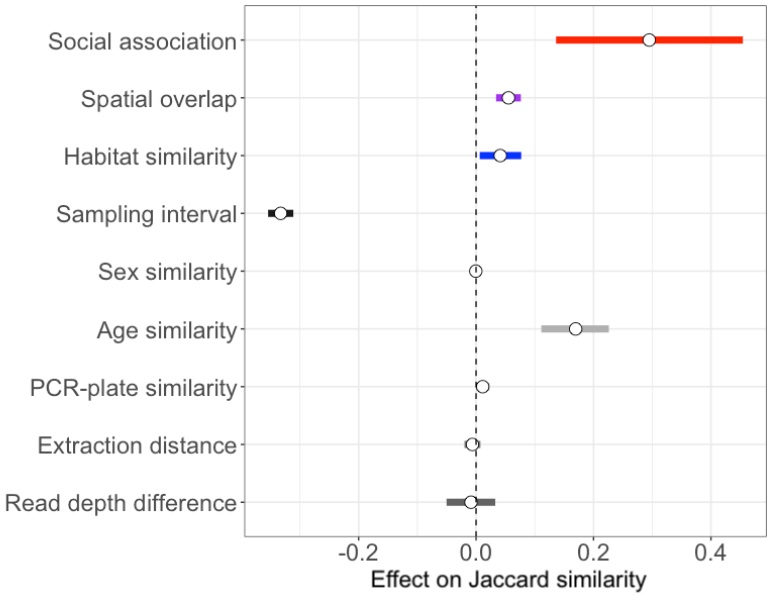

Figure 2. **Effects of different predictors on microbiota similarity**. Posterior means (points) and their 95% credible intervals (coloured lines) are plotted from Bayesian regression (brms) models (Supplementary Table S4.2) with pairwise microbiota similarity among hosts (Jaccard Index) as the response. Where credible intervals do not overlap zero, a variable significantly predicts microbiota similarity.

The social effect on microbiota similarity varied between seasons and according to the sex combination of interacting individuals. Specifically, in spring (Feb-Jun) there was a significant interaction whereby the social association effect was stronger and only significant in same-sex pairs, compared to opposite-sex pairs where it was non-significant (Figure 3A, Supplementary Table S4.3A). In the fall, this pattern was similar, but less strong (Figure 3A, Supplementary Table S4.3B). The strongest effect, same-sex social associations during spring, meant that while pairs of mice that were never observed together shared on average 17% of their gut microbial taxa, pairs of mice with intense social associations (mice observed associated>50% of the time they were observed) were predicted to share on average up to 50% of their gut microbial taxa (Figure 3A), and this effect was independent of the extent to which their home ranges overlapped.

In the spring, the same-sex effect was driven by both male-male and female-female pairs having more similar microbiotas the more socially associated they were, though the effect was stronger in female-female pairs (Figure 3B). In the fall, the same-sex social association effect on microbiota was driven by, and only significant for, male-male pairs (Figure 3B).

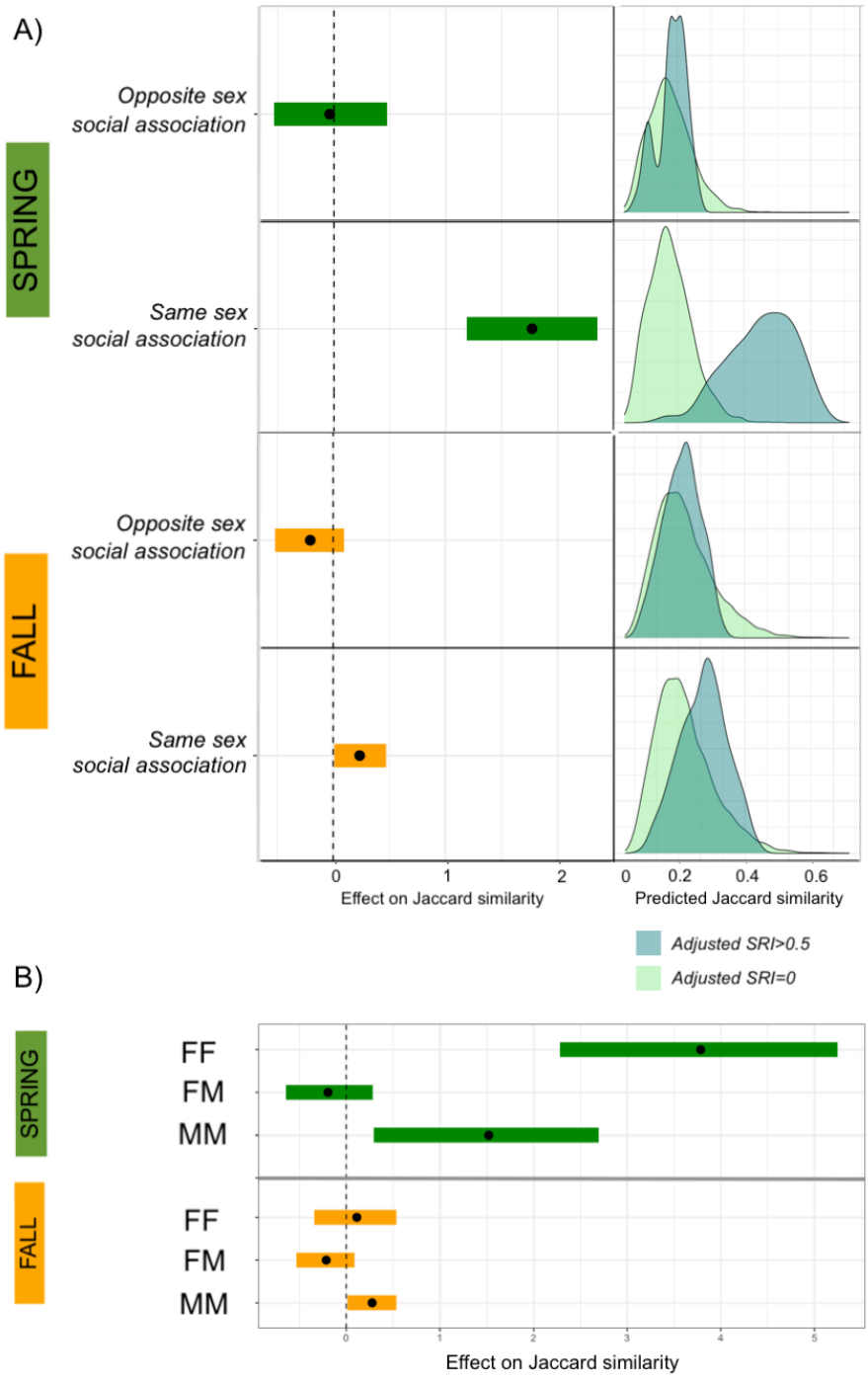

Figure 3. **Effects of social association on microbiota similarity across seasons and sexes**. A) Social effect on microbiota similarity (Jaccard Index) in same- or opposite sex pairs (y-axis) during spring (green, top half) and fall (orange, bottom half). Left panel: Effect size estimates (points along x-axis) and their 95% credible intervals (lines along x-axis), plotted from Bayesian regression (brms) models (Supplementary Table S4.3) with pairwise microbiota similarity among hosts (Jaccard Index) as the response and an interaction term between sex-similarity (same/different) and either spring or fall social network as the main predictor. Where credible intervals do not overlap with zero, a variable significantly predicts microbiota similarity. Right panel: Distribution of Jaccard Index values (x-axis) across pairs of mice who were never observed associated (Adjusted SRI =0, light turquoise) vs. pairs of mice who were observed associated more than 50% of the time they were observed (Adjusted SRI>0.5, dark turquoise), as predicted by the model. B) Social effect on microbiota similarity (Jaccard Index) across specific sex categories and seasons: female-female, female-male (FM) and male-male (MM) pairs (y-axis) during spring (green) and fall (orange).

Indicator taxa for environmental and social transmission signals

All drop-models yielded significant effects for each predictor on microbiota, with effect estimates similar to the full model with all genera, implying that no single genus drove any of the observed transmission signals. However, the uncertainty around these effects fluctuated between the genera dropped (Supplementary Figure S4.5). Consequently, different bacterial genera had varying importance for the social, spatial and habitat effects on microbiota, in that dropping them from the data either increased or decreased precision in our ability to estimate these effects. Compared to the genera with high importance for spatial of habitat effects (spatial and habitat indicators) the genera with high importance for the social effect (social indicators) were more numerous and less overlapping with the other indicator taxa (Figure 4A). Across genera, importance scores for spatial and habitat signal were significantly positively correlated (Pearson's correlation r =0.28, $p<0.001$), while neither of these were correlated with importance scores for social signal (Pearson's correlation between social and spatial importance r=0.03, p=0.68; between social and habitat importance r= -0.11, p=0.14), implying that different set of taxa were influenced by social vs environmental associations between mice.

Indicator taxa for the social signal comprised a taxonomically and ecologically diverse set, with genera of highest importance belonging to multiple phyla (top five genera belonged to Actinobacteria, Firmicutes, Tenericutes and Spirochaetes) and social indicators exhibiting aerobic and anaerobic as well as spore-forming and non-spore forming lifestyles (Figure 4B, Supplementary Figure S4.6). In contrast, the spatial and habitat indicators were more taxonomically uniform (top five genera in both belonged to Proteobacteria and Firmicutes) and significantly enriched in aerobic life-styles, and in the case of spatial indicators, especially aerobic spore-formers (Figure 4B, Supplementary Figure S4.6). Paralleling these findings, when Jaccard indices were recalculated using only aerobic taxa the social signal lost significance but the spatial signal remained significant while the opposite was true when keeping only anaerobic taxa in the Jaccard similarity index (Figure 4C). We ensured these findings were not driven by the greater number of anaerobic ASVs and genera in the data (aerobic taxa: n=71 genera, 164 ASVs; anaerobic taxa: n= 90 genera, 1262 ASVs), by calculating the aerobic and anaerobic Jaccard index using a similar sized subsample of taxa, chosen randomly to have the same number of genera and ASVs as aerobic taxa. This stratified Jaccard index was first calculated for all taxa, aerobic as well as anaerobic, to get a baseline effect of transmission signals on microbiota with this subset of taxa. We then calculated Jaccard indices based on the anaerobic subset of taxa, and compared social association and spatial overlap effects for aerobic vs anaerobic taxa. In this subsample, social and spatial signals on microbiota in the baseline model were similar to those for the full dataset, but similarity of aerobic taxa was only significantly predicted by spatial overlap whereas similarity of anaerobic taxa was only significantly predicted by social association (Supplementary Table S4.5, Figure 4C). In this stratified subsample of taxa, habitat similarity was not a significant predictor of microbiota similarity overall.

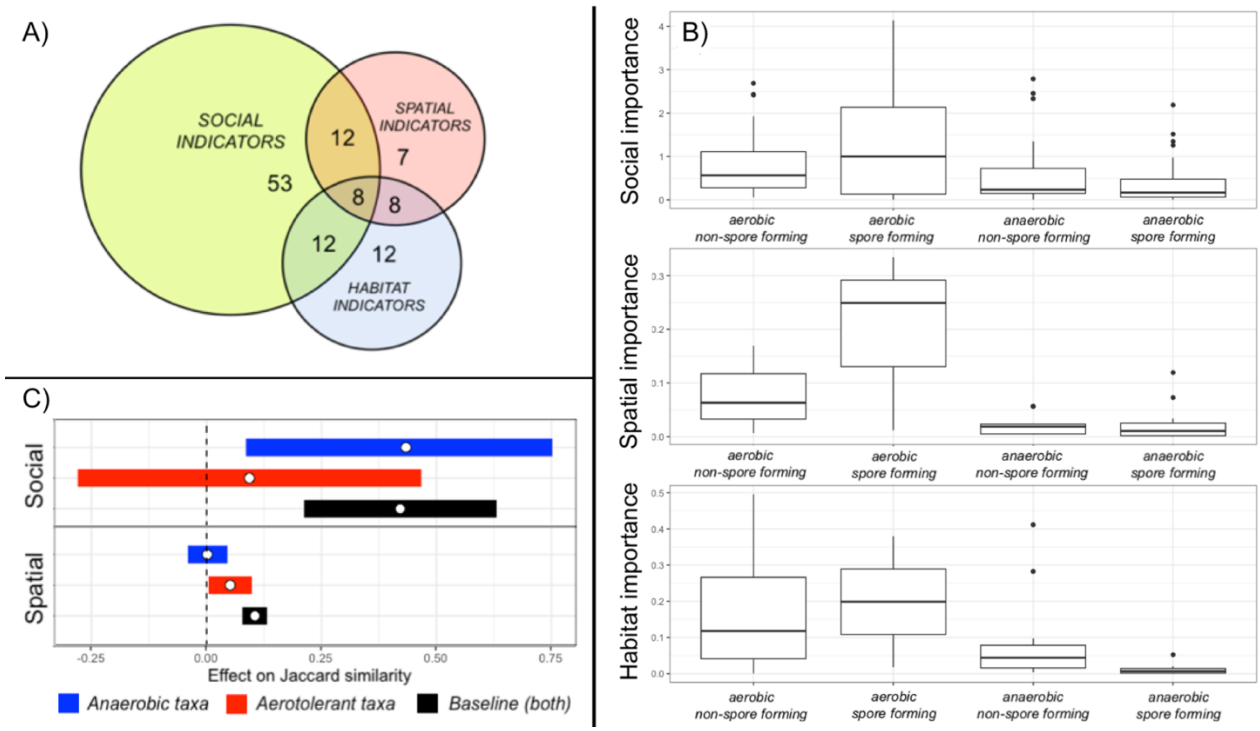

Figure 4. **Indicator taxa of social and environmental transmission signals and their aerotolerance**. A) Venn diagram of the number and overlap of the indicator genera (microbial genera with positive importance scores for a given effect on microbiota similarity). B) Distributions of social (top-panel), spatial (middle panel) and habitat (bottom panel) importance scores for bacterial genera, across categories of bacterial phenotypes of aerotolerance and spore-forming ability (x-axis). C) Social and spatial effects on microbiota similarity for aerotolerant vs anaerobic microbes. Posterior means (points) and their 95% credible intervals (coloured lines) for the effects of social association (top panel) and spatial overlap (bottom panel) effect on microbiota Jaccard similarity, based on a model (Supplementary Table S4.5) using equal-sized sub-sample of anaerobic taxa (blue), aerotolerant taxa (red) or both (baseline, black). Where confidence intervals do not touch zero, a variable significantly predicts microbiota similarity. Social association significantly predicts sharing of anaerobic, but not aerotolerant taxa, while the opposite is true for spatial overlap.

4.4 Discussion

Recent research has shown that the composition of the mammalian gut microbiota is greatly influenced by transmission of microbes from outside the host, such as through social behaviours (Sarkar et al., 2020) and contacts with the natural living environment (Ruokolainen et al., 2016). However, the effects of microbial transmission from other hosts vs. from the environment has been difficult to separate, because social contacts that transmit microbes between hosts are generally confounded with shared exposure to environmental transmission of microbes. Disentangling the effects of social and environmental transmission is important if we want to understand how the contact landscape of host animals translates into different microbiota compositions, subsequently influencing host health and fitness (Browne et al., 2017; Hanski et al., 2012; Sarkar et al., 2020). Here we provide strong evidence for parallel, independent effects of environmental and social contact transmission in shaping the gut microbiota composition of wild mice. While the microbiota of wood mice was affected by both their overlap in space use and by social associations with other conspecifics, these transmission pathways generated very different effects, both in terms of magnitude and in terms of the microbial taxa transmitted. Specifically, the social signal in wood mouse gut microbiota was over five times stronger than effects of either spatial overlap or habitat similarity, meaning that mice who were often observed together (in the same place close in time) hosted much more similar gut microbiota compared to mice who only shared living space or were exposed to similar microhabitats. These results concord well with our earlier findings on transmission effects on the microbiota from another wood mouse population, where we showed that social networks strongly predicted the composition of microbiota, independent of spatial proximity (a simple distance), temporal and genetic distance between mice (Raulo et al., 2021). Notably, unlike in this earlier study, the findings here are based on spatio-temporal co-occurrence data collected with un-lured RFID loggers recording the natural space-use behaviour of wild mice, suggesting that strong social effects on microbiota are general across wood mouse populations and different tracking methods. The finding that spatial overlap and habitat similarity had generally small effects on microbiota sharing is also in line with our earlier findings from this same population, where we show that geographic location and local soil microbiota

explain a small but significant amount of variation in wood mouse gut microbiota across sites in the same woodland (unpublished, Chapter 3).

Social contacts have been shown to homogenize the microbiota among co-habiting (McCafferty et al., 2013; Pajarillo et al., 2015; Song et al., 2013; Torres et al., 2017) and socially interacting (Dill-McFarland et al., 2019; Moeller et al., 2016; Raulo et al., 2018; Tung et al., 2015) individuals of highly social species. For example, the rate at which pairs of baboons touched each other predicted the similarity of their gut microbiota (Tung et al., 2015) and after experimental cohabitation, the gut microbiota of pigs became more similar (Pajarillo et al., 2015). Despite these well-documented effects, the fact that social contact transmission can have such a strong effect independent of the effect of shared space and even in a relatively non-social species as wood mouse, is striking. This raises the question of how important processes of social microbiota transmission actually are across animal species, in creating phenotypic variation especially in microbiome-mediated traits with adaptive value, such as immunological resilience, metabolic capacity or thermal tolerance. Future research is needed to estimate the importance of microbiota transmission in ecology and evolution of the host species and vice versa: The importance of the host social system and ecological niche of the host in structuring the ecology and evolution of the microbiota.

When gut microbes are transmitted through social contact, their distribution across the host population can reflect population-level patterns of social behaviour among hosts. Consistent with this, we found that the social signal in microbiota composition varied between seasons, in a way that appears to be consistent with known variation in social behaviour of this species. Specifically, the effect of social association on the microbiota was stronger in the spring (Feb-Jun) than the fall (Jul-Nov), when social associations were stronger and consequently gut microbial profiles were also more homogeneous across the whole population. This reflects the known behavioural differences of wood mice across their breeding cycle. During the breeding season (approx. June-November in Wytham), wood mice, especially females, are known to be more solitary and territorial compared to the non-breeding season, when multiple mice may co-nest together in same-sex groups (Gurnell, 1978; Montgomery, 2009; Wolton, 1985; Zgrabczyñska & Pilacinska, 2002.). It is worth noting that wood mouse gut microbiota

is also known to vary remarkably between seasons (Maurice et al., 2015) as a result of a major dietary shift (Marsh, 2020) but this dietary variation is unlikely to be confounded with the social or spatial signals in microbiota due to multiple reasons: Firstly, we found that social associations (and in fact spatial and habitat effects) predicted microbiota similarity not only across the year but also within seasons separately, making microbiota variation between seasons an unlikely confounder of the observed effects. Secondly, based on earlier research in this same mouse population, diet-effects on microbiota seem to be mostly explained by time, i.e. there exist no detectable individual variation in diets among mice inhabiting the same habitat during the same time (Marsh, 2020). In our models, social and environmental effects on microbiota similarity are independent of time as they are controlled against the effect of temporal distance (sampling interval) between samples on microbiota similarity. Lastly, in a previous study on another wood mouse population (Chapter 2, Raulo et al., 2020), we found that social effect on microbiota strengthened when social association was defined with increasingly intimate/narrow time windows. Thus, pairs of mice who coincided in space within 2 minutes of each other had more similar microbiome than mice who coincided in space within 30 minutes of each other, and when association threshold was set to 1 hour, 4 hours or 12 hours, the effect weakened linearly. This kind of pattern is consistent with the hypothesis of social transmission driving microbiota homogenization. Since food sources of this species (plants, nuts or insects) do not shift in the matter of hours, this pattern is unexpected if microbiota homogenization would be caused by similar diets among more associated mice.

Interestingly, we also found that social associations predicted microbiota sharing differentially depending on the sex of the interacting mice. During both seasons, social contact was associated with increased microbiota similarity only between same sex pairs of mice (male-male or female-female) but not between opposite sex pairs. The lack of social signal in microbiota of opposite-sex pairs of mice was not a consequence of less strong social associations between these pairs, as measured by spatiotemporal co-occurrence, since associations were on average equally strong across all sex combinations. These findings differ from our earlier findings from another wood mouse population, where social association was an influential driver of microbiota variation only in male-male and male-female, but not female-female pairs (Raulo et al., 2021). These sex patterns in social microbiota sharing and the between-

population inconsistencies therein suggest that social transmission of microbiota may happen through very specific social behaviours (e.g. grooming, licking, huddling or aggressive encounters) that can vary according to the sex of interacting partners, and across seasons and populations. This further emphasizes that microbial transmission links between mice can differ greatly depending on fine-scale variation in social relationships, even among individuals utilizing the same space.

Social contacts between hosts seem to function as an effective way to share gut microbiota among wood mice, but the importance of social transmission pathways seems not to be limited to quantity microbial transmission, but also the quality of microbes transmitted. In this study, possibly the most striking evidence of the separate nature of social and environmental transmission processes in shaping gut microbiota was the fact that different microbial taxa were shared by mice in response to social associations compared to spatial overlap and habitat similarity. This means that separate parts of the gut microbial community may be transmitted through social contact and a through a shared environment respectively. Consistent with this, the spatial transmission signal was dominated by aerotolerant and spore-forming bacteria, which can readily survive outside the host whereas the social transmission signal was specifically driven by sharing of anaerobic bacteria, in that it was no longer significant when anaerobic taxa were removed from analysis. Similar evidence was found in wild baboons, where social associations based on the intensity of physical social contacts between a pair of individuals (grooming social networks) predicted microbiota similarity and this effect was driven by anaerobic and non-spore forming bacteria (Tung et al., 2015). A follow-up study further showed that baboon populations living in different geographic locations differed specifically in the aerobic microbiota they hosted (Grieneisen et al., 2019). A recent study investigating the aerotolerance of human gut microbes transmitting through the built environment also found that the majority of human-derived microbiota detected on bathroom floors that were viable belonged to aerobic taxa, though some methanogenic anaerobes could also stay alive on building surfaces for up to 6 hours (Pausan et al., 2020). Alternative evidence comes from a laboratory rodent experiment, where aerotolerance was shown to be associated with transmission pathways of gut microbiota among 17 inbred laboratory mouse lines derived from geographically distinct wild populations (Moeller et al., 2018). Here, microbial transmission between adult mice (horizontal transmission) was in fact driven by aerotolerant taxa, while obligate anaerobes were found to be strongly

vertically transmitted (passed from mothers to offspring in birth). However, in this study, horizontal transmission of microbes was mostly limited to transmission through the aerobic environment, as caged mice did not socially interact with each other. Future research could usefully assess whether vertically transmitted gut bacteria overlap with those taxa transmitted horizontally through social contact later in life. If so, this would mean that the same (likely strictly anaerobic) microbial taxa would have populations bound both by the social networks as well as phylogenetic trees of their hosts.

If aerobic and anaerobic microbes spread from host to host through different transmission pathways as our data suggest, this has two important implications. First, if anaerobic microbes are in fact more likely to evolve a more mutualistic relationship with their host as they are more dependent on their host species (as suggested by Moeller et al., 2018), this would mean that compared to environmental transmission, social transmission might be expected to spread microbes more important to the host. These key symbionts might include microbes specialized in breaking down complex compounds of their host's diet as in the case of some but not all wild woodrat population hosting tannin-degrading gut microbes that enable members of these populations to digest otherwise indigestible creosote bush (Kohl & Dearing, 2016). Key symbionts could also include microbes protecting their host against pathogen infections, as has been shown in some insect systems. For instance, specific socially spread gut microbes were found to protect their bumblebee hosts against parasite infection (Koch & Schmid-Hempel, 2011). Social transmission of key symbionts is an important possibility to consider in weighing the pros and cons of social life style in theorizing over the evolution of sociality, especially if socially acquired microbes include the same gut microbes that are known to further influence social behaviour (Wu et al., 2021), as this could potentially lead to positive feedback loops of social spread of social behaviour boosting microbes, accelerating social behaviour on population or even evolutionary scale (Davidson et al., 2020). Secondly, the fact that some microbes only live in and transmit between hosts while others readily spread between the host and microbial pools in the external environment calls in question the relevance of viewing host associated microbiotas as classic cases of metacommunities, such as in island ecosystems. While some of the assumptions of metacommunity ecology, such as the idea of completely inhabitable matrix separating the habitat patches, have been updated to model microbiota (Miller & Bohannan, 2019), considering all members of a microbiota as able to persist in the environment between hosts may

be also unrealistic. To properly separate the transmission ecologies of different members of microbiota, we need more research on the aerotolerance, spore-formation and other abilities of different gut microbes in persisting outside the host. Current understanding is limited to anecdotal phenotypic evidence, which is still largely lacking from many of the gut microbial genera of wild animals. For example, the most common genera in wood mouse gut were unidentified genera in Muribaculaceae, which is in itself only a recently described family of common mouse gut symbionts (Lagkouvardos et al., 2019).

Understanding more about gut microbial phenotypes can help us study the various parallel transmission dynamics of gut microbe in parallel. While the populations of microbes enclosed within a gut clearly live in a metacommunity to some extent similar to an island, the microbial taxa within the community can vary greatly in the extent to which they experience the host as a true island. Specifically, anaerobic microbes may well live in a strict metacommunity, structured by the host social network, while the aerotolerant microbes may experience a much more continuous landscape, more analogous to valleys amidst hills than islands amidst the sea. These variations in the restrictiveness of a metacommunity landscape across community members are of course present in macroecological metacommunities as well, for instance due to varying dispersal ability across species (Leibold & Chase, 2018), yet microbiotas differ from these because the variation arises not from varying abilities in crossing the matrix but rather a gradient of abilities of living in it. Metacommunity ecology is a promising framework for understanding the assembly of gut microbial communities (Miller et al., 2018), but caution is required in drawing simplistic analogies between macroecological communities with real geographic boundaries and host-associated microbiotas with a gradient of more or less strict ecological boundaries varying across community members.

For a microbe spreading among hosts, the basic principles of transmission ecology are likely to be similar regardless of host species, raising the question whether we are underestimating the role of social transmission in shaping gut microbial profiles in general. This highlights the need for more attention to our own practises of social contact and isolation, and their potential implications for the transmission dynamics not only of pathogens but of potentially beneficial members of the microbiota. Humans are a socially flexible species capable of large-scale modifications of their own social contact network, as

made evident by the intense social contact avoidance many human communities have gone through recently in attempts to control viral spread. Reducing social contacts is an effective way to reduce pathogen spread, but multiple studies have also raised the point that we know essentially nothing about the long-term consequences of social isolation on natural microbiota transmission and subsequent health of humans (Brett Finlay et al., 2021; Browne et al., 2017; Sarkar et al., 2020). This might be of specific importance when considering young children, whose microbiota and immune system are still in a critical stage of development (Romano-Keeler et al., 2021). For example, a growing body of evidence suggests that diminishing contacts with the natural environment coupled with urban life style can have negative health consequences for children, through a lack of microbiota transmission and consequent natural immune development (Lehtimäki et al., 2017; Ruokolainen et al., 2015, 2017, 2020). For example, correlational studies have suggested green areas around the home of human children have a protective effect over atopic sensitization (Ruokolainen et al., 2015), and accumulating evidence implies this is driven by environmentally acquired microbes, such as *Acinetobacter* (Ruokolainen et al., 2020), which have protective effects over both infections and allergic reactions on skin (Fyhrquist et al., 2014). Paralleling these findings, a laboratory mouse model of asthma was shown to gain a more diverse gut microbiota and more realistic immune function after exposure to soil (Ottman et al., 2019) and another study showed that exposure to natural soil shaped gut microbiota of laboratory mice with downstream consequences on their butyrate-metabolism and anxiety-like behaviour (Liddicoat et al., 2020). If isolation from natural sources of environmental microbiota transmission can bias host health towards autoimmune diseases, what might be the consequence of isolating from natural sources of social microbiota transmission? Based on our results, social isolation may have even more dramatic consequences on microbiotas than environmental isolation. Both reduced natural contact and reduced social contact may independently disrupt the co-evolved transmission networks of human microbiota (Browne et al., 2017), reflected by the observation that modern lifestyles seem to be depleting the diversity of human gut microbiota (Sonnenburg et al., 2016; Wibowo et al., 2021).

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Supplementary Material S4 for

Chapter 4

Social and environmental transmission spread distinct sets of gut microbes in wild mice

Index

Supplementary Appendices

Supplementary Figures

Supplementary Tables

Appendix S4.1. Custom functions in R for social network analysis

https://github.com/nuorenarra/Social-Network-Analysis

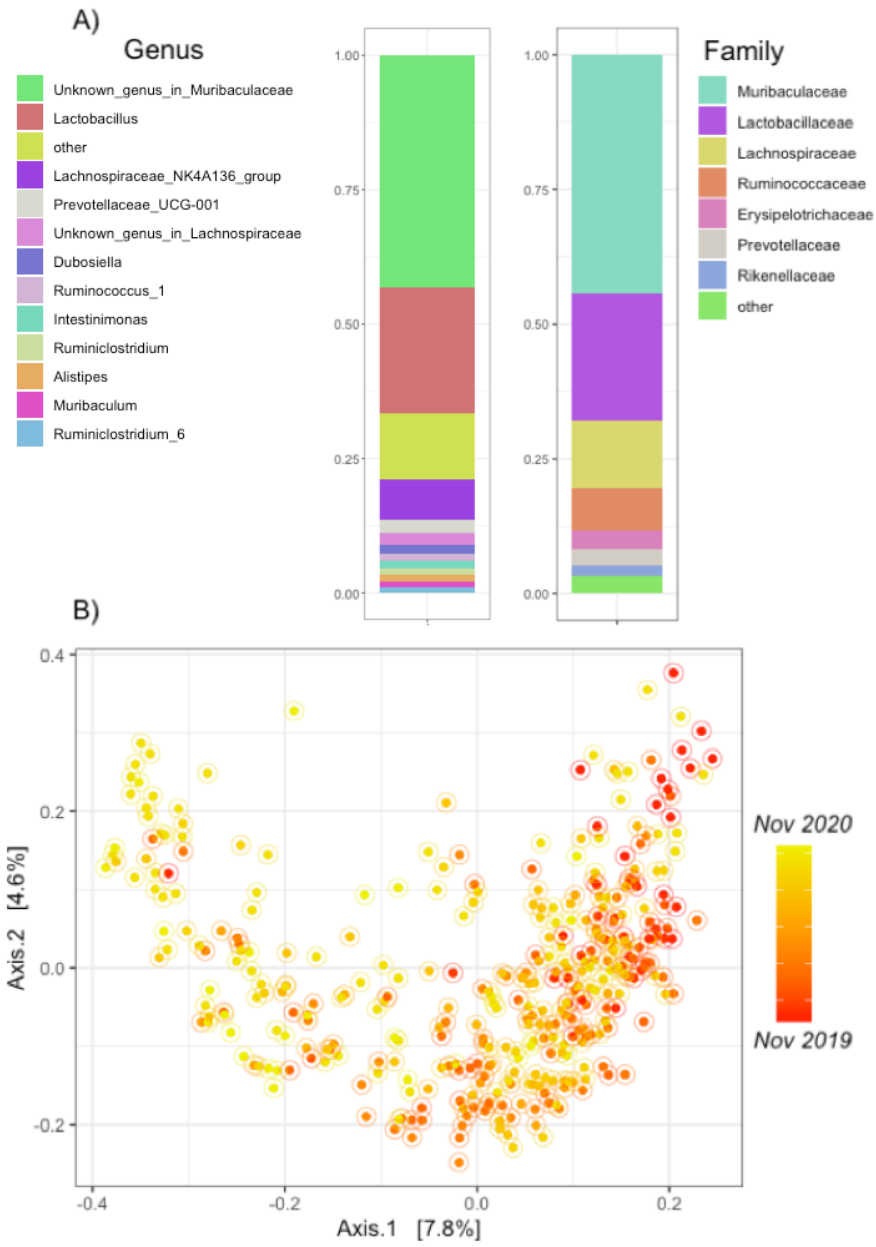

Figure S4.1. Composition and variation in wood mouse gut microbiota.

A) Taxonomic composition of wood mouse gut microbiota in terms of bacterial genera (left) and families (right). Note that while Muribaculaceae is the most common family, genus Muribaculum is not one of the most common genera. This was because most of the ASVs assigned to Muribaculaceae were of unknown genera within this Family. B) PCoA plot shows that the first axis of variation in wood mouse gut microbiota composition (Jaccard Index) reflects temporal changes in microbiota composition across the study period.

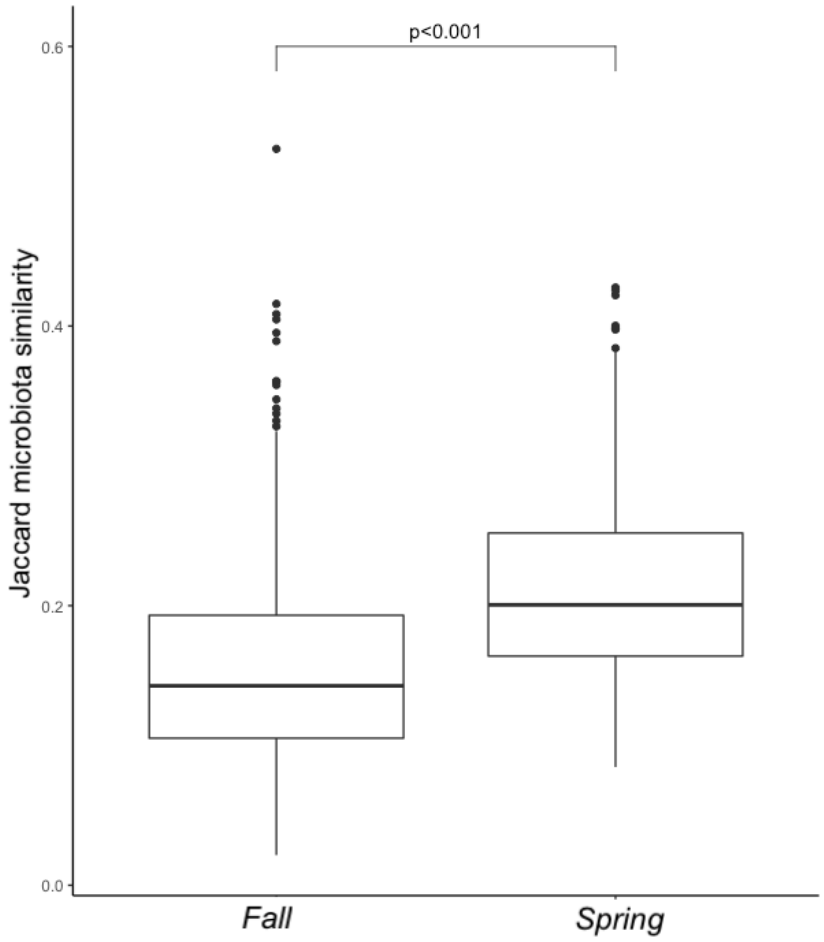

Figure S4.2. Distribution of Jaccard microbiota similarities among mice in Spring and Fall.

Jaccard microbiota similarity values between mice during spring months (Feb-June) were on average significantly higher than during fall (July-November).

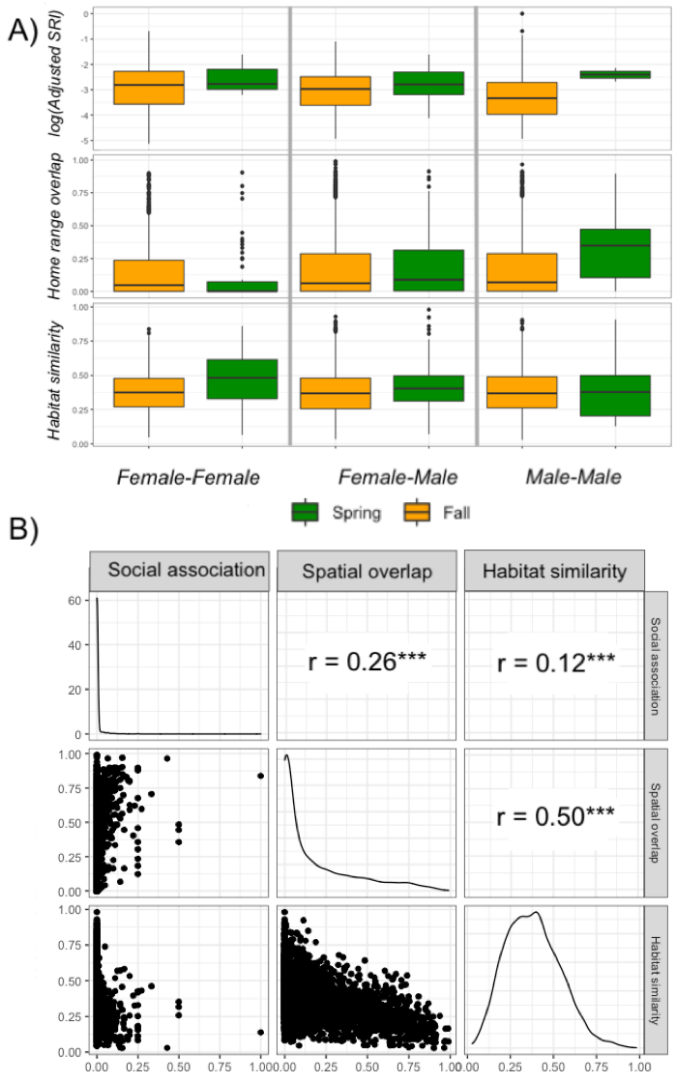

Figure S4.3. Variation and co-variation among social association, spatial overlap and habitat similarity

A) Distribution of social association (upper panel), spatial overlap (middle panel) and habitat similarity (bottom panel) values across categories of sex-combinations (x-axis) and seasons (color). Heavily left-skewed values of social association values are reported on log-scale for ease of comparison. B) Correlation plot depicting associations among measures of social association (Adjusted SRI), spatial overlap (Bhattacaryya overlap between 75%? kernel utilization distributions) and habitat similarity (Bray-Curtis habitat similarity). Lower triangle illustrates covariation among the two intersecting variables, diagonal depicts the distribution of raw values in the intersecting variable and upper triangle reports the correlation among the intersecting variables, as measured by Mantel test statistic.

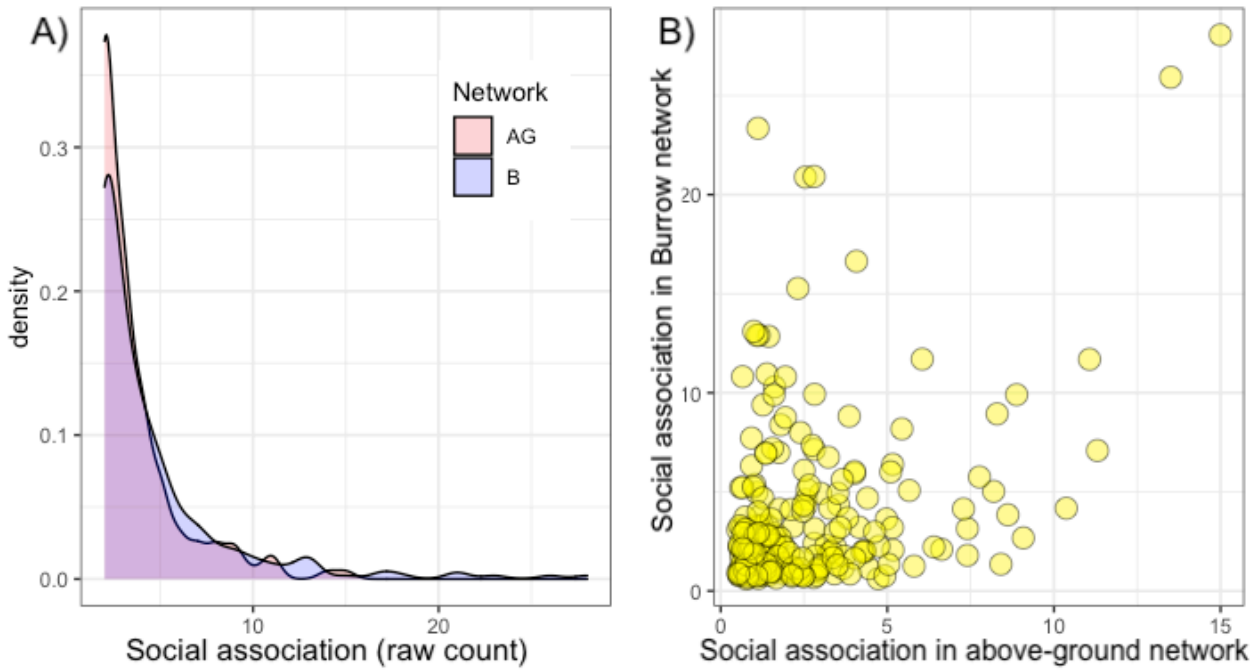

Figure S4.4. Comparison of raw count social networks based on burrow logger and above-ground logger data.

A) Distribution of raw count social association values (numbers of time two individuals were observed at the same burrow entrance on the same night) from burrow and above-ground networks are similar. B) Raw count social association in the burrow network is correlated with raw count social association in the above-ground network, though the correlation is far from complete (Mantel test, r=0.52, p=0.001).

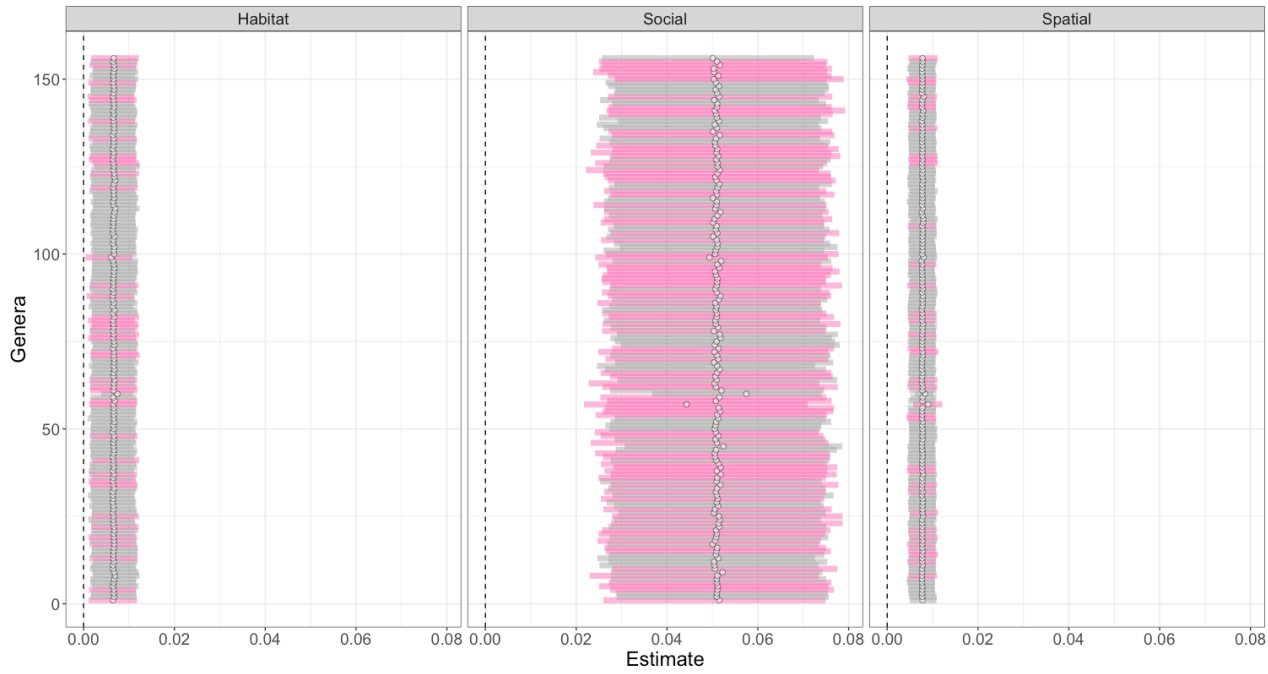

Figure S4.5. Variation in effect estimates and uncertainty across 186 models in which a single bacterial genus was dropped.

Posterior means (points) and their 95% credible intervals (lines) of habitat similarity (left panel), social association (middle panel) and spatial overlap (right panel) effect on microbiota Jaccard similarity (x-axis), are plotted from brms model dropping each of the 186 bacterial genera in turn away from the calculation of Jaccard index (y-axis). Effect size estimates (posterior means=points) are minimally affected by dropping any single genus, but models yield different uncertainty (credible interval breadth=length of line) for effects after each drop. Genera, whose exclusion increases uncertainty around an effect estimate are considered indicator genera important for that effect (pink lines).

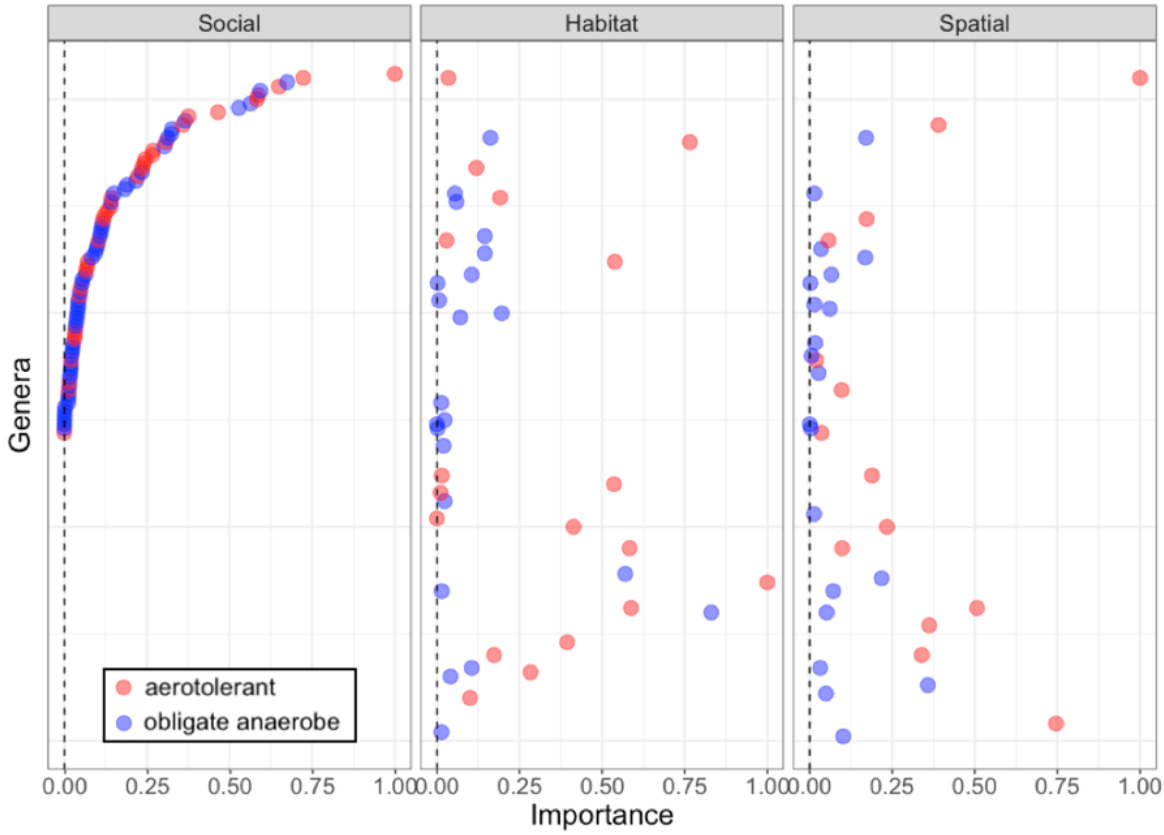

Figure S4.6. Indicator genera for social, spatial and habitat signals in microbiota similarity.

The distribution of social, spatial and habitat signal importance values (x-axis) across the 186 bacterial genera (y-axis) ordered according to their importance for the social signal. Apart from few cases, genera with high importance for spatial and habitat signals in microbiota have no or little importance for the social signal in microbiota. Genera with high importance for social signal comprise equally aerotolerant (in red) as well as anaerobic taxa (in blue), while genera with high importance for spatial or habitat signal are mostly aerotolerant.

Table S4.1. Aerotolerance and spore-forming ability of bacterial genera together with their importance scores in models where excluded. We used Bergey's Manual of Systematics of Archaea and Bacteria alongside other individual scientific publications to find information about the aerotolerance and spore-formation abilities of bacterial genera present in our data. We imitated the protocol described in (Suzuki et al., 2019), whereby for genera with unknown aerotolerance of spore-formation ability (or for unknown genera within a known family), we labelled them according to a majority rule of other genera in the same family, unless there was known to be considerable variation in phenotypes across genera within a family. For some genera with missing information on these phenotypic traits, we also labelled them after another name for the same genus present in the literature (e.g. "UBA1819" as "Faecalibacterium") or according to the genus that they had been previously classified as belonging to (e.g. *Lysinibacillus* as part of *Bacillus*). All genera assigned an aerotolerance or spore-formation label in these indirect manners, have been marked with *, and the taxon after which they were labelled is specified in the "References" column. Spore-formation column labels taxa as spore-forming (SF) or non-spore-forming (NSF). Aerotolerance column labels taxa as obligate anaerobe (OA), facultative anaerobe (OA), microaerobe (MA) or aerobe (A). In the analyses, as in Suzuki et al., 2019, all taxa with any aerotolerance (all except obligate anaerobes) where considered aerotolerant. Other columns describe the number of ASVs belonging to that genus, and the importance of that genus to social, spatial and habitat signals in microbiota ("IMP.SOC", "IMP.SPA", "IMP.HAB").

Genus	Aero-tolerance	Sporulation	Reference	ASVs	IMP. SOC	IMP. SPA	IMP. HAB
A2	?	?		4	NA	NA	NA
Ac37b	?	?		1	NA	NA	NA
Acetatifactor	OA	NSF	(Pfeiffer et al., 2012)	31	0.001	NA	0.023
Achromobacter	OA	NSF	Bergey's Manual	2	NA	NA	NA
Acidibacter	OA-MA	NSF	Bergey's Manual	1	NA	NA	NA
Acidiphilium	OA	NSF	Bergey's Manual	1	0.108	NA	0.145
Acinetobacter	A	NSF	Bergey's Manual	3	0.141	NA	NA
Actinoplanes	A	SF	Bergey's Manual	2	0.015	NA	NA
Aeromonas	FA	NSF	Bergey's Manual	2	NA	NA	NA
Alistipes	OA	NSF	Bergey's Manual	15	0.026	0.016	NA
Allobaculum	OA	NSF	Bergey's Manual	2	NA	NA	NA
Alloprevotella	OA	NSF	Bergey's Manual	1	0.19	NA	NA

Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	A	NSF	Bergey's Manual, based on Rhizobium and Allorhizobium	3	NA	NA	NA
Amaricoccus	A	NSF	Bergey's Manual	1	NA	0.362	NA
Aminobacter	A	NSF	Bergey's Manual	1	NA	NA	0.394
Anaeroplasma	OA	?	Bergey's Manual	1	NA	NA	NA
Anaerosporobacter	OA	SF	(Jeong et al., 2007)	1	NA	0.358	NA
Anaerostipes	OA	NSF	Bergey's Manual	4	0.042	0.014	NA
Angelakisella	OA	NSF	(Mailhe, Ricaboni, Vitton, et al., 2017)	2	0.001	NA	NA
Bacteroides	OA	NSF	Bergey's Manual	6	NA	0.071	0.015
Bifidobacterium	OA	NSF	Bergey's Manual	4	0.02	NA	NA
Bilophila	OA	NSF	Bergey's Manual	1	NA	0.051	0.83
Blastocatella	A	NSF	Bergey's Manual	1	0.268	NA	NA
Brevundimonas	A	NSF	Bergey's Manual	1	0.359	0.39	NA
Butyricicoccus	OA	SF	(Trachsel et al., 2018)	9	NA	NA	0.023
Candidatus_Arthromitus	OA	SF	(Schnupf et al., 2015)	1	0.528	NA	NA
Candidatus_Saccharimonas	?	?		2	NA	NA	NA
Candidatus_Soleaferrea	?	?		1	NA	NA	NA
Candidatus_Stoquefichus	?	?		4	NA	NA	NA
Caproiciproducens	OA	NSF	(Kim et al., 2015)	3	NA	NA	NA
Carnobacterium	FA	NSF	Bergey's Manual	2	0.235	NA	0.12
Cellulosilyticum	OA	SF	(Cai & Dong, 2010)	2	NA	0.05	NA
Chryseobacterium	A	NSF	Bergey's Manual	5	0.07	NA	NA
Clostridium_sensu_stricto_1	OA	SF	Bergey's Manual	2	NA	NA	NA
Coprococcus_2	OA	SF	Bergey's Manual, (Browne et al., 2016)	2	NA	NA	NA
Coriobacteriaceae_UCG-002*	OA	NSF	Bergey's Manual, based on Coriobacteriaceae	2	0.047	NA	NA
Corynebacterium_1	A-FA	NSF	Bergey's Manual	4	NA	NA	NA
Curtobacterium	A	SF	Bergey's Manual	1	0	0.036	NA
Cutibacterium*	OA-MA	NSF	Bergey's Manual, based on Propionibacterium	1	NA	NA	NA
Desulfoplanes*	OA	NSF	Bergey's Manual, based on Desulfomicrobiaceae	1	0.313	0.17	0.162
Desulfovibrio	OA	NSF	Bergey's Manual	20	NA	NA	0.02
Diplorickettsia	OA (Intra-cellular)	NSF	(Mediannikov et al., 2010)	3	0.1	NA	NA
Distigma	?	NA		1	NA	NA	NA
DNF00809	?	?		2	NA	NA	NA
Dubosiella	OA	NSF	(Cox et al., 2017)	6	0.037	NA	0.071
Duganella	A	NSF	Bergey's Manual	4	0.013	0.096	NA
Eisenbergiella*	OA	SF	Bergey's Manual, based on Lachnospiraceae	1	0.097	0.034	NA
Ellin6067*	A	?	(Prosser et al., 2014), based on Nitrosomonadaceae	1	NA	NA	NA
Elusimicrobium	OA	NSF	(Geissinger et al., 2009)	1	0.218	NA	NA
Enterococcus	FA	NSF	Bergey's Manual	5	NA	NA	0
Enterorhabdus	FA	NSF	(Clavel et al., 2010)	9	0.031	NA	NA
Erysipelatoclostridium	OA	NSF	(Yutin & Galperin, 2013)	1	0.674	NA	NA
Escherichia/Shigella	FA-A	NSF	Bergey's Manual	3	NA	NA	NA
Faecalibaculum	OA	NSF	(Chang et al., 2015)	10	NA	NA	NA
Family_XIII_AD3011	?	?		2	NA	NA	NA

Family_XIII_UCG-001	?	?		1	NA	NA	NA
Flavobacterium	A	NSF	Bergey's Manual	7	0.122	NA	NA
Flavonifractor	OA	SF	Bergey's Manual, based on Eubacterium;(Browne et al., 2016), based on Flavonifractor	9	NA	NA	NA
Frigoribacterium	A	NSF	Bergey's Manual	1	0.07	NA	0.538
GCA-900066225*	OA	SF	Bergey's Manual, based on Lachnospiraceae	1	0.111	NA	NA
GCA-900066575*	OA	SF	Bergey's Manual, based on Lachnospiraceae	14	NA	NA	NA
Helicobacter	MA	NSF	Bergey's Manual	3	NA	NA	NA
Intestinimonas	OA	SF	(Kläring et al., 2013)	19	NA	NA	NA
Janthinobacterium	A	SF	Bergey's Manual	1	NA	0.746	NA
Lachnoclostridium	OA	SF	(Yutin & Galperin, 2013)	23	0.022	0.006	NA
Lachnospiraceae_FCS020	OA	SF	Bergey's Manual	4	0.116	NA	NA
Lachnospiraceae_NC2004	OA	SF	Bergey's Manual	1	0.366	NA	NA
Lachnospiraceae_NK4A136	OA	SF	Bergey's Manual	148	0	0.004	0.003
Lachnospiraceae_UCG-001	OA	SF	Bergey's Manual	12	0.011	NA	0.013
Lachnospiraceae_UCG-006	OA	SF	Bergey's Manual	16	0.015	NA	NA
Lactobacillus	FA	NSF	Bergey's Manual	27	NA	NA	NA
Lactococcus	FA	NSF	Bergey's Manual	1	NA	NA	0.1
Lelliottia	FA	?	(Brady et al., 2013)	3	NA	NA	NA
Lysinibacillus*	A	SF	Bergey's Manual, based on Bacillus	1	0.723	1	0.035
Marvinbryantia*	OA	SF	Bergey's Manual, based on Lachnospiraceae	2	0.234	NA	NA
Massilia	A	NSF	Bergey's Manual	1	NA	0.506	0.588
Mesorhizobium	A	NSF	Bergey's Manual	1	0.049	NA	NA
Methylobacterium	A	NSF	Bergey's Manual	1	0.465	NA	NA
Microbacterium	A	NSF	Bergey's Manual	2	NA	NA	NA
Microvirga	A	NSF	(Kanso & Patel, 2003)	1	NA	NA	NA
Millionella	OA	NSF	(Mailhe, Ricaboni, Benezech, et al., 2017)	3	NA	NA	NA
Moraxella	A	NSF	Bergey's Manual	1	0.066	NA	NA
Morganella	FA	NSF	Bergey's Manual	1	NA	NA	1
Mucispirillum	OA	NSF	Bergey's Manual	3	0.15	0.014	0.055
Muribaculum	OA	?	Bergey's Manual, (Lagkouvardos et al., 2019; Ormerod et al., 2016)	4	NA	NA	NA
Mycobacterium	A	NSF	Bergey's Manual	1	0.02	0.02	NA
Myroides	A	NSF	Bergey's Manual	1	NA	NA	NA
Nakamurella	A	NSF	Bergey's Manual	1	NA	NA	NA
Nocardioides	A	NSF	Bergey's Manual	1	NA	NA	0.536
Novosphingobium	A	NSF	(Takeuchi et al., 2001)	1	NA	0.098	0.583
Odoribacter	OA	NSF	Bergey's Manual	4	0.024	NA	NA
Oerskovia	A	NSF	Bergey's Manual	1	NA	NA	0.283
Oscillibacter	OA	NSF	(Iino et al., 2007)	20	0.043	NA	0.007
Oscillospira	OA	SF	Bergey's Manual	1	NA	0.032	0.105
Oxalobacter	OA	NSF	Bergey's Manual	2	0.093	NA	0.145
Paenarthrobacter*	A	NSF	Bergey's Manual, based on Arthrobacter	1	0.118	0.172	NA
Paenibacillus	OA-FA	SF	Bergey's Manual	4	NA	NA	NA
Paeniglutamicibacter*	A	NSF	Bergey's Manual based on Micrococcaceae	1	NA	NA	NA
Paenisporosarcina	A	SF	(Krishnamurthi, Bhattacharya, et al., 2009)	2	0.047	NA	NA

Pantoea	FA	NSF	Bergey's Manual	1	NA	NA	NA
Parabacteroides	OA	NSF	Bergey's Manual	2	0.04	NA	0.196
Paraclostridium*	OA	SF	Bergey's Manual, based on Clostridium	1	0.325	NA	NA
Paraprevotella	OA	NSF	Bergey's Manual	1	NA	NA	NA
Parasutterella	OA	NSF	(Nagai et al., 2009)	8	0.065	0.066	0.105
Parvibacter	OA	NSF	(Clavel et al., 2013)	3	0.034	NA	NA
Pedobacter	A	NSF	Bergey's Manual	4	0.034	NA	NA
Pelosinus	OA	NSF	(Shelobolina et al., 2007)	1	0.325	NA	NA
Peptococcus	OA	NSF	Bergey's Manual	1	0.564	NA	NA
Polaromonas	A	NSF	Bergey's Manual	1	0.222	NA	NA
Prevotella_6	OA	NSF	Bergey's Manual	1	0.041	0.061	NA
Prevotellaceae_Ga6A1	OA	NSF	Bergey's Manual	3	NA	NA	NA
Prevotellaceae_UCG-001	OA	NSF	Bergey's Manual	4	0.142	NA	0.059
Providencia	FA	NSF	Bergey's Manual	1	0.587	NA	NA
Pseudomonas	A	NSF	Bergey's Manual	4	NA	NA	NA
Pseudonocardia	A	SF	Bergey's Manual	2	NA	NA	NA
Pseudoxanthomonas	A	NSF	Bergey's Manual	2	NA	NA	NA
Rickettsiella	OA	NSF	Bergey's Manual	5	0.012	NA	NA
Rikenella	OA	NSF	Bergey's Manual	6	NA	NA	NA
Rikenellaceae_RC9_gut_group	OA	NSF	Bergey's Manual	2	0.183	NA	NA
Rodentibacter	FA	NSF	Bergey's Manual, (Benga et al., 2018)	1	NA	NA	NA
Romboutsia	OA	mix	(Gerritsen et al., 2019)	1	NA	0.101	NA
Roseburia	OA	NSF	Bergey's Manual	40	0.002	NA	NA
Ruminiclostridium	OA	SF	(Yutin & Galperin, 2013)	29	0.012	NA	NA
Ruminiclostridium_5	OA	SF	(Yutin & Galperin, 2013)	13	NA	NA	NA
Ruminiclostridium_6	OA	SF	(Yutin & Galperin, 2013)	6	0.026	NA	NA
Ruminiclostridium_9	OA	SF	(Yutin & Galperin, 2013)	19	NA	NA	NA
Ruminococcaceae_NK4A214*	OA	SF	Bergey's Manual, (Browne et al., 2016), based on Ruminococcaceae	2	0.303	NA	NA
Ruminococcaceae_UCG-003*	OA	SF	Bergey's Manual, (Browne et al., 2016), based on Ruminococcaceae	1	NA	NA	NA
Ruminococcaceae_UCG-005*	OA	SF	Bergey's Manual, (Browne et al., 2016), based on Ruminococcaceae	1	NA	NA	NA
Ruminococcaceae_UCG-009*	OA	SF	Bergey's Manual, (Browne et al., 2016), based on Ruminococcaceae	1	0.113	NA	NA
Ruminococcaceae_UCG-010*	OA	SF	Bergey's Manual, (Browne et al., 2016), based on Ruminococcaceae	5	0.001	NA	NA
Ruminococcaceae_UCG-013*	OA	SF	Bergey's Manual, (Browne et al., 2016), based on Ruminococcaceae	2	NA	NA	0.041
Ruminococcaceae_UCG-014*	OA	SF	Bergey's Manual, (Browne et al., 2016), based on Ruminococcaceae	97	NA	NA	NA
Ruminococcus_1*	OA	SF	Bergey's Manual, (Browne et al., 2016), based on Ruminococcaceae	16	0.019	NA	NA
Saccharibacillus	FA	SF	(Rivas et al., 2008)	1	0.241	NA	NA
Solibacillus	A	SF	(Krishnamurthi, Chakrabarti, et al., 2009)	1	0.307	NA	0.766
Sphaerochaeta	OA-FA	NSF		1	0.593	NA	NA
Sphingobium*	A	NSF	Bergey's Manual, based on Sphingomonas	2	NA	NA	NA
Sporosarcina	OA-FA	SF	Bergey's Manual	10	0.053	0.002	0.002

Staphylococcus	FA	NSF	Bergey's Manual	2	NA	0.189	0.015
Streptococcus	FA	NSF	Bergey's Manual	4	0.104	0.057	0.029
Streptomyces	A	SF	Bergey's Manual	1	1	NA	NA
Tardiphaga	A	NSF	(de Meyer et al., 2012)	1	0.244	NA	NA
Terrimonas	A	NSF	Bergey's Manual	2	0.266	NA	NA
Tessaracoccus	FA	NSF	Bergey's Manual	1	NA	NA	NA
Treponema_2	OA	NSF	Bergey's Manual	1	NA	NA	0.57
Tritrichomonas	OA	NSF	Bergey's Manual	1	0.037	NA	NA
Tyzzerella	OA	mix	(Yutin & Galperin, 2013)	2	0.055	NA	NA
Tyzzerella_3	OA	mix	(Yutin & Galperin, 2013)	3	NA	0.218	NA
UBA1819*	OA	NSF	Bergey's Manual, based on Faecalibacterium	1	NA	NA	NA
UC5-1-2E3*	OA	SF	Bergey's Manual, based on Clostridia	1	NA	NA	0.014
Unknown genus in Aphagea_fa	?	NA		1	NA	NA	NA
Unknown genus in Burkholderiaceae*	mix	mix	Bergey's Manual, based on Burkholderiaceae	3	NA	NA	NA
Unknown genus in Carnobacteriaceae*	FA-A	NSF	Bergey's Manual, based on Carnobacteriaceae	1	0.131	NA	NA
Unknown genus in Chitinophagaceae*	?	NSF	Bergey's Manual, based on Chitinophagaceae	2	NA	NA	NA
Unknown genus in Clostridiales_vadinBB60_group*	OA	SF	Bergey's Manual, Based on Clostridia	54	0.016	NA	NA
Unknown genus in Coriobacteriales_Incertae_Sedis	?	?		1	NA	NA	NA
Unknown genus in Desulfovibrionaceae*	OA	NSF	Bergey's Manual, based on Desulfovibrionaceae	1	0.082	0.168	NA
Unknown genus in Eggerthellaceae*	OA	NSF	Bergey's Manual, based on Eggerthellaceae	17	NA	0.013	NA
Unknown genus in Enterobacteriaceae*	FA	NSF	Bergey's Manual, based on Enterobacteriaceae	8	NA	NA	0.011
Unknown genus in Entotheonellaceae	?	?		1	NA	NA	NA
Unknown genus in Erysipelotrichaceae	?	?		2	NA	NA	NA
Unknown genus in Family_XIII	?	?		5	NA	NA	NA
Unknown genus in JG30-KF-CM45	?	?		1	NA	NA	NA
Unknown genus in Lachnospiraceae*	OA	SF	Bergey's Manual, based on Lachnospiraceae	179	0	0	0
Unknown genus in Micrococcaceae*	A	NSF	Bergey's Manual, based on Micrococcaceae	1	NA	NA	NA
Unknown genus in Microscillaceae	?	?		2	NA	NA	NA
Unknown genus in Muribaculaceae*	OA	?	Bergey's Manual, (Lagkouvardos et al., 2019; Ormerod et al., 2016), based on Muribaculaceae	276	NA	NA	NA
Unknown genus in Unknown family	?	?		59	NA	NA	NA
Unknown genus in Neisseriaceae*	A	NSF	(Cohen, J., Powderly, W., & Opal, 2017), based on Neisseriaceae	1	NA	NA	NA

Unknown genus in Nitrososphaeraceae*	A	NSF	Bergey's Manual, based on Nitrososphaeraceae	1	NA	NA	NA
Unknown genus in Paracaedibacteraceae	?	?		1	NA	NA	NA
Unknown genus in Pasteurellaceae	?	?		1	NA	NA	NA
Unknown genus in Peptococcaceae*	OA	?	Bergey's Manual, based on Peptococcaceae	3	NA	NA	NA
Unknown genus in Rhizobiaceae*	A	NSF	Bergey's Manual, based on Rhizobiaceae	1	NA	0.234	0.414
Unknown genus in Rikenellaceae*	OA	?	Bergey's Manual, based on Rikenellaceae	2	NA	NA	NA
Unknown genus in Ruminococcaceae*	OA	SF	Bergey's Manual, (Browne et al., 2016), based on Ruminococcaceae	20	0.018	0.027	NA
Unknown genus in Xanthobacteraceae*	A	NSF	Bergey's Manual, based on Xanthobacteraceae	3	0.032	NA	NA
Ureaplasma	FA	NSF	Bergey's Manual	1	0.649	NA	NA
Variovorax	A	NSF	Bergey's Manual	1	0.583	NA	NA
Verticia	FA	?	Bergey's Manual	1	NA	NA	NA
Vitreoscilla	A-MA	NSF	Bergey's Manual	1	0.143	NA	0.192
Xanthomonas	A	NSF	Bergey's Manual	1	0.376	NA	NA
Yokenella	FA	NSF	Bergey's Manual	2	NA	0.339	0.173

Table S4.2. Effects of Social association, spatial overlap and habitat similarity on microbiota similarity.

Results of *brms* models testing the effect of social association, spatial overlap, habitat similarity and covariates on microbiota similarity (Jaccard Index). Significant terms (where 95% credible intervals do not include zero) are shown in bold. Est.Error indicates the standard deviation of the posterior distribution.

Table S4.2.				
	Estimate	Est.Error	l-95% CI	u-95% CI
Intercept	-1.794	0.049	-1.891	-1.699
Sample read depth difference	-0.009	0.021	-0.050	0.033
Extraction distance	-0.006	0.007	-0.020	0.007
PCR-plate similarity	**0.011**	**0.006**	**0.000**	**0.023**
Age-similarity	**0.170**	**0.029**	**0.111**	**0.226**
Sex similarity	0.000	0.004	-0.008	0.007
Sampling interval	**-0.333**	**0.011**	**-0.354**	**-0.311**
Habitat similarity	**0.041**	**0.018**	**0.006**	**0.077**
Spatial overlap	**0.055**	**0.011**	**0.034**	**0.076**
Social association	**0.295**	**0.082**	**0.136**	**0.454**

Table S4.3. Effects of same- and opposite sex social association in the Spring and Fall social networks on microbiota similarity. Results of *brms* models testing the effect of social association, spatial overlap, habitat similarity and covariates on microbiota similarity (Jaccard Index). Significant terms (where 95% credible intervals do not include zero) are shown in bold. Est.Error indicates the standard deviation of the posterior distribution. For ease of interpretation, interaction effects are *NOT* reported as slopes relative to each other but as independent effects within each group.

Table S4.3. A. Effects of association in the spring social network on microbiota similarity				
	Estimate	Est.Error	l-95% CI	u-95% CI
Intercept	-1.452	0.099	-1.642	-1.251
Sample read depth difference	-0.014	0.063	-0.138	0.109
Extraction distance	-0.011	0.016	-0.042	0.021
PCR-plate similarity	0.004	0.014	-0.022	0.031
Sampling interval	**-0.582**	**0.043**	**-0.667**	**-0.498**
Habitat similarity	**0.140**	**0.049**	**0.044**	**0.236**
Spatial overlap	**-0.089**	**0.035**	**-0.158**	**-0.020**
Sex similarity	**-0.023**	**0.011**	**-0.044**	**-0.001**
Opposite sex: Spring social association	-0.038	0.259	-0.535	0.475
Same sex: Spring social association	**1.770**	**0.366**	**1.187**	**2.354**
Table S4.3. B. Effects of association in the fall social network on microbiota similarity				
	Estimate	Est.Error	l-95% CI	u-95% CI
Intercept	-1.832	0.055	-1.941	-1.726
Sample read depth difference	-0.045	0.030	-0.104	0.014
Extraction distance	-0.005	0.008	-0.021	0.011
PCR-plate similarity	0.010	0.007	-0.004	0.023
Age similarity	**0.202**	**0.031**	**0.140**	**0.262**
Sampling interval	-0.489	0.029	-0.547	-0.432
Habitat similarity	0.042	0.025	-0.007	0.091
Spatial overlap	**0.109**	**0.016**	**0.078**	**0.139**
Sex similarity	0.002	0.005	-0.008	0.013
Opposite sex: Fall social association	-0.204	0.159	-0.519	0.097
Same sex: Fall social association	**0.237**	**0.189**	**0.010**	**0.471**

Table S4.5. Effects social association on Jaccard similarity based on aerotolerant vs. anaerobic taxa. Results of brms models testing the effect of social association, spatial overlap, habitat similarity and covariates on microbiota similarity (Jaccard Index), based on an equal-sized set (71 genera, 164 ASVs) chosen randomly from a) all taxa, b) aerotolerant taxa or c) anaerobic taxa. Significant terms (where 95% credible intervals do not include zero) are shown in bold. Est.Error indicates the standard deviation of the posterior distribution.

Table S4.4.A. Effects of social association on microbiota similarity (baseline effects)				
	Estimate	Est.Error	l-95% CI	u-95% CI
Intercept	-1.837	0.058	-1.951	-1.723
Sample read depth difference	0.001	0.027	-0.052	0.053
Extraction distance	-0.007	0.009	-0.024	0.011
PCR-plate similarity	**0.021**	**0.007**	**0.006**	**0.036**
Age-similarity	0.042	0.034	-0.026	0.108
Sex similarity	-0.003	0.005	-0.012	0.007
Sampling interval	**-0.300**	**0.014**	**-0.328**	**-0.272**
Habitat similarity	0.021	0.023	-0.023	0.065
Spatial overlap	**0.105**	**0.014**	**0.078**	**0.132**
Social association	**0.421**	**0.106**	**0.211**	**0.630**
Table S4.4. B. Effects of Social association aerotolerant microbiota similarity				
	Estimate	Est.Error	l-95% CI	u-95% CI
Intercept	-1.538	0.094	-1.721	-1.353
Sample read depth difference	-0.035	0.048	-0.128	0.059
Extraction distance	-0.006	0.016	-0.038	0.026
PCR-plate similarity	-0.012	0.013	-0.038	0.014
Age-similarity	**0.252**	**0.070**	**0.111**	**0.388**
Sex similarity	-0.001	0.009	-0.020	0.016
Sampling interval	-0.381	0.025	-0.431	-0.332
Habitat similarity	0.008	0.041	-0.070	0.090
Spatial overlap	**0.051**	**0.024**	**0.004**	**0.099**
Social association	0.093	0.191	-0.282	0.467

Table S4.4. C. Effects of Social association anaerobic microbiota similarity				
	Estimate	Est.Error	l-95% CI	u-95% CI
Intercept	-2.711	0.079	-2.865	-2.558
Sample read depth difference	0.002	0.043	-0.082	0.089
Extraction distance	-0.020	0.014	-0.049	0.008
PCR-plate similarity	0.023	0.012	-0.001	0.047
Age-similarity	**0.358**	**0.054**	**0.250**	**0.463**
Sex similarity	0.002	0.008	-0.014	0.017
Sampling interval	-0.393	0.023	-0.437	-0.349
Habitat similarity	**0.091**	**0.036**	**0.021**	**0.160**
Spatial overlap	0.002	0.022	-0.042	0.045
Social association	**0.434**	**0.170**	**0.094**	**0.764**

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5

Cross-species transmission of gut microbes in a wild rodent community

List of contributions

- *Aura Raulo* designed the study, helped develop the new RFID tracking technology, collected the data from Wytham, completed all laboratory analyses on gut microbiota profiling prior to sequencing, developed analytical methods, analysed the data and wrote the manuscript
- *Emma Dale* helped collect field data using RFID loggers
- *Holly English* helped collect field data using RFID loggers and provided feedback on home range analyses
- *Bryony Allen* helped collect and clean the tracking data set from Silwood
- *Curt Lamberth* led development of RFID tracking devices and helped collect field data from Wytham
- *Josh Firth* supervised the research project, developed social network analysis methods and provided feedback on the analyses and the manuscript
- *Tim Coulson* supervised the research project and provided feedback on the analyses and the manuscript
- *Sarah Knowles* supervised the research project, helped develop the tracking technology and design the study, collected data from Silwood and Wytham, planned and supervised laboratory methods, developed analytical methods and provided feedback on analyses and the manuscript.

Abstract

Gut microbiota composition of the microbiota thus provides an important source of adaptive plasticity for their hosts but little is known about how microbiota variation between individuals, populations or species arise. Mounting evidence suggests that an important process in shaping this variation is transmission of microbes from the environment, especially through direct social contact with others. The effects of social behaviour and environmental exposure on microbial transfer have been shown in multiple species of mammals, but an important open question remains in whether such behaviourally driven individual-to-individual transmission processes also transmit bacteria across species boundaries. Here, in two separate woodlands, we use RFID-tracking data to monitor the fine-scale spatial and social interactions of three common sympatric wild rodent species: wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicollis*) and bank vole (*Myodes glaerolus*). Using over 10 months of tracking data from each population, we link multi-species social networks and spatial overlap matrices with faecal microbiota profiles to explore how social association or spatial overlap with conspecific and interspecific individuals might differentially affect the gut microbiota of the most common species, wood mice. Additionally, for each wood mouse we test how measures of con- and heterospecific social connectedness and density predict the extent to which their gut microbiota harbours microbes typically associated with other species. Across both populations, we reveal a consistent pattern in which pairwise social associations predict overall microbiota similarity between conspecific individuals only, while space-sharing is a strong predictor of microbiota similarity between heterospecific pairs. However, while similarity in overall microbiota composition was not predicted by heterospecific social association, we found evidence for cross-species social transmission when focusing on indicator taxa for each host species; in both study populations, direct and indirect social connectedness of wood mice to their sister species yellow-necked mice positive predicted the richness and abundance of yellow-necked mouse-typical gut microbes in their guts. These results highlight the potential for individual-level variation in behaviour to drive processes of gut microbial spread across species boundaries, especially between closely related species with overlapping niches.

5.1 Introduction

All vertebrates harbour a variety of symbiotic microbes (the ‘microbiota'). In mammals, the most diverse of these microbial communities, containing hundreds to thousands of microbial species, resides in the gut. The composition of the mammalian gut microbiota is highly individualized, varies across populations, between species and shows marked dynamics within individuals over time. These highly variable symbiotic communities are a key modulator of host biology, and are known to help animals digest specific foods (Delsuc et al., 2014; Duron & Gottlieb, 2020; Kohl et al., 2014), regulate immunity (Hooper et al., 2012; Round & Mazmanian, 2009), metabolism (Visconti et al., 2019) and thermoregulation (Chevalier et al., 2015; Fontaine et al., 2021), and even affect mood and behaviour through hormonal (Sandrini et al., n.d.) and neural pathways (Montiel-Castro et al., 2013; Wu et al., 2021). Consequently, the gut microbiota is increasingly seen as a source of adaptive phenotypic variation (Alberdi et al., 2016), that can enhance a host’s capacity to adapt to different environments and lifestyles. Through such effects, the gut microbiota ultimately shapes the ecological niche of their host.

Because of this biological significance, an ever-growing body of research aims to map the forces shaping gut microbiota composition. Variation in the microbiota will be shaped both by ecological processes operating within the microbial community (e.g. competition and cooperation between microbial species) and those occurring between microbes and their host (e.g. selection imposed by the host immune system; Coyte et al., 2015). Host-driven selection on the microbiota is underpinned by heritable differences in host physiology and behaviour, and consequently genetic effects on the mammalian gut microbiota have been widely documented (Goodrich et al., 2014; Grieneisen et al., 2021; Knowles et al., 2019; Suzuki et al., 2019). A host’s environment also typically has strong effects on the microbiota, for example with factors like diet and habitat affecting which microbes can thrive within the gut (Amato et al., 2014; Bennett et al., 2016; David et al., 2014; Lehtimäki et al., 2017). However, alongside these factors operating at a within-host scale, it is increasingly recognised that between-host processes (microbial transmission) play a critical role in shaping individual microbiota composition, such that a

metacommunity perspective on these communities is valuable (Miller et al., 2018; Miller & Bohannan, 2019; Robinson et al., 2019).

Transmission among hosts is a key process affecting gut microbiota variation in mammals. This can occur through vertical transmission from mother to offspring during birth and rearing (Dominguez-Bello et al., 2010; Moeller et al., 2018) or via horizontal transmission, either during direct social contact or indirectly through contact with microbes in the host's environment (e.g. contact with contaminated soil). Recent research has highlighted the powerful role that horizontal transmission through social interactions, or 'social transmission' can play in shaping the mammalian gut microbiota. For example, intimate social contacts have been shown to lead to more similar gut microbiotas among conspecific social partners in humans (Brito et al., 2019; Dill-McFarland et al., 2019) wild primates (Perofsky et al., 2017; Raulo et al., 2018; Tung et al., 2015) and wild rodents (Raulo et al., 2021). Further, sharing the same living environment has been shown to homogenise the gut microbiota of cohabiting humans (Sharma et al., 2019; Song et al., 2013), laboratory rodents (McCafferty et al., n.d.), domestic animals (Pajarillo et al., 2015; Torres et al., 2017) and animals in natural populations such as baboons (Grieneisen et al., 2019) and horses (Antwis et al., 2018; Stothart et al., 2021). Such social influences or household effects often outweigh the influence of individual-level factors in explaining microbiota community composition within species (Grieneisen et al., 2019; Raulo et al., 2018, 2021; Rothschild et al., 2018; Tung et al., 2015).

While there is mounting evidence that social transmission among members of a single species or population is an important driver of microbiota variation, a major outstanding question is to what extent such transmission processes occur across host species boundaries and could therefore shape interspecific microbiota variation. Specifically, most studies into the social transmission of microbiota have focussed on effects within single-species populations, but in reality, these populations often exist in larger mixed-species communities where heterospecifics interact. As such sympatric animal species, and especially those with strongly overlapping niches, may share gut microbes directly through social contacts (e.g. aggressive encounters or affiliative interactions) or indirectly through shared exposure to, and transmission through, environmental pools of microbes (e.g. faeces in the general environment or at

shared feeding or nesting sites). Gut microbiota composition is well known to differ between mammalian species, but these distinctions are far from complete. Importantly, while there exists evidence for a strong host phylogenetic signal in the mammalian gut microbiota (a pattern termed 'phylosymbiosis'; Brooks et al., 2016; Knowles et al., 2019; Kohl et al., 2018; Moeller et al., 2016), there is also often considerable overlap in the microbial taxa found within sympatric populations of different species, more so among closely related species. Indeed, sharing living habitat has been shown to correlate with similarity of gut microbiota between humans and their pets (Song et al., 2013) as well as among sympatric wild species of small mammals and primates (Baxter et al., 2015; Grieneisen et al., 2019; Knowles et al., 2019; Moeller et al., 2013). For example, co-occurring species of voles, mice and shrews, while hosting clearly species-specific microbiotas, were shown to have more similar microbiota with locally sympatric heterospecific individuals, than heterospecific individuals caught at other nearby sites (Knowles et al., 2019). Similarly, gorillas and chimpanzees inhabiting the same broad geographic area shared more gut microbes than gorillas and chimpanzees living far apart (Moeller et al., 2013). Further, humans were shown to share more gut microbes with their own dogs than other dogs (Song et al., 2013). Further, in a study comparing gut microbiota of multiple mammalian species across the whole western hemisphere, sympatric populations of different mammalian species had significantly more similar microbiota than allopatric (Moeller et al., 2017). When sympatric species are phylogenetically close relatives, the effect of sharing space on the microbiota might even erode species specificity in the microbiota. For instance, gut microbial profiles were found to be indistinguishable between sympatric populations of closely related *Peromyscus* mouse species (Baxter et al., 2015) and similar microbiota homogeneity existed among sympatric populations of closely related baboon subspecies (Grieneisen et al., 2019). Furthermore, in addition to niche overlap, other types of ecological interactions, such as trophic relationships can create transmission routes for gut microbiota. For instance, while the gut microbiota of mammalian carnivores is compositionally different (e.g. less diverse) than that of herbivores (Milani et al., 2020), predatory mammals were shown to host more similar gut microbiota to their common prey animals than to other sympatric herbivores (Moeller et al., 2017).

Despite the fact that most natural populations exist within a larger community, no studies of wild animals have resolved patterns of cross-species microbiota transmission at an individual-level within a single

host community, and assessed to what extent microbiota similarity between sympatric arises from direct (e.g through physical contact) vs. indirect microbial transmission (e.g. through the environment). Here, we do this by using RFID technology to intensively track social interactions and space use of individuals belonging to three sympatric wild rodent species at two distinct locations, while collecting parallel data on their gut microbiota. Using this data, we assess how social and spatial interactions predict gut microbiota similarity among con- and heterospecific pairs of individuals. Specifically, we test three predictions about cross-species sharing of gut microbes: (1) we predict that both direct (via social contact) and indirect (via the environment) mechanisms could drive gut microbe transmission among heterospecifics. Transmission by direct social contact is expected to generate a positive correlation between heterospecific social association (proximity in a social network) and gut microbiota similarity, that is independent of spatial proximity between individuals. Indirect transmission is expected to generate a positive relationship between spatial proximity and gut microbiota similarity, that is independent of social association. Overall, therefore, we predict positive and independent effects of social association and spatial proximity on pairwise microbiota similarity among heterospecifics. (2) Since each host species is expected to harbour some 'indicator taxa' (microbes enriched in that particular host species), we predict that those individuals most strongly socially connected to members of another species should harbour either a greater diversity or relative abundance of that species' indicator taxa. Finally, (3) since host species should share only a proportion of microbial taxa, we predict a positive relationship between an individual's social association with heterospecifics, and overall microbiota richness.

5.2 Methods

Study species

We studied two communities of three common woodland rodent species, that commonly co-occur across Europe: wood mice (*Apodemus sylvaticus*, AS), yellow-necked mice (*Apodemus flavicollis,* AF) and bank voles (*Myodes glaerolus,* MG). These species have overlapping home ranges and partially overlapping diets, and nest in (occasionally the same) underground burrows. They can engage in a range of ecological and social interactions that could facilitate gut microbe transmission, including i) shared

exposure to environmental microbes (e.g. from soil, diet or vegetation) where home ranges overlap, ii) direct transmission of microbes through social behaviours such as aggression (Hoffmeyer, 1973; Montgomery, 1978; Simeonovska-Nikolova et al., 2016) and iii) indirect transmission of gut microbes through exposure to faeces in the shared environment.

Field data collection

We used a combination of trapping, PIT-tagging and PIT-tag loggers to collect high-resolution spatiotemporal data on individual rodent whereabouts in two ~2.5 ha study plots in separate woodlands: Wytham Woods in Oxfordshire (hereafter "Wytham", 160 x 160 m, 51.77 °N, -1.33°S) and Nash's Copse, Silwood Park (hereafter 'Silwood", 200 x 120 m 51.41 °N, -0.64°S) in Berkshire, UK. Species were present in similar relative abundances at both sites, with wood mice dominating in numbers. At both sites, trapping was carried out approximately fortnightly throughout the study. At first capture, all rodents were injected subcutaneously with a passive integrated transponder (PIT)-tag for permanent identification and tracking purposes. At each trapping, demographic data on rodent individuals was recorded and faecal samples for gut microbiota analysis (~50-300 mg) were collected from the traps with sterilized tweezers and frozen at -80°C within 12 hours of collection. Traps were sterilized by washing in bleach solution between trappings (with additional autoclave of traps in Silwood) to avoid cross-contamination of samples. Rodents were tracked for approximately one year at each site using custom-build PIT-tag loggers - devices that record the time-stamped presence of any tagged rodent within the range of their detection coil. Loggers were evenly spaced and rotated around study areas to enable even spatial coverage in rodent monitoring. While the general principles of data collection were comparable between sites, the study areas, logger design and logger rotation protocols differed somewhat between the two sites, with an improved logger design and greatly increased logger density in Wytham (n=60 loggers in Wytham and n=9 loggers in Silwood, each across a ~2.5ha plotm, see Table S5.1, Figure S5.1).

Constructing social networks and spatial matrices

We used spatiotemporal co-occurrence data from loggers to build networks describing both social interactions (social networks) and spatial proximity (spatial matrices) among all sympatric rodent individuals, at each of the two sites. Networks and matrices were built using data from all tagged rodents that were recorded by loggers in each site. All analyses were conducted in R version 4.0.3 (R Core Team, 2020).

Social network construction

Following the principles of animal social network analysis (Croft et al., 2008; Krause et al., 2015). individuals were nodes in the social network, and edge weight was based on the frequency of incidents when two individuals were observed 'associated' relative to the frequency they were observed in general. Here, 'associated' meant observed within 12 hours of each other at the same logger location during the same night, where night was defined as the 16-h period from 4pm to 8am, since this is the daily period when all three rodent species are active (Figure S5.2). From the raw counts of co-occurrence incidents, association matrices were derived by calculating a customized index of association strength, similar to the Simple Ratio Index (SRI), but adjusted for variable overlap in individual lifespans (Firth & Sheldon, 2016; Raulo et al., 2021). Individual 'lifespans' were taken as the time between their first and last logger observation. This association index (hereby "Adjusted SRI") was defined as:

$$I = \frac{X}{[X + y_{AB} + yA + yB],}$$

where X = the number of instances (number of night-location combinations) in which individuals A and B were observed associated (within 12 h of each other), y_{AB} is the number of instances in which A and B were *both* observed but not associated (observed in the same location on the same night but not within 12h of each other), y_A and y_B are the number of instances in which both were known to be alive but only A or B were observed respectively. Using an association criterion based on observed in the same locating within 12h of each other during a 16h "night" allowed us to specify association in a way comparable to earlier research on wood mouse social networks (Raulo et al., 2021) while simultaneously use all data from the time of the day when rodents were most active. Social networks were plotted using the *igraph* package (Csardi, 2014).

Spatial matrix construction

To explore space use patterns of our study species, we estimated home ranges as the 75% core of the kernel density utilization distribution of each individual's tracking records using the R packga ctmm, and used the Bhattacaryya coefficient to estimate overlap between any two ranges (See Chapter 4 for details on this method). Since accurate home ranges (and therefore measures of home range overlap) could only be estimated for individuals with high enough numbers of logger records (52% of AF, 81% of AS, 73% of MG that had more than five unique observations in more than three unique locations and whose logger records satisfied the variogram criteria described in Chapter 3), we adopted a simpler metric of *spatial overlap* that could be calculated for all individuals, based on distance between the centroids of each rodent's logger records. Metric distances between centroids were scaled between 0 and 1 and the complement taken to derive spatial proximity (spatial proximity = 1-scaled distance). Preliminary analyses showed that this spatial proximity measure correlated well with home range overlap (Bhattacaryya coefficients) up to ~100m (Pearson's correlation coefficient= 0.66, $p<0.01$, Supplementary Appendix S5.1, Supplementary Figure S5.3). Therefore, we used spatial proximity as a proxy for spatial overlap for animals whose centroids were up to 100 meters apart, and set spatial overlap to 0 for those with spatial proximity values >100m.

Estimating individual social connectedness

In order to test our second and third predictions, we calculated node-based metrics reflecting each individuals' degree of social connectedness to different types of rodents (social centrality). For these analyses we used wood mice as a focal species, as they were the most numerous at both sites. To measure overall social connectedness, we first calculated each individual's "Full degree" in the multi-species social network, which is the number of social connections each wood mouse has to individuals of any rodent species. Second, we calculated conspecific and heterospecific degree (CD and HD), that is the number of social connections each mouse has to individuals of its own species or to individuals of all other species, respectively. Third, we calculated species-specific heterospecific degrees: AF-degree (number of connections to *A. flavicollis* individuals) and MG-degree (number of connections to *M. glareolus* individuals).

Finally, we complemented these measures of direct heterospecific connectedness with a set of custom indices additionally capturing secondary connections to heterospecific individuals through other wood mice. These extended heterospecific connectedness indices (Extended HD) were defined as:

$$Extended\ HD_a = HD_a + \sum HD_b w_{ab}$$

where HD_a is individual a's heterospecific degree and $\sum HD_b w_{ab}$ is the sum of the heterospecific degrees of all direct contacts of individual a (HD_b) multiplied by social association strength between individual a and each direct contact b respectively (w_{ab}). Extended heterospecific degree was calculated for all wood mice, separately to describe each individual's connectedness to yellow-necked mice ("Extended AF-degree"), bank voles ("Extended MG-degree") and heterospecific individuals in general ("Extended HD").

We also derived a parallel set of metrics to capture each individual wood mouse's indirect exposure to other rodents through shared space. We used the spatial proximity matrices to calculate a set of spatial rodent density measures, comparable to the social centrality metrics, for each individual wood mouse. Variation in conspecific and heterospecific rodent density around each wood mouse's location centroid was captured by calculating the sum of all spatial proximity values for each wood mouse individual. Similar to social centrality metrics, this was done separately to measure summed proximity to all rodents ("Full density"), conspecific rodents ("Conspecific density"), heterospecific rodents ("Heterospecific density"), yellow-necked mice ("AF-density") and bank voles ("MG-density").

Laboratory work and microbial data pre-processing

We successfully characterised the gut microbiota from 411 faecal samples belonging to 221 rodent individuals in Wytham (297 AS samples, 21 AF samples, 22 MG samples) and from 269 samples belonging to 88 individuals in Silwood (239 AS samples, 30 AF samples). Vole microbiota samples from Silwood were not collected, and thus all our data on vole microbiota comes from Wytham. The gut microbiota was profiled with amplicon sequencing of the 16S rRNA marker gene (V4 or V4-5 region),

sequenced on the Illumina MiSeq platform. Samples from the two sites were processed and analysed similarly, though a different reverse primer was used in Wytham. Full details of library preparation, sequencing and bioinformatics are given in Chapter 2 (for Silwood data) and Chapter 3 (for Wytham data) with methodological differences between the two studies summarised in Supplementary Table S5.1. For both data sets, sequence data were processed through the DADA2 pipeline ((Callahan et al., 2016); versions 1.6 and 1.14), to infer amplicon sequence variants (ASVs). Using package *phyloseq* (McMurdie & Holmes, 2013), ASV counts were normalized to proportional abundance within each sample (McKnight et al., 2019) and singleton ASVs as well as those belonging to probable non-gut microbial taxa (Cyanobacteria) were removed. Lastly, we used sample completeness and rarefaction curves made with package *iNEXT* (Hsieh et al., 2016) to decide read depth thresholds below which samples were dropped from the dataset (6000 in Wytham and 10,000 in Silwood, See Figure S3.7). Despite no visible bands in negative controls when run on a gel, the Wytham dataset had detectable levels of contamination in sequenced negative controls, and plate-wide patterns of spatial autocorrelation in beta diversity were consistent with some sample-to-sample cross-contamination. We minimized the effect of this cross-contamination by removing taxa labelled as contaminants using the *decontam* algorithm (Davis et al., 2018), and by having the extraction and PCR plate of each sample as technical covariates in all models. A full description of this contamination issue and mitigation can be found in Supplementary Appendix S3.4. For consistency, extraction/PCR plate (the two are equivalent in this dataset) was also included as a covariate in models using Silwood data.

Describing the gut microbiota of woodland rodents

To estimate microbiota alpha diversity, we calculated asymptotic richness and asymptotic Shannon diversity using package iNEXT. To characterise beta diversity, we estimated the Jaccard Index for all pairs of individuals (the proportion of observed ASVs that are shared). Finally, to estimate the extent to which each individual wood mouse harboured microbial taxa (ASVs) characteristic of another host species (AF or MG), we first used Random Forest Classifier (RFC) algorithms (Breiman, 2001), as implemented in R package *randomForest,* (Andy Liaw et al., 2018), to identify host species indicator taxa, i.e those with high importance in allowing host species classification. Each RFC model included

wood mice and one other species (AF or MG) and was performed with 1500 trees and repeated 3 times, pruning away taxa with importance values of zero for distinguishing host species between each run. Taxon importance was assessed using the mean Decrease in Accuracy (the mean change in out-of-bag error estimates when data for that ASV was permuted). We considered indicator taxa for AF or MG be all those with importance values >0 in the final RFC model, that had a higher relative abundance in that species compared to wood mice. Thus AF-indicators were those ASVs important for distinguishing AF from AS which were enriched in AF compared to AS. MG-indicators were those ASVs important for distinguishing MG from AS which were enriched in MG compared to AS. For each wood mouse, we calculated the richness of these species-specific indicator taxa (AF-indicator or MG-indicator taxon richness) as well as an enrichment score that captures how much higher the relative abundance of these indicator taxa is in a given mouse compared to the population average. This AF or MG enrichment score was calculated as follows:

1. Average abundance of taxon t across all samples

$$C_t = \frac{\sum_{samples} A_t}{N_{samples}}$$

2. Enrichment value for taxon t in sample s:

$$R_{ts} = \frac{A_{ts}}{C_t}$$

3. Enrichment score for sample s

$$ES_s = \frac{\sum_{taxa} R_{ts}}{N_{taxa}}$$

Here, C_t is the average of all relative abundances (A) of indicator taxon t (either AF or MG indicator) across all wood mouse samples in the population ($N_{samples}$). Each taxon t then was given an enrichment value in each sample s (R_{ts}), based on its abundance in that sample relative to the average abundance of that taxon across all samples (C_t). Finally, each sample was given an enrichment score (ES_s) as the mean of all enrichment values across indicator taxa in that sample. To test our second prediction, we then used the richness or abundance enrichment (Enrichment score) of AF- or MG-indicators in each wood mouse sample as the response variable in our models. As a null comparison, we also calculated and modelled the same metrics based on the contrasting AS-indicators of either indicator set (the complementary AS-indicators in comparison to AF-indicators and MG-indicators respectively).

Statistical analyses

Estimating host species specificity in rodent microbiota

To estimate how different gut microbiotas were across the host species we used a marginal PERMANOVA, implemented with the adonis2 function of the vegan package (Oksanen et al., 2008), to predict Jaccard similarity by host species. Models were run separately for Wytham and Silwood data and host species specificity in microbiota was visualized with Principal Components Analysis (PCoA).

Social and spatial effects on microbiota similarity

To test whether dyadic microbiota similarity between all pairs of rodents was predicted by spatial overlap or social association strength in conspecific vs heterospecific pairs of rodents, we performed Bayesian regression models with the R package *brms* (Bürkner, 2017b). This model framework was used because it allows random effect structures that can account for the types of dependence inherent to pairwise comparisons, thus meaning it can be used to predict pairwise microbiota similarity with measures of social or spatial proximity between pairs. Further, it also allows for the inclusion of multiple samples (microbiota profiles) per individual, making it possible to include all data from these longitudinal studies for increased statistical power (Bürkner, 2017a; Raulo et al., 2021). Here again, we focused on the conspecific and heterospecific associations of the most numerous rodent species in the data, the wood mouse, and thus our model only considered pairs involving wood mice (AS-AS, AS-AF and AS-MG pairs). We used Jaccard Index as the response variable, excluding comparisons of samples from the same individual. As main predictors of interest, we fitted interaction terms between social association (Adjusted SRI) and species combination of a pair (3-way factor: AS-AS, AS-AF or AS-MG), as well as and spatial proximity and species combination. To control for potential confounding variables that could drive an effect of social or spatial proximity on microbiota similarity, we fitted several pair-level dyadic control variables: similarity in sex (binary 0/1; same or different), similarity in age class (binary 0/1; same or different) and sampling interval (time in days between which samples were taken, scaled between 0 and 1). We also fitted technical covariates: difference in sample read depth (absolute difference, scaled between 0 and 1, PCR plate similarity (binary 0/1; same or different), and for Wytham

data also extraction plate similarity (binary 0/1; same or different). To control for non-independence among dyadic measures, the intercept was allowed to vary as defined by two random effects: i) a multi-membership random effect capturing the individuals in each dyad (Individual A + Individual B) and ii) a multi-membership random effect capturing the samples in each dyad (Sample A + Sample B). This random effect structure controls both for the inherent non-independence of the dyadic response variable and the presence of repeat microbiota measurements for some individuals in the dataset.

Social and spatial effects on indicator taxa richness and abundance

To test whether each individual wood mouses' social connectedness to heterospecifics, or the spatial density of heterospecifics they experienced affected the richness or abundance of heterospecific indicator microbes in their gut, we used a Bayesian mixed model (*MCMCglmm package* in R (Hadfield, 2010). Here we used our measures of social or spatial heterospecific connectedness (either AF-degree and AF-density or MG-degree and MG-density) to predict either the richness or abundance enrichment (enrichment score) of AF- or MG- indicator taxa, respectively. In addition to these two main predictors of interest, each model included conspecific degree and conspecific density alongside other covariates (month, sex, age class, read depth) and random effects for extraction plate, PCR plate and individual ID, as here we used the full data with multiple microbiota samples per individual. Additionally, to ensure that any effect of connectedness to other species on indicator taxon richness or abundance was not a mere by-product of higher alpha diversity in more connected individuals, microbiota richness or Shannon diversity was used as a control variable in predicting indicator taxa richness or abundance enrichment respectively.

Social and spatial effects on alpha diversity

Similar to above, to test whether a wood mouse's heterospecific connectedness predicted their overall microbiota alpha diversity, we used a MCMCglmm model to predict asymptotic microbiota richness or Shannon diversity with overall heterospecific degree and heterospecific density. These models included the same set of covariates as the brms models described above, apart from the fact that full degree and full density were used instead of conspecific degree and density. Inclusion of an individual's full degree

and density allows us to test whether an individual's heterospecific connectedness, independent of its overall level of social connectedness or local rodent density, enriches their microbiota.

5.3 Results

Social structure and spatiotemporal distribution of sympatric rodent species

We followed the behaviour of 211 rodent individuals in Wytham (157 AS, 18 AF, 36 MG) and 159 in Silwood (83 AS, 26 AF, 50 MG) over a 10- and 12-month period respectively. Social networks of sympatric rodents showed low but significant levels of social assortment according to species (Figure 1 A-B) i.e. individuals associated slightly more strongly with members of their own species than of others (permutation test on assortativity: r=0.18, se=0.04, p=0.03). Home range sizes and social connectedness levels were generally comparable across the three study species, with slight species differences: yellow-necked mice had more social connections (higher full degree) compared to wood mice while bank voles had fewer social connections to other rodents (Figure S5.4A). Similarly, average home-range size varied significantly between species (Figure S5.4B) with yellow-necked mice having the largest home ranges (Wytham: mean 2199.0 m^2, sd=1720.4; Silwood: mean 4321.2 m2, sd=2632.5), wood mice having second largest (Wytham: mean 1909.0 m2, sd=1661.2; Silwood: mean 3366.9 m2, sd=1456.6), and bank voles having the smallest home ranges (Wytham: mean 549.7 m2, sd=550.1; Silwood: mean 1921.7 m2, sd=2047.1). All three rodent species shared living space with each other (home ranges overlapped across species, Figure S5.5) and individuals of all sympatric species were frequently detected at the same logger within the same hour (Figure S5.2). These co-occurrences followed the general activity patterns of rodent species, with heterospecific co-occurrences generally happening during the time of the day when all three rodent species were active (Figure S5.2). Across individuals of our focal species, the wood mouse, measures of heterospecific and conspecific degree were significantly positively correlated implying that wood mice who had social contacts with many other wood mice has also tended to have more contacts with yellow-necked mice and bank voles. Paired correlations between social and spatial connectedness measures are summarised in Supplementary Figure S5.6.

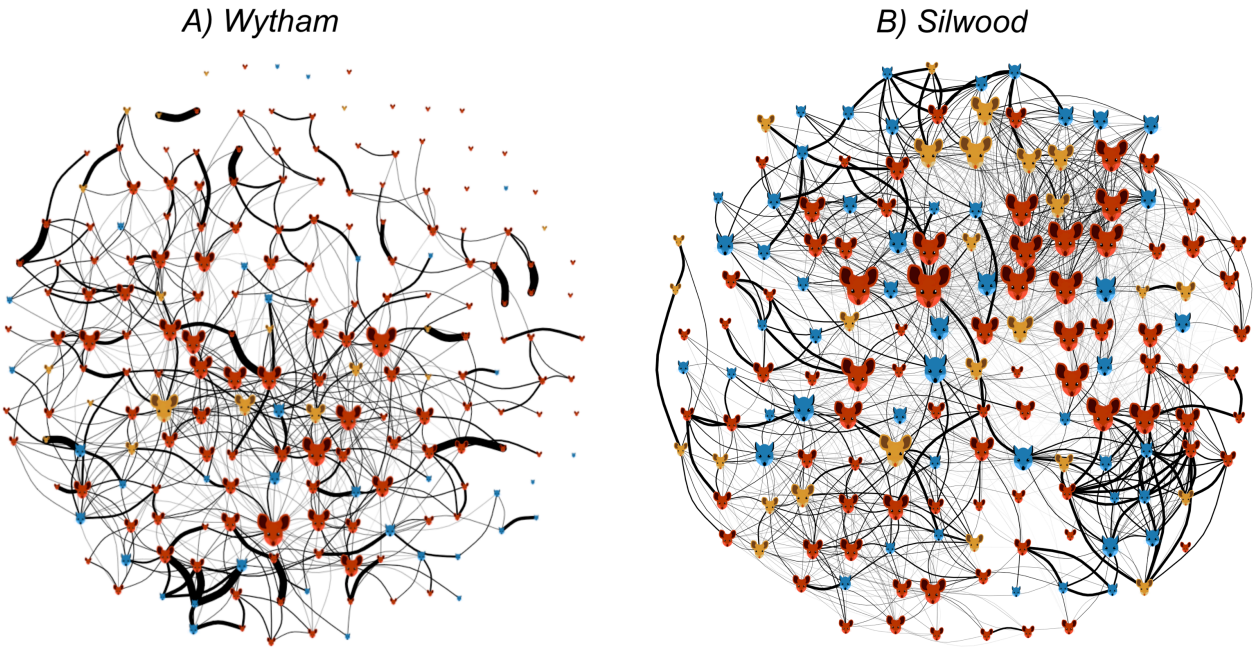

Figure 1. Mixed-species social network of rodents in A) Wytham and B) Silwood. Nodes are individual wood mice (red), yellow-necked mice (yellow) and bank voles (blue) and edges are measures of social association (Adjusted SRI). Node size depicts the full degree of an individual, i.e. the number of social connections (larger = more connections). Line thickness denotes social association bond weight between individuals (thicker = higher SRI value).

Microbiota differences between host species

Within both sites, microbiota composition differed significantly between the two *Apodemus* mouse species (PERMANOVA on Jaccard distance, AS vs AF: Wytham R^2=0.02, F=2.05 p<0.01, Silwood R^2=0.02, F=1.73, p=0.01), though clustering of the two mouse species was not obvious on the first two axes in a principle coordinates analysis (Figure 2). In Wytham, differences in mouse and vole microbiota composition were greater than those among the two species of mice (PERMANOVA on Jaccard distance, AS vs MG in Wytham: R2=0.05, F=10.53, p<0.01).

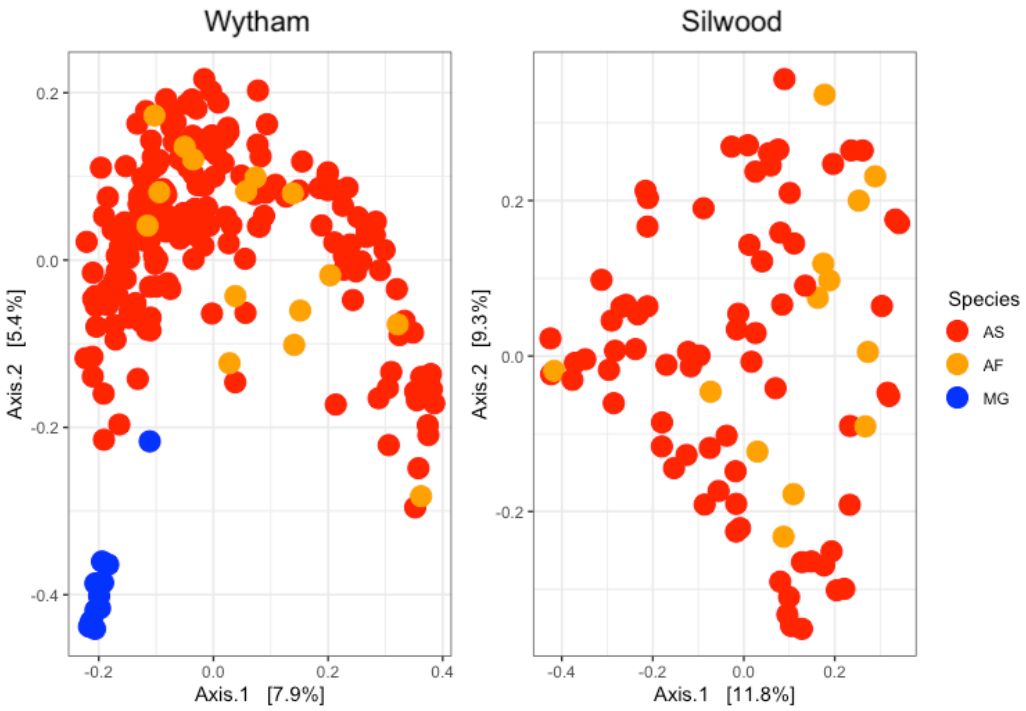

Figure 2. PCoA clustering of microbiota composition across host species (colour) in both study populations. The two mouse species (AS, red, and AF, orange) cluster together while vole microbiota (MG, blue) clusters separately from mice along axis 2.

Social and spatial effects on microbiota similarity

In both study sites, social and spatial effects on the wood mouse microbiota varied significantly between conspecific and heterospecific pairs (interactions between social and spatial terms and pair type were significant, Table 1). Overall, gut microbiota similarity (Jaccard Index, the proportion of shared taxa) was predicted by both social association and spatial proximity between conspecific pairs, while only spatial proximity predicted microbiota similarity in heterospecific pairs (Table 1). Among conspecific (AS-AS) pairs, social association strongly positively predicted microbiota similarity (brms model, Wytham: Posterior mean 0.30, CI=0.19 to 0.40; Silwood: Posterior mean 0.43, CI=0.37 to 0.50; Figure 3), while spatial proximity had a much weaker yet significant effect on microbiota similarity among wood mice (brms, Wytham: Posterior mean 0.03, CI=0.02 to 0.04; Silwood: Posterior mean 0.03, CI=0.01 to 0.05; Figure 3). Conversely, for heterospecific mice (AS-AF), spatial proximity predicted microbiota similarity in both populations (brms, Wytham: Posterior mean 0.07, CI=0.02 to 0.11; Silwood: Posterior mean 0.23, CI=0.16 to 0.30; Figure 3), while social association between AS-AF pairs

had no significant effect on microbiota similarity in either population (brms, Wytham: Posterior mean 0.26, CI= -0.24 to 0.75; Silwood: Posterior mean 0.09, CI= -0.13 to -0.32; Table 1, Figure 3). Between wood mice and bank voles (AS-MG pairs), neither spatial nor social proximity significantly predicted microbiota similarity.

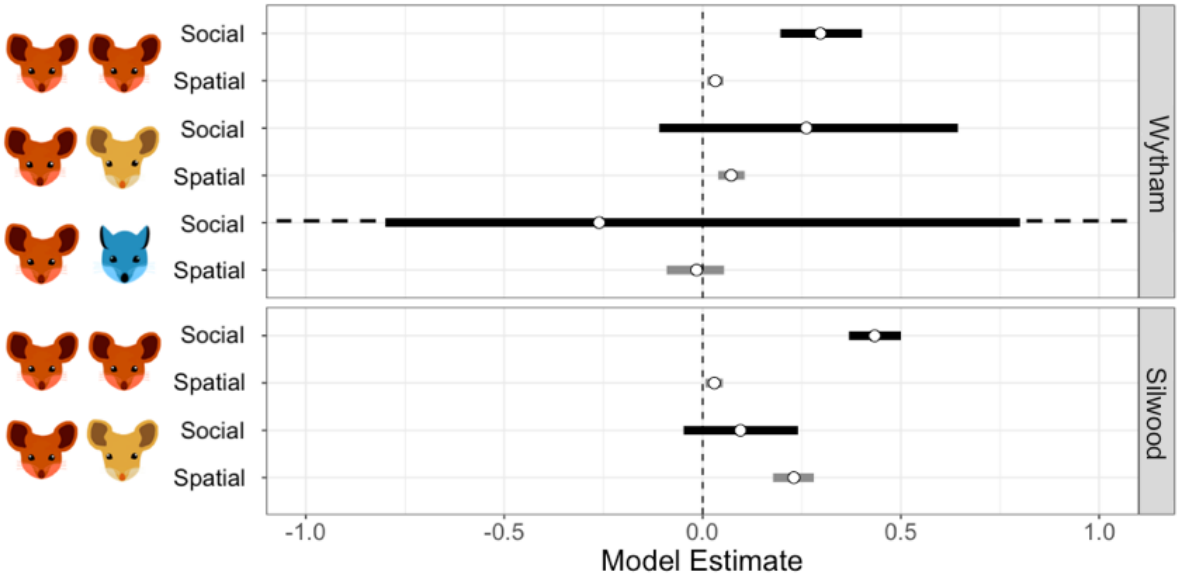

Figure 3. Effects of social association and spatial proximity on microbiota similarity between conspecific (AS-AS) and heterospecific (AS-AF & AS-MG) pairs of sympatric rodents in Wytham and Silwood. Effect size estimates (points) and their 95% credible intervals (black and grey lines for social and spatial effects respectively) are plotted from the above reported Bayesian regression (brms) models with pairwise microbiota similarity among hosts (Jaccard Index) as the response and an interaction term between social association* species or spatial overlap*species as main predictors. Where credible intervals do not overlap zero, a variable significantly predicts microbiota similarity. The credible intervals of AS-MG social association effect (Posterior mean -0,26, CI= -1.9 to 1.3) extend beyond the x axis boundaries (dashed line).

Table 1: Results of *brms* models testing the effect of social association, spatial proximity and covariates on microbiota similarity (Jaccard Index) of conspecific (AS-AS) and heterospecific (AS-AF & AS-MG) rodent pairs in Wytham (above) and Silwood (below). Significant terms (where 95% credible intervals do not include zero) are shown in bold. For ease of interpretation, interaction effects are *NOT* reported as slopes relative to each other but as independent effects within each group. Est.Error indicates the standard deviation of the posterior distribution.

Table 1.				
Wytham				
	Estimate	Est.Error	l-95% CI	u-95% CI
Intercept	-1.84	0.05	-1.94	-1.73
Read depth difference	**-0.13**	**0.02**	**-0.17**	**-0.10**
PCR plate similarity	**0.01**	**0.01**	**0.00**	**0.02**
Extraction-plate similarity	0.00	0.01	-0.01	0.01
Sex similarity	-0.00	0.00	-0.01	0.00
Age similarity	**0.17**	**0.03**	**0.11**	**0.23**
Sampling interval	**-0.32**	**0.01**	**-0.33**	**-0.30**
Species difference AS-AF	-0.14	0.08	-0.30	0.03
Species difference AS-MG	-2.30	0.10	-2.49	-2.10
AS-AS: Social association	**0.30**	**0.05**	**0.19**	**0.40**
AS-AS: Spatial overlap	**0.03**	**0.01**	**0.02**	**0.04**
AS-AF: Social association	0.26	0.20	-0.11	0.64
AS-AF: Spatial overlap	**0.07**	**0.02**	**0.04**	**0.11**
AS-MG: Social association	-0.26	0.84	-1.9	1.3
AS-MG: Spatial overlap	-0.02	0.04	-0.09	0.05
Silwood				
	Estimate	Est.Error	l-95% CI	u-95% CI
Intercept	-1.53	0.06	-1.65	-1.41
Read depth difference	-0.01	0.02	-0.05	0.03
Pcr-plate similarity	0.01	0.01	-0.00	0.02
Sex similarity	-0.00	0.00	-0.01	0.01
Age similarity	0.04	0.04	-0.03	0.12
Sampling interval	**-0.44**	**0.01**	**-0.46**	**-0.42**
Species difference AS-AF	**-0.19**	**0.07**	**-0.32**	**-0.05**
AS-AS: Social association	**0.43**	**0.03**	**0.37**	**0.50**
AS-AS: Spatial overlap	**0.03**	**0.01**	**0.01**	**0.05**
AS-AF: Social association	0.10	0.08	-0.05	0.24
AS-AF: Spatial overlap	**0.23**	**0.03**	**0.18**	**0.28**

Social and spatial effects on indicator taxa

At both study sites, the social connectedness of wood mice to yellow-necked mice individuals (AF-degree) positively predicted the richness and abundance enrichment of AF-indicator taxa in their gut microbiota (Figure 4A-D, Table 2). These effects were independent of social connectedness to other wood mice (conspecific degree), spatial density of yellow-necked mice or conspecifics and other covariates (Table S5.2-S5.3). The positive effects of AF-degree on AF taxon richness and enrichment scores remained of a similar magnitude or became even stronger when using the extended AF-degree as a predictor instead of standard AF-degree (Figure S5.7, Table S5.4-S5.5). By contrast, neither richness nor abundance enrichment of the complementary indicator taxa of wood mice themselves (AS-indicators) were positively predicted by AF-degree in either population (Figure 4E-H, Tables S5.6-S5.7), and in fact in Silwood AF-degree had a negative relationship with AS indicator taxon richness (Figure 4F, Table S5.7). Mirroring this, in Silwood, conspecific degree negatively predicted the abundance enrichment of AF-indicator taxa (Table 2, Table S5.3B). Finally, spatial density of yellow-necked mice had no significant effect on richness or enrichment of AF-indicator taxa in wood mice (Table S5.2-S5.3), and no measure of connectedness to bank voles, neither social nor spatial, significantly predicted MG-indicator taxon richness or abundance enrichnment in wood mice (Table S5.8).

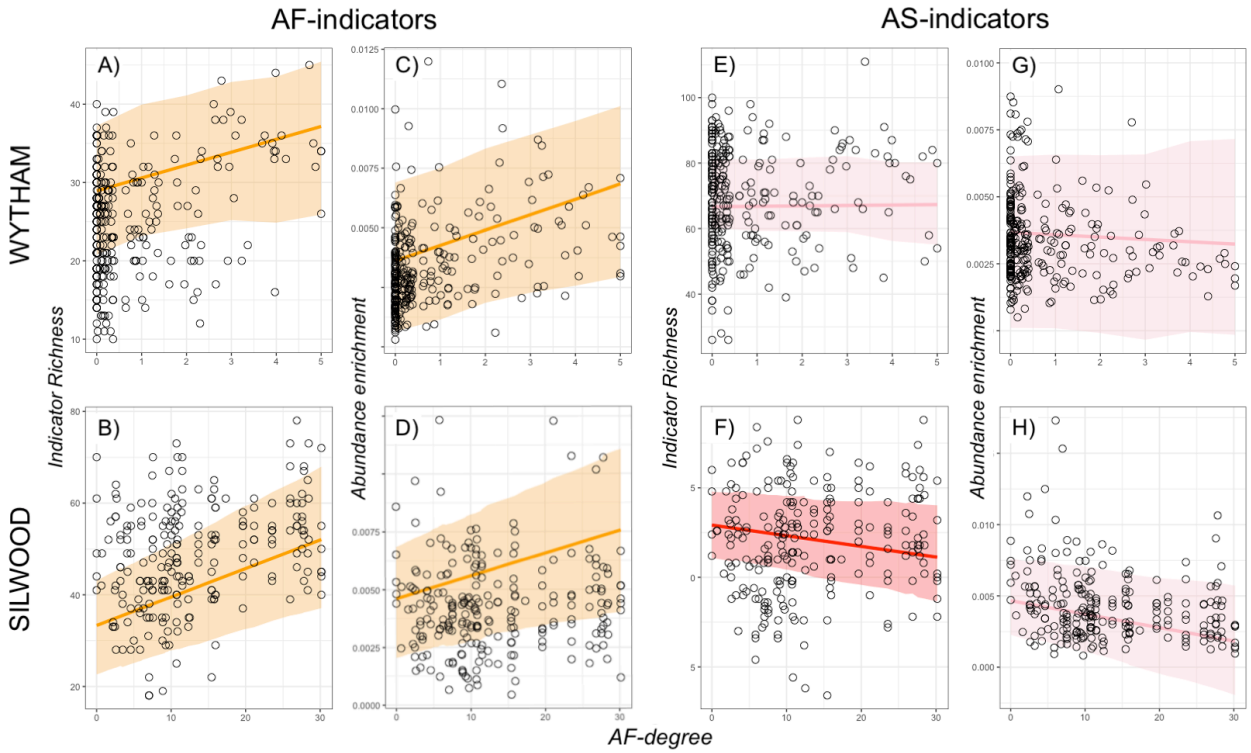

Figure 4. Effects of social connectedness to yellow-necked mice on host species indicative taxa in the gut microbiota of wood mice. In both Wytham (top panel) and Silwood (bottom panel), social connectedness to yellow-necked mice (AF-degree, x-axis) positively predicts the richness and abundance enrichment (y-axis) of yellow-necked mouse indicator taxa (A-D, in orange), but not of wood-mouse indicator taxa (E-H, in red). Estimated slopes and their 95% credible intervals from MCMCglmm models are depicted together with raw data points (circles). Brighter coloured lines (orange, red) indicate significant effects, lighter colours (light pink) non-significant trends.

Table 2. Summary of results from MCMCglmm models predicting richness or abundance enrichment score for host species indicative taxa, as a function of social network metrics. All models included a common set of covariates (see *Statistical Analysis*). Models predict either richness or enrichment scores for taxa indicative of either yellow-necked mice (AF-taxa) or wood mice (AS-taxa) as a function of the number of connections within the social network (degree) to either other AS or AF individuals. Full model results are presented in Supplementary Tables S5.2-S5.3 and S5.6-S5.7, and significant effects are indicated in bold text.

Response	*Predictor*	*Table*	*Post. mean*	*lCI*	*uCI*
Wytham					
AF-taxa richness	**Conspecific (AS) degree**	S2A	**0.177**	**0.063**	**0.288**
AF-taxa richness	AF-degree	S2A	0.046	-0.067	0.166
AF-taxa abundance enrichment	**Conspecific (AS) degree**	S2B	**0.237**	**0.135**	**0.343**
AF-taxa abundance abundance enrichment	AF-degree	S2B	-0.060	-0.171	0.049
AS-taxa richness	Conspecific (AS) degree	S6A	-0.020	-0.071	0.033
AS-taxa richness	AF-degree	S6A	0.010	-0.048	0.067
AS-taxa abundance enrichment	Conspecific (AS) degree	S6B	-0.057	-0.156	0.036
AS-taxa abundance enrichment	AF-degree	S6B	-0.047	-0.148	0.048
Silwood					
AF-taxa richness	**Conspecific (AS) degree**	S3A	**0.164**	**0.037**	**0.318**
AF-taxa richness	AF-degree	S3A	-0.086	-0.218	0.056
AF-taxa abundance enrichment	**Conspecific (AS) degree**	S3B	**0.210**	**0.074**	**0.346**
AF-taxa abundance enrichment	**AF-degree**	S3B	**-0.178**	**-0.311**	**-0.046**
AS-taxa richness	**Conspecific (AS) degree**	S7A	**-0.108**	**-0.202**	**-0.026**
AS-taxa richness	AF-degree	S7A	0.072	-0.009	0.153
AS-taxa abundance enrichment	Conspecific (AS) degree	S7B	-0.094	-0.202	0.011
AS-taxa abundance enrichment	AF-degree	S7B	0.021	-0.083	0.126

Social and spatial effects on alpha diversity

No measure of social or spatial connectedness to other species individuals significantly affected the overall alpha diversity (asymptotic Richness or Shannon diversity) of the wood mouse gut microbiota in either study population (Table S5.9-S5.10), implying that heterospecific connectedness did not enrich the wood mouse microbiota.

5.4 Discussion

Using individual tracking within a mixed-species system of wild rodents, we present the first evidence for individual-to-individual level cross-species transmission of microbiota among sympatric wild mammals. While other research has emphasized that sympatric host populations may harbour more similar microbiota than allopatric (Baxter et al., 2015; Knowles et al., 2019; Moeller et al., 2013, 2017), whether this pattern between species arises through convergent exposure to the same environments or more direct exposure to each other has so far not been directly investigated. We show that individual-level variation in social and space-use behaviour can drive microbiota spread across species boundaries, and that some individuals have stronger transmission links to heterospecific individuals than others, consequently harbouring more of the gut microbes typical of other species. When considering pairwise interactions and overall microbiota composition, sharing living space, but not direct social contacts, were found to homogenize microbiota between heterospecific neighbours, while direct social interactions influenced microbiota strongly only among conspecifics. However, when considering overall connectedness of individual wood mice to other rodents, we found that being socially connected to many yellow-necked mice did in fact seem to introduce yellow-necked mouse - typical bacteria into the wood mouse gut. This effect was independent of the effect of sharing space with many yellow-necked mice, implying a more direct transmission link than just exposure to the same environmental pool of microbes. This effect was strong and independent of technical covariates (including extraction plate effects) and as such it is unlikely to have been affected by the observed contamination across microbiota samples on same extraction plates. However, as the contamination effect was random across samples, the increased

noise in the data can mean that we may be slightly under-estimating the actual sharing of microbes between rodent species.

These results emphasize the importance of a network perspective in understanding the effects of transmission on microbiota composition. Specifically, microbiota compositions may indeed emerge as result of an individual's position in a network of microbial transmission, even in ways that are not evident by just comparing microbiota sharing between pairs. An example of such situation is non-symmetrical transmission between individuals, whereby one individual's microbiome is shaped by interaction with the other but not the other way around. This kind of phenomen could explain why wood mice were enriched with yellow-necked mouse-typical microbes through frequent interactions with them while pairwise associations between the two mouse species were not predictive of the (symmetrical) similarity of their microbiotas.

Gut microbiota may be shared between wood mice and yellow-necked mice through more or less intimate contact behaviours, such as aggressive encounters or exposure to each other's fresh faeces while sharing burrows. Encounter experiments, where by wild mouse individuals of different species are introduced to each other in a temporary cage, have implied that yellow-necked mice are generally dominant over the smaller wood mice (Hoffmeyer, 1973; Montgomery, 1978) and that individuals of both mouse species behave more agonistically towards each other than towards conspecifics (Montgomery, 1978). However, high aggressiveness is still rare and also more amicable social behaviours, such as nose-to-nose contact and allogrooming may happen between wood mice and yellow-necked mice (Simeonovska-Nikolova et al., 2016). Higher connectedness to yellow-necked mice in wood mice might also drive variation in stress levels, which could in turn affect the microbiota (Arcidiacono et al., 2018; Bailey et al., 2011; Foster et al., 2017), but this would not explain the effect of connectedness to a specific species enriching the indicator taxa of that species in the gut of the recipient. Interestingly, while microbiota seems to be transmitted through contacts between wood mice and yellow-necked mice, this was not true between wood mice and sympatric bank voles. This was not driven by fewer social associations between wood mice and bank voles compared to the two mouse species, as wood mice had just as strong and many associations with voles as with yellow-necked mice.

In fact, earlier research on wood mouse-bank vole interactions in a natural habitat found that bank voles were more often observed at a feeding site with wood mice than with other bank voles (Lambin, 1988). However, the two mouse species have more overlapping niches and may be even more likely to be directly exposed to each other than mice and voles. Alternatively, contact transmission between the mouse species might have more pervasive effects on their microbiota because they are phylogenetically related sister species with likely more similar gut physiology or immune system compared to the voles. Consequently, bacteria transmitted across mouse species may more easily colonize and persist in wood mice, while vole-typical taxa might only pass through mouse gut. In line with this, earlier research has shown that the gut microbiota sharing between sympatric species seems to be particularly influential between closely related species. For example, sympatry was associated with gut microbiota homogenization between closely related species of *Peromyscus* mice to such an extent that host species could not be distinguished based on gut microbial profiles (Baxter et al., 2015). Similarly, while many primate species are known to harbour a highly species-specific microbiota even when inhabiting the same environment (Gogarten et al., 2018), among populations of very closely related baboon sub-species, environmental variation was a strong and significant predictor of microbiota composition, while subspecies identity was not (Grieneisen et al., 2019).

Microbiota sharing between sympatric species may not be similar across all species pairs and even in species pairs where it happens, microbiota sharing may involve more than one transmission route, such as environmental or social contact transmission. Importantly, cross-species microbiota transmission through interactions of varying intimacy, from mere niche overlap to actual spatial overlap to (direct or indirect) social contacts, may have different effects on the microbiota. This is because different transmission routes, such as exposure to the environmental pool of microbes, indirect gut-to-gut transmission through contact with faeces in the environment or direct social transmission through physical contact, may serve as transmission pathways for different sets of microbes. Specifically, for generalist bacteria that can live in or outside of an animal gut, transmission from or through the environment is likely, whereas bacteria that cannot readily persist outside their host (e.g. strictly anaerobic non-spore forming taxa), physical contact might present the only possible transmission route. Importantly, as these latter taxa cannot persist outside the host, they are likely highly adapted to live as

part of their hosts and may thus be more important key symbionts playing specific roles in host physiological pathways. Consequently, in addition to convergent exposure to the same sources of environmental transmission, physically close behaviours between individuals of different species may be particularly influential for microbiota sharing, as they may spread key gut symbionts with important metabolic properties from one species to another.

Sharing gut microbes through individual-level interactions between species holds potential for shaping larger-scale biological processes. For instance, cross-species transmission of symbiotic microbes, especially those that cannot spread through environment, may result in horizontal transfer, or "lending", of metabolic capacity, immune resilience, thermal tolerance or other microbiota-related traits between sympatric host species. In this way, microbiota transmission among sympatric species might shape the niche of these species, making their social and ecological interactions potentially important drivers of the adaptive variation provided by symbiotic microbiota, with possibly important consequences for species ability to adapt to changing environments. For example, gut microbiota spread among sympatric populations could transfer digestive abilities between species, resulting in broadening of dietary niche and better adapting to changing diet sources. Evidence hinting towards this kind of possibility comes from laboratory research on the toxin-degrading microbiota of wild wood rats. Specific gut microbes present in some wood rat populations but not others were shown to enable them to digest a highly tannin-rich plant (Kohl et al., 2014) and experimental transplantation of this tannin-degrading gut microbiota from wild wood rat donors to domestic laboratory rats made them capable of digesting and extracting energy out of a tannin-rich diet that otherwise damaged their liver (Kohl et al., 2016). This kind of lending of metabolic properties through cross-species microbiota transmission may shape species niches over evolutionary timescales, presenting a new link between ecological interactions and evolution of populations. For example, many domesticated farm animals have evolved not only under human selection but also under continuous exposure to each other and microbiota transfer between domestic species such as cows and dogs may have enhanced the latter's ability to digest more plant-based diets while adapting from the ancestral carnivore diets of wolves to more omnivore diets of dogs living off the leftovers of humans (Arendt et al., 2016; Axelsson et al., 2013).

The effect of cross-species microbiota sharing on adaptive plasticity of wild animal individuals and population could be tested in the future by comparing microbiota and metabolic differences between populations of same species living with or without closely interacting sympatric species. If niche-overlap, spatial overlap or direct interactions serve as means for sharing valuable gut symbionts, populations living with ecologically similar species may benefit from this contact by for example gaining the ability to utilizing a broader range of diets. Notably, however, our results did not support the idea that individuals most connected to other-species individuals would harbour an overall more diverse microbiota. Rather, we found evidence that interspecific connectedness shifts the microbiota composition more towards that of the other species, at the expense of other microbes; while wood mice that were well-connected to yellow-necked mice hosted more yellow-necked-mouse typical taxa, they simultaneously hosted fewer of the bacterial taxa indicative of their own species in comparison to yellow-necked mice. This did not arise from trade-offs between conspecific vs heterospecific social contacts since conspecific and AF-degree were in fact positively correlated, i.e. wood mice with many contacts with yellow-necked mice also had more contacts with conspecifics. More likely this pattern may reflect gut microbial intra-community dynamics, whereby some taxa mutually exclude one another and cannot readily co-exist in the same microbiota.

Our findings here concerning fine-scale individual variation in behaviour driving patterns of microbiota sharing across species provides one starting point for future research linking broader ecosystem dynamics with metacommunity dynamics of host-associated microbiota. This study has demonstrated the power of network perspective on mapping the metacommunity dynamics of gut microbiota transmission. Next steps would be to expand this network thinking from social contact networks to ecological networks considering not only parallel (competitive/cooperative) ecological interactions but also hierarchical species associations, such as trophic or parasitic interactions. Instead of a flat and symmetrical transmission network, multidimensional networks with directed edges could be used in mapping the transmission of microbiota across the ecosystem. Understanding how microbiota spreads on all levels of host organisation, from vertical transmission along maternal lines to horizontal transmission through social contact networks or ecological interaction networks of sympatric hosts, will help us see the shape of the ecological landscape in which these microbes live and evolve. Ultimately,

this will enhance our understanding of the role microbiotas play in tying together, or separating, individuals, populations and species assemblages within ecosystems.

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Supplementary Material S5 for

Chapter 5

Cross-species transmission of gut microbes in a wild rodent community

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Supplementary Appendices

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Appendix S5.1. Rationale behind the simplified spatial overlap metric

Spatial networks were constructed by first calculating a matrix of distances in meters between observation record centroids (mean locations) for each individual rodent. An "observation" is defined as the detection of an individual at a location (logger) within a ten-minute period. To assess whether spatial distance between the mean location of each rodent could provide a good measure of home range overlap, and whether this applied up to a particular distance threshold, we examined the relationship between these two variables for the subset of rodents with dense enough spatial sampling to accurately estimate home range size. As described in Chapter 3, we determined the minimum number of unique observations (five) and locations (three) needed to derive stable estimates of home range size by inspecting variograms. Home ranges were inferred as the 75% core of the autocorrelated kernel utilization density distribution (Fleming et al., 2015), based on spatiotemporal (path-constructing) movement models implemented in R package *ctmm,* (Calabrese et al., 2016). Home range overlap was calculated using the Bhattacaryya coefficient of overlap between probability distributions (See Chapter 4). For the 128 individuals (98 AS, 9 AF and 21 MG) from Wytham with sufficient home range estimates, we found that centroid distance (in meters) between pairs of (multiple species of) rodents was correlated with their Bhattacaryya home range overlap up to ~100-130 meters of centroid distance, after which the correlation broke down (Figure S5.3). Based on this, we derived a measure of spatial overlap that was equal to spatial distance up to 100m between centroids, and zero for distances >100m. Spatial proximity values were then scales between 0 and 1, and subtracted from 1 to obtain a measure of proximity rather than distance for use in analyses.

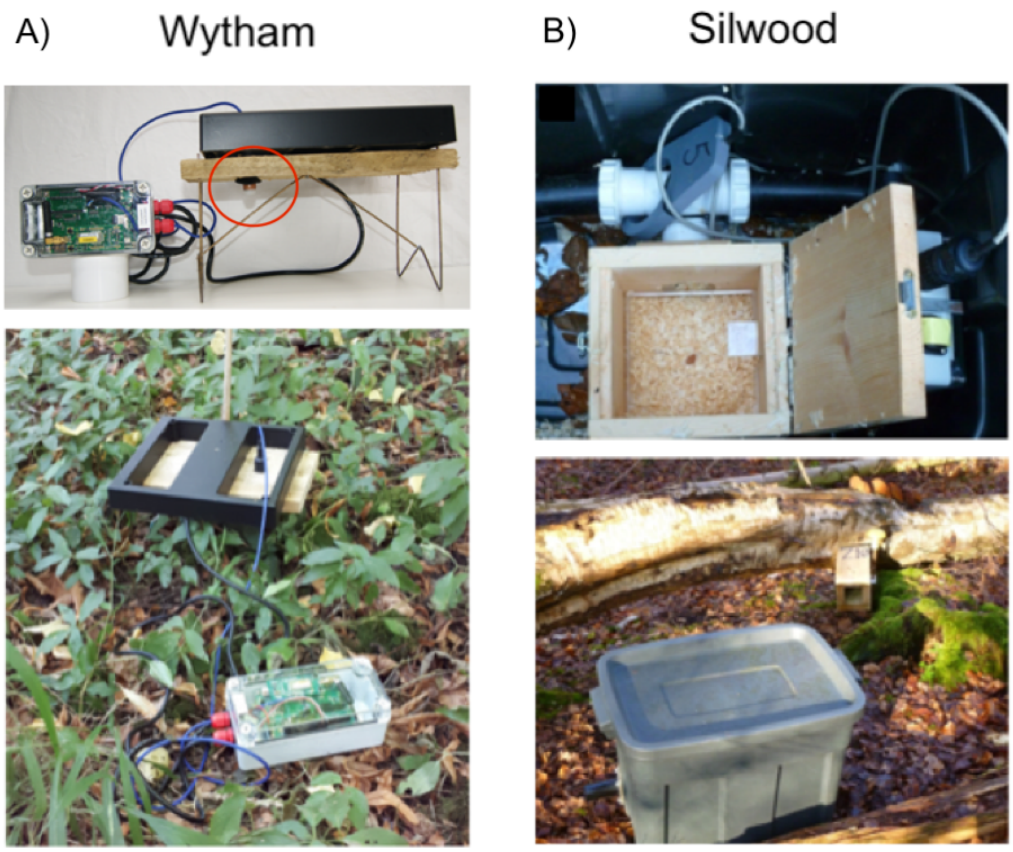

Figure S5.1. The two types of RFID-loggers used in the two studies.

A) In Wytham, the logger was unbaited and consisted of a motion sensor (red circle, figure top left), that woke up the detection coil (black square) whenever a warm object, such as a rodent came nearby. The coil, sitting on a wooden "table" to allow rodents to pass under as well as around it, then created a ~1m^2 oscillating detection field that would interfere – and subsequently log - any 124 kHz RFID tag within this field. Exploiting this, wild rodents were tagged with a subcutaneous passive integrated transponder (PIT) tags containing a 10-digit HEX-code identification number readable in this exact oscillation frequency. When a rodent tag was read by the coil, this identification number was saved on an SD-card (in the grey box) and additionally transmitted into and interactive internet database through an antenna (in the grey box) connected to the broadband network covering Wytham Woods (Wytham Data Net).

B) In Silwood, the logger took the form of a large plastic box (figure bottom right) with two entrance tubes leading to a central wooden box containing sawdust and a single peanut (figure top right). An RFID reader coil surrounding the entrance to the central box was on continuously and recorded the

PIT-tag ID of any tagged rodent present under it every 0.3 seconds. Peanut oil around the tube entrances and a single peanut inside the box, renewed at each logger rotation, was used as a minor lure. Upon each logger rotation, each logger box and entrance tubing were thoroughly cleaned with 70% ethanol and the sawdust was replaced. Cleaning was aided by the box's removable plastic floor that could be cleaned thoroughly. Loggers were powered using 12V lead acid rechargeable batteries. Further details about these loggers can be found in (Godsall et al., 2014).

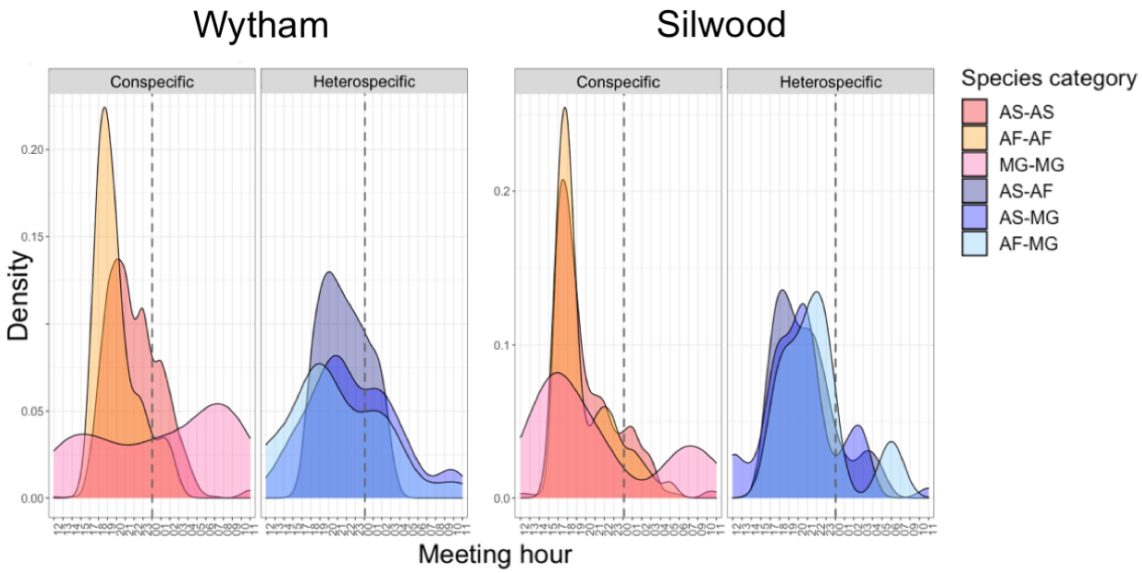

Figure S5.2. Temporal overlap of conspecific and heterospecific rodents in the tracking data. Distribution of co-occurrences by hour of day. Here, co-occurrences are defined as observations of two individuals at the same location (PIT-tag logger) within the same hour. Conspecific co-occurrences (warm colors) tend to happen during the general activity period of the species: During night hours for nocturnal mouse species (AS-AS, and AF-AF co-occurrences) and during light hours for more diurnal bank voles (MG-MG co-occurrences). Heterospecific co-occurrences (cold colors, AS-AF, AS MG, and AF-MG co-occurrences) tend to happen during the overlapping activity period of all three rodent species, a few hours before midnight (dashed line).

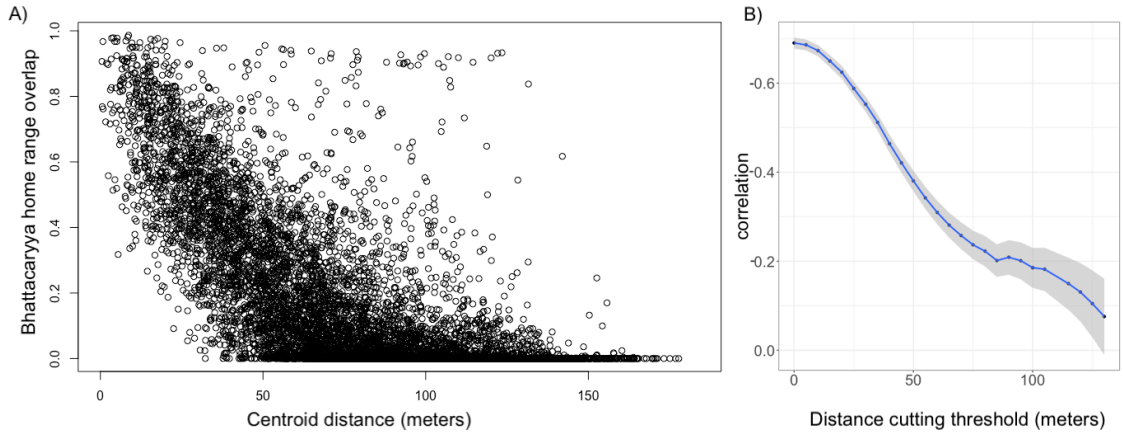

Figure S5.3. Relationship between centroid distance and kernel home range overlap

A) Centroid distance is strongly negatively associated with home range overlap up to ~100 meters, but the correlation degrades thereafter. B) Considering increasingly faraway distances (x-axis, distance threshold below which rodent pairs were dropped from data), correlation (y-axis, Pearson's correlation coefficient) decreases linearly. After 130 meters of centroid distance, the correlation (line) is not significant anymore (confidence intervals touch zero).

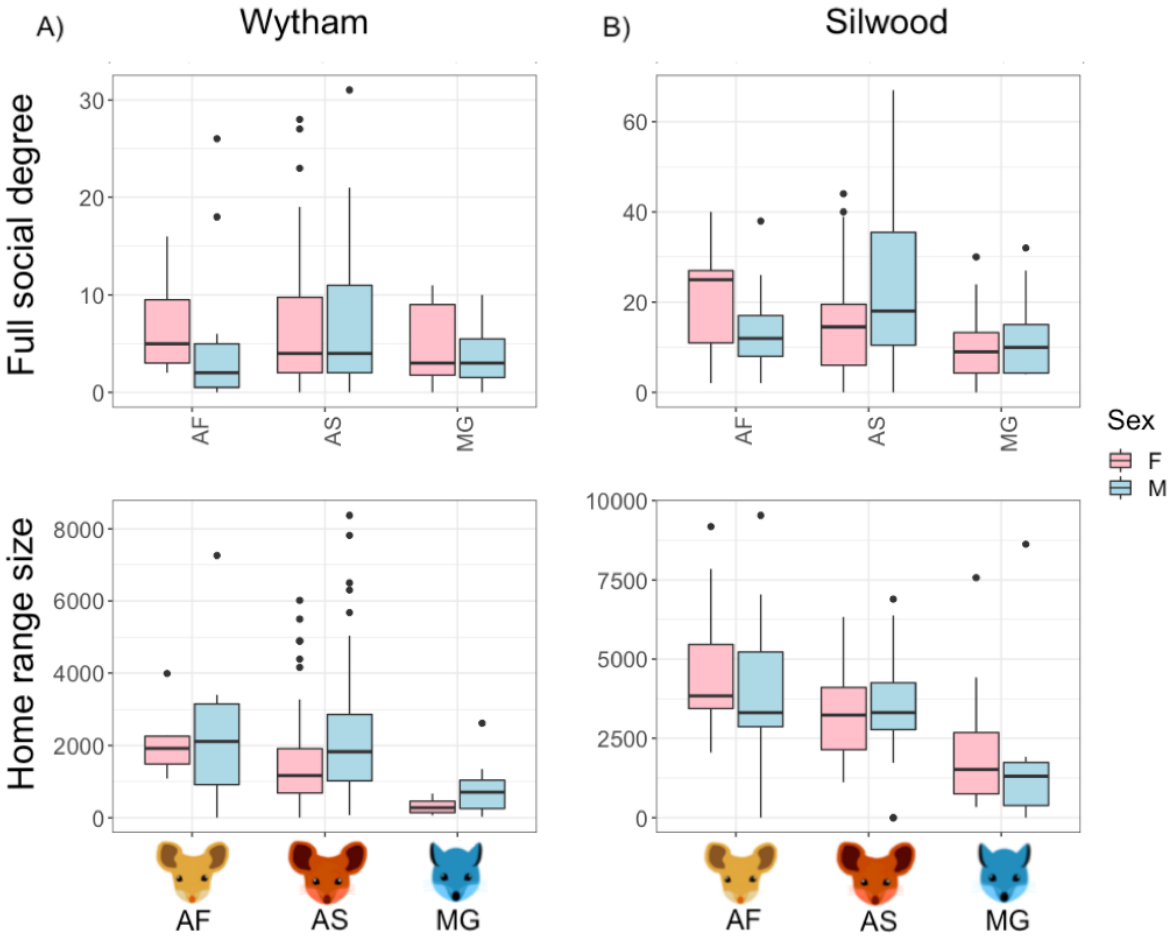

Figure S5.4. Distributions of social and spatial node metrics across species and sexes.

A) Full social degree (total number of direct social network connections to rodents of any species) had overlapping distributions across rodent species and in both Wytham and Silwood was highest in yellow-necked mouse females compared to other rodents. B) Home range sizes were greater for the two mouse species compared to voles and slightly larger for yellow-necked mice compared to wood mice.

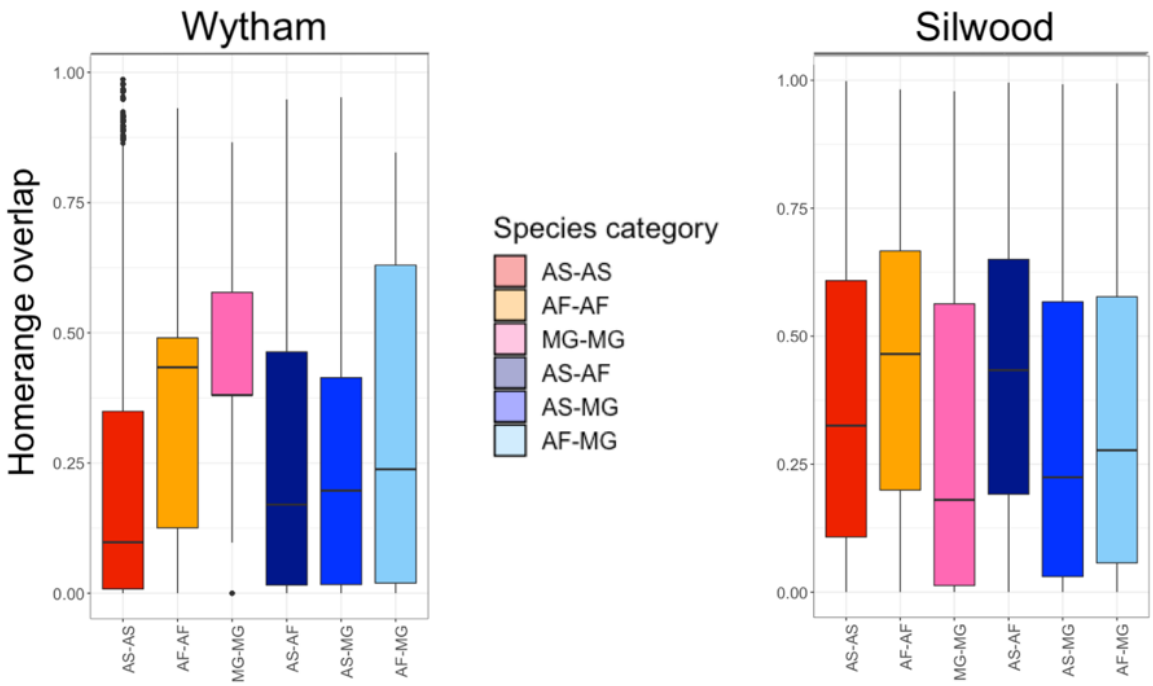

Figure S5.5. Spatial overlap of conspecific and heterospecific rodents in the tracking data.

Levels of home range overlap (Bhattacaryya coefficient) vary between species categories and study sites with more overlap in Silwood than in Wytham and yellow-necked mice (AF) generally having more overlapping home ranges with all other rodents.

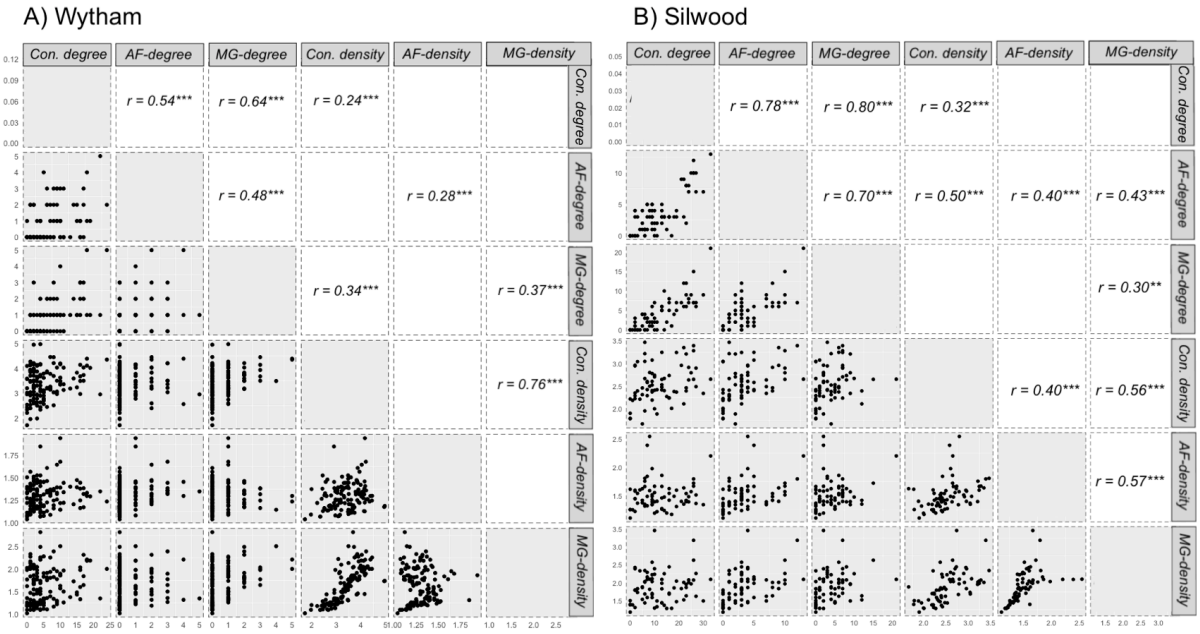

Figure S5.6. Pairplot of correlations between social and spatial connectedness measures

Covariation among connectedness metrics in A) Wytham data and B) Silwood data. Lower triangle visualizes correlation between intersecting variables and upper triangle reports significant pairwise correlations (Pearson's correlation coefficient).

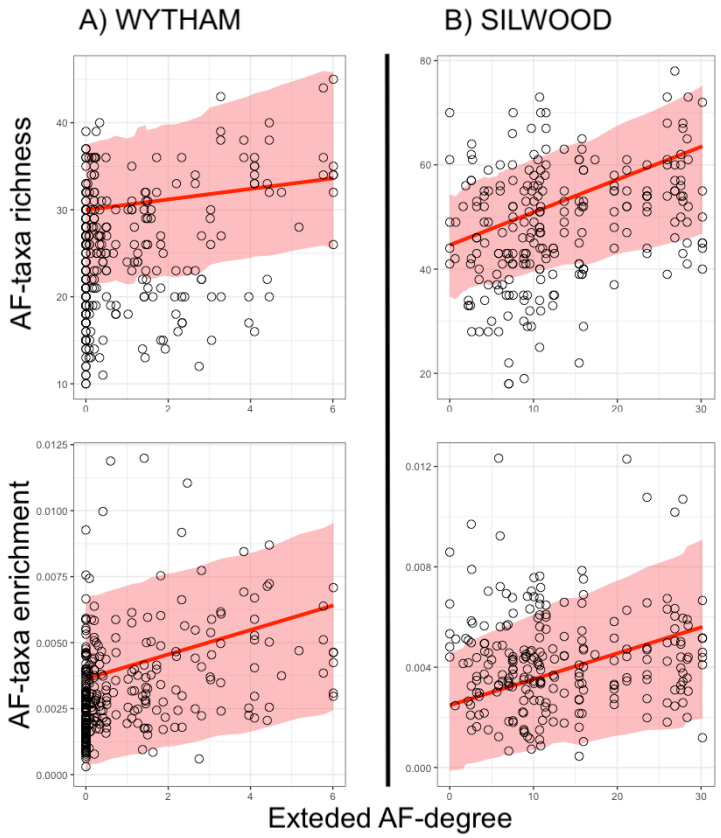

Figure S5.7. Effects of extended social connectedness to yellow-necked mice on the prevalence of AF-indicator taxa in wood mouse gut

A) Wytham and B) Silwood study population. Extended social connectedness to yellow-necked mice (Extended AF-degree, x-axis) strongly and significantly predicts richness and abundance enrichment (y-axis) of yellow-necked mouse indicator taxa. Slopes (lines) and their credible intervals (band) based on the MCMCglmm models are projected on top of values in the raw data (points).

Table S5.1. Additional information on the two study sites and data sets.

Summarized differences between the (A) field data collection, (B) laboratory analyses and (C) resulting sample sizes in the two parallel studies.

A) Field data collection:		
	Wytham	***Silwood***
Study plot area	2.56 ha	2.47 ha
Study plot habitat type	open deciduous woodland, bramble bushes	open deciduous woodland, rhododendron, bamboo
Study duration and period	10 months (Feb-Nov 2019)	12 months (Nov 2014-Nov 2015)
Loggers used	60	9
Logger density	~24.3 per ha	~3.5 per ha
Logger design	• Large flat coil on a wooden table in open woodland. Coil reads PIT-tag of any rodent passing under or near the table within ~28cm • Loggers activated by body heat, when a warm object moves close to the PIR sensor *See Figure S5.1A*	• Plastic box with entrance tube. Circular coil around entrance tube, that reads the PIT-tag of any rodent that enters the chamber in the box centre • Loggers constantly on and recording data; Frequent battery recharging *See Figure S5.1A*
	• **Baiting**: no bait • **Logger placement and rotation**: Each logger had much smaller ($400m^2$) territorty of 4 contiguous 10mx10m grid cells. Loggers were rotated fortnightly in a systematic design among the 4 grid cells, so that each logger covered its entire territory over a 2-month period. At any given moment, all loggers were positioned in an exactly even, chequerboard-like design across the site, with maximum distance to a logger being 15 m from any given point.	• **Baiting**: Loggers baited with a single peanut in the inner chamber, and peanut oil around the rim of the entrance tube. • **Logger placement and rotation**: Each logger had a 2700 m^2 territory of ~27 10x10m grid cells, within which it was rotated on average ~4 times per week. Upon rotation, a logger was positioned to a random cell within its territory (sampling cells without replacement) until the whole territory had been covered, before the process started again. Details can be found from Raulo et al. 2021 (Chapter 2).

B) Laboratory analyses				
	Wytham		***Silwood***	
Microbiota sample DNA extraction	Using Zymo fecal/Soil DNA plate format DNA extraction kit with Qiagen Tissuelyzer II		Using Zymo fecal/Soil plate-format DNA extraction kit with a tube format Tissulyzer	
Microbiota sample Library preparation	Using 16S V4-5 primers 515F and 926R (Walters et al., 2015) and a two-step (tailed-tag) approach with dual-indexing. Details in Chapter 3		Using 16S V4 primers 515F and 805R (Caporaso et al. 2011), and a two-step (tailed-tag) approach with dual-indexing. Details in Raulo et al., 2012 (Chapter 2)	
Bioinformatics	Sample preprocessing done with DADA2 pipeline (1.14.0) Taxonomy assigned against Silva data base (ref)		Sample preprocessing done with DADA2 pipeline (1.6.0) Taxonomy assigned against GreenGenes Database (GreenGenes Consortium 13.8)	
C) Sample size summary				
	Wytham		***Silwood***	
	Unique individuals recorded on loggers	Microbiota samples from these individuals	Individuals recorded on loggers	Microbiota samples from these individuals
Wood mice (AS)	157	297	83	239
Yellow-necked mice (AF)	18	21	26	30
Bank voles (MG)	36	22	50	0

Tables S5.2-S5.6. Results of MCMCglmm-models predicting indicator taxa prevalence with social and spatial connectedness.

The response variable (richness or enrichment score of AF-, AS- or MG-indicator taxa) was predicted with social and spatial connectedness to others (Conspecific degree, conspecific density, AF/MG-degree, AF/MG density) in Wytham (Table S5.2-S5.4) and Silwood (Table S5.5-S5.6). All models include the same set of covariates: sex, age, sampling month (as factors), sample read depth and sample alpha diversity (Richness or Shannon diversity). Post.mean indicates the mean of the posterior distribution of effect estimates, while l-95% CI and u-95% CI showing the lower and upper limits of 95% credible intervals. Effective sample size (eff.samp) is the number of samples taken, adjusted for autocorrelation in the chain. An effect is considered significant if 95% credible intervals do not overlap zero, which is also indicated by p.MCMC-values. Specifically, p.MCMC values indicate the proportion of posterior samples (out of 1000) that fall on the other side of zero from the majority. The effect is considered significant when p.MCMC<0.05.

TABLE S5.2
A) Effects of AF-degree on AF-taxa richness in Wytham

	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	-0.005	-0.144	0.129	1000.000	0.932
Sample read depth	0.176	0.030	0.314	1000.000	0.020
month: 2018-12	-0.021	-0.248	0.166	1000.000	0.872
month: 2019-01	0.217	0.034	0.388	1000.000	0.010
month: 2019-02	0.096	-0.002	0.195	1489.077	0.056
month: 2019-03	0.186	0.083	0.297	1000.000	0.001
month: 2019-04	0.269	0.156	0.384	799.212	0.001
month: 2019-05	0.320	0.096	0.542	1000.000	0.002
month: 2019-06	0.177	0.058	0.301	1000.000	0.008
month: 2019-07	0.211	0.084	0.329	1000.000	0.001
month: 2019-08	0.235	0.108	0.356	545.448	0.001
month: 2019-09	0.181	0.060	0.299	743.623	0.006
month: 2019-10	0.149	0.032	0.260	750.284	0.012
month: 2019-11	0.221	0.098	0.337	781.509	0.001
Sex: Male	0.001	-0.039	0.042	1000.000	0.962

Age: Juvenile	-0.133	-0.242	-0.026	1000.000	0.014
Microbiota Richness	0.460	0.334	0.584	1000.000	0.001
Conspecific degree	0.048	-0.063	0.167	1000.000	0.420
AF-degree	0.175	0.058	0.269	1156.026	0.002
Conspecific proximity sum	0.018	-0.102	0.137	1000.000	0.756
AF proximity sum	-0.015	-0.144	0.128	1000.000	0.842
B) Effects of AF-degree on AF-taxa enrichment in Wytham					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.040	-0.072	0.168	1105.737	0.560
Sample read depth	0.045	-0.075	0.173	1000.000	0.458
month: 2018-12	0.027	-0.189	0.266	807.862	0.814
month: 2019-01	0.118	-0.061	0.301	948.104	0.204
month: 2019-02	0.056	-0.052	0.166	1000.000	0.294
month: 2019-03	0.095	-0.002	0.206	1000.000	0.080
month: 2019-04	0.123	0.010	0.248	1402.564	0.054
month: 2019-05	0.279	0.059	0.513	900.925	0.012
month: 2019-06	0.098	-0.035	0.222	1000.000	0.146
month: 2019-07	0.155	0.023	0.262	1000.000	0.016
month: 2019-08	0.132	0.026	0.258	1000.000	0.036
month: 2019-09	0.179	0.064	0.287	968.455	0.004
month: 2019-10	0.142	0.033	0.249	885.158	0.012
month: 2019-11	0.117	0.008	0.242	864.725	0.058
Sex: Male	0.014	-0.025	0.053	727.234	0.504
Age: Juvenile	-0.025	-0.138	0.092	1000.000	0.664
Microbiota Shannon diversity	0.090	0.014	0.171	912.033	0.032
Conspecific degree	-0.059	-0.172	0.056	1000.000	0.302
AF-degree	**0.238**	**0.121**	**0.331**	**1000.000**	**0.001**
Conspecific proximity sum	0.110	-0.014	0.226	847.589	0.106
AF proximity sum	-0.091	-0.216	0.054	1102.534	0.198

TABLE S5.3 A) Effects of AF-degree on AF-taxa richness in Silwood					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.238	0.143	0.327	1000.000	0.001
Sample read depth	-0.048	-0.149	0.047	1000.000	0.344
month: 2015-02	0.042	-0.032	0.112	894.833	0.232
month: 2015-03	0.015	-0.049	0.079	1000.000	0.618
month: 2015-04	0.053	-0.017	0.121	1042.977	0.160
month: 2015-05	0.007	-0.081	0.094	1000.000	0.878
month: 2015-06	-0.025	-0.091	0.038	1000.000	0.478
month: 2015-07	-0.071	-0.198	0.070	1000.000	0.310
month: 2015-08	-0.054	-0.143	0.017	1000.000	0.174
month: 2015-09	-0.050	-0.147	0.038	1000.000	0.314
month: 2015-10	-0.101	-0.171	-0.028	1000.000	0.006
month: 2014-11	-0.034	-0.081	0.014	1000.000	0.158
month: 2014-12	-0.006	-0.079	0.057	883.729	0.860
Sex: Male	-0.002	-0.047	0.042	1000.000	0.946
Age: Juvenile	-0.134	-0.240	-0.041	1000.000	0.004
Microbiota Richness	0.669	0.587	0.752	1000.000	0.001
Conspecific degree	-0.084	-0.240	0.038	1000.000	0.240
AF-degree	0.165	0.020	0.311	1000.000	0.040
Conspecific proximity sum	-0.113	-0.213	0.008	1000.000	0.052
AF proximity sum	0.055	-0.074	0.188	1112.977	0.428
B) Effects of AF-degree on AF-taxa enrichment in Silwood					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.270	0.180	0.357	1000.000	0.001
Sample read depth	-0.099	-0.211	0.007	1000.000	0.074
month: 2015-02	0.039	-0.050	0.116	1000.000	0.362
month: 2015-03	-0.011	-0.080	0.058	1000.000	0.780
month: 2015-04	0.005	-0.079	0.081	1000.000	0.886
month: 2015-05	-0.030	-0.128	0.061	1000.000	0.516
month: 2015-06	-0.020	-0.082	0.057	1000.000	0.618
month: 2015-07	-0.062	-0.213	0.085	1119.573	0.394
month: 2015-08	-0.067	-0.152	0.007	1000.000	0.086

month: 2015-09	-0.021	-0.122	0.081	1091.012	0.666
month: 2015-10	-0.065	-0.144	0.009	1000.000	0.104
month: 2014-11	-0.008	-0.061	0.045	1000.000	0.796
month: 2014-12	-0.040	-0.106	0.051	1000.000	0.336
Sex: Male	0.003	-0.034	0.038	1000.000	0.880
Age: Juvenile	-0.036	-0.140	0.073	1000.000	0.474
Microbiota Shannon diversity	0.584	0.505	0.671	1000.000	0.001
Conspecific degree	-0.175	-0.306	-0.054	1281.733	0.002
AF-degree	0.208	0.086	0.335	1000.000	0.001
Conspecific proximity sum	-0.003	-0.102	0.088	1000.000	0.970
AF proximity sum	-0.114	-0.246	0.006	1000.000	0.068

TABLE S5.4
A) Effects of extended AF-degree on AF-taxa richness in Wytham

	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.011	-0.119	0.140	793.215	0.866
Sample read depth	0.177	0.026	0.323	1000.000	0.026
month: 2018-12	-0.027	-0.235	0.182	1000.000	0.794
month: 2019-01	0.217	0.039	0.396	1000.000	0.020
month: 2019-02	0.089	-0.013	0.189	1000.000	0.094
month: 2019-03	0.183	0.079	0.296	1000.000	0.001
month: 2019-04	0.265	0.141	0.377	1000.000	0.001
month: 2019-05	0.302	0.099	0.519	713.752	0.010
month: 2019-06	0.165	0.035	0.298	1000.000	0.008
month: 2019-07	0.202	0.080	0.315	613.670	0.001
month: 2019-08	0.216	0.087	0.333	356.551	0.001
month: 2019-09	0.160	0.048	0.277	384.692	0.012
month: 2019-10	0.127	0.021	0.244	482.508	0.022
month: 2019-11	0.202	0.073	0.313	474.691	0.002
Sex: Male	0.000	-0.044	0.039	1000.000	0.986
Age: Juvenile	-0.136	-0.245	-0.036	758.572	0.012
Microbiota Richness	0.462	0.332	0.573	929.516	0.001
Conspecific degree	0.046	-0.071	0.165	879.208	0.436

Extended AF-degree	0.167	0.064	0.279	1161.631	0.014
Conspecific proximity sum	0.000	-0.125	0.111	1000.000	0.994
AF proximity sum	-0.006	-0.136	0.135	1092.224	0.942
B) Effects of extended AF-degree on AF-taxa enrichment in Wytham					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.050	-0.078	0.179	695.018	0.442
Sample read depth	0.051	-0.068	0.171	1000.000	0.420
month: 2018-12	0.026	-0.210	0.252	477.333	0.812
month: 2019-01	0.121	-0.066	0.307	1000.000	0.224
month: 2019-02	0.058	-0.053	0.165	1000.000	0.310
month: 2019-03	0.098	-0.019	0.206	811.292	0.090
month: 2019-04	0.120	0.001	0.238	1165.544	0.040
month: 2019-05	0.274	0.075	0.510	1000.000	0.018
month: 2019-06	0.098	-0.019	0.233	1328.317	0.142
month: 2019-07	0.152	0.038	0.279	1000.000	0.020
month: 2019-08	0.125	0.009	0.229	1000.000	0.034
month: 2019-09	0.165	0.050	0.276	1000.000	0.004
month: 2019-10	0.126	0.018	0.235	799.942	0.022
month: 2019-11	0.108	-0.013	0.217	1243.700	0.076
Sex: Male	0.012	-0.028	0.050	1000.000	0.538
Age: Juvenile	-0.022	-0.137	0.090	859.428	0.698
Microbiota Shannon diversity	0.087	0.009	0.178	1000.000	0.052
Conspecific degree	-0.062	-0.177	0.064	1000.000	0.310
Extended AF-degree	0.230	0.118	0.331	1230.732	0.001
Conspecific proximity sum	0.085	-0.041	0.200	1000.000	0.170
AF proximity sum	-0.080	-0.226	0.044	1000.000	0.206

TABLE S5.5

A) Effects of extended AF-degree on AF-taxa richness in Silwood					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.227	0.146	0.305	1099.570	0.001

Sample read depth	-0.038	-0.146	0.055	1000.000	0.456
month: 2015-02	0.048	-0.021	0.124	906.606	0.182
month: 2015-03	0.021	-0.039	0.079	1000.000	0.536
month: 2015-04	0.061	-0.008	0.137	1000.000	0.106
month: 2015-05	0.021	-0.062	0.099	1000.000	0.608
month: 2015-06	-0.020	-0.085	0.041	798.565	0.564
month: 2015-07	-0.052	-0.178	0.068	1000.000	0.452
month: 2015-08	-0.035	-0.106	0.048	1000.000	0.366
month: 2015-09	-0.023	-0.104	0.073	1094.404	0.606
month: 2015-10	-0.087	-0.152	-0.013	1000.000	0.012
month: 2014-11	-0.025	-0.074	0.023	1000.000	0.306
month: 2014-12	-0.007	-0.074	0.056	1123.878	0.814
Sex: Male	0.006	-0.026	0.042	1000.000	0.710
Age: Juvenile	-0.117	-0.213	-0.025	590.219	0.016
Microbiota Richness	0.676	0.596	0.766	1052.368	0.001
Conspecific degree	-0.184	-0.288	-0.057	1000.000	0.001
Extended AF-degree	0.304	0.186	0.416	1000.000	0.001
Conspecific proximity sum	-0.169	-0.275	-0.083	1000.000	0.002
AF proximity sum	0.045	-0.056	0.163	1000.000	0.388
B) Effects of extended AF-degree on AF-taxa enrichment in Silwood					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.254	0.157	0.339	1000.000	0.001
Sample read depth	-0.095	-0.190	0.020	1000.000	0.072
month: 2015-02	0.045	-0.037	0.121	1859.382	0.288
month: 2015-03	-0.011	-0.078	0.059	1000.000	0.726
month: 2015-04	0.016	-0.057	0.099	1603.701	0.690
month: 2015-05	-0.015	-0.113	0.075	1000.000	0.762
month: 2015-06	-0.004	-0.074	0.067	1000.000	0.938
month: 2015-07	-0.041	-0.191	0.085	1488.955	0.566
month: 2015-08	-0.045	-0.128	0.039	1000.000	0.348
month: 2015-09	0.007	-0.096	0.106	1000.000	0.894
month: 2015-10	-0.044	-0.127	0.027	1000.000	0.258
month: 2014-11	0.004	-0.048	0.059	693.337	0.866
month: 2014-12	-0.035	-0.112	0.044	1000.000	0.374

Sex: Male	0.011	-0.025	0.048	1000.000	0.526
Age: Juvenile	-0.025	-0.117	0.086	1000.000	0.636
Microbiota Shannon diversity	0.591	0.506	0.676	1000.000	0.001
Conspecific degree	-0.203	-0.326	-0.093	816.010	0.002
Extended AF-degree	0.259	0.120	0.386	1000.000	0.001
Conspecific proximity sum	-0.059	-0.157	0.037	909.677	0.280
AF proximity sum	-0.088	-0.200	0.029	1000.000	0.134

TABLE S5.6
A) Effects of AF-degree on AS-taxa richness in Wytham

	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.169	0.098	0.239	389.250	0.001
Sample read depth	-0.008	-0.084	0.078	1000.000	0.824
month: 2018-12	-0.008	-0.122	0.128	1000.000	0.924
month: 2019-01	-0.036	-0.146	0.059	1106.899	0.500
month: 2019-02	-0.012	-0.076	0.040	1000.000	0.712
month: 2019-03	0.028	-0.027	0.087	1121.467	0.344
month: 2019-04	-0.017	-0.087	0.041	1000.000	0.598
month: 2019-05	-0.061	-0.190	0.065	1000.000	0.334
month: 2019-06	0.047	-0.026	0.117	1000.000	0.204
month: 2019-07	-0.019	-0.084	0.050	1000.000	0.600
month: 2019-08	-0.020	-0.085	0.046	1000.000	0.526
month: 2019-09	-0.038	-0.106	0.023	420.769	0.248
month: 2019-10	-0.029	-0.092	0.040	260.618	0.342
month: 2019-11	0.003	-0.071	0.070	305.063	0.948
Sex: Male	-0.006	-0.027	0.013	1000.000	0.572
Age: Juvenile	0.004	-0.062	0.058	946.843	0.908
Microbiota Richness	0.860	0.796	0.932	473.773	0.001
Conspecific degree	0.011	-0.045	0.069	781.037	0.724
AF-degree	-0.021	-0.068	0.030	1000.000	0.386
Conspecific proximity sum	0.045	-0.007	0.103	1000.000	0.116
AF proximity sum	-0.034	-0.095	0.038	1000.000	0.298

B) Effects of AF-degree on AS-taxa enrichment in Wytham					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.249	0.139	0.359	936.028	0.001
Sample read depth	0.000	-0.107	0.105	1000.000	0.986
month: 2018-12	0.057	-0.115	0.258	1000.000	0.524
month: 2019-01	-0.097	-0.252	0.041	1000.000	0.192
month: 2019-02	-0.102	-0.190	-0.013	1000.000	0.028
month: 2019-03	-0.160	-0.247	-0.064	1000.000	0.004
month: 2019-04	-0.162	-0.257	-0.068	1000.000	0.002
month: 2019-05	-0.168	-0.349	0.012	1000.000	0.084
month: 2019-06	-0.149	-0.254	-0.036	1000.000	0.002
month: 2019-07	-0.159	-0.258	-0.058	1000.000	0.002
month: 2019-08	-0.199	-0.296	-0.091	286.587	0.001
month: 2019-09	-0.094	-0.186	0.008	203.550	0.048
month: 2019-10	-0.129	-0.222	-0.032	263.762	0.004
month: 2019-11	-0.113	-0.215	-0.004	332.097	0.022
Sex: Male	-0.005	-0.046	0.031	1000.000	0.796
Age: Juvenile	0.010	-0.081	0.118	1000.000	0.850
Microbiota Shannon diversity	0.474	0.404	0.545	1000.000	0.001
Conspecific degree	-0.049	-0.155	0.052	1000.000	0.368
AF-degree	-0.054	-0.148	0.043	1000.000	0.262
Conspecific proximity sum	-0.028	-0.133	0.071	1000.000	0.568
AF proximity sum	0.031	-0.088	0.138	1000.000	0.610

TABLE S5.7 A) Effects of AF-degree on AS-taxa richness in Silwood					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.127	0.071	0.199	1133.434	0.002
Sample read depth	-0.010	-0.088	0.068	1000.000	0.800
month: 2015-02	0.024	-0.032	0.076	1000.000	0.394
month: 2015-03	-0.009	-0.053	0.035	1000.000	0.692
month: 2015-04	-0.011	-0.063	0.037	821.515	0.742
month: 2015-05	-0.020	-0.083	0.042	1000.000	0.528

month: 2015-06	-0.059	-0.106	-0.013	910.395	0.012
month: 2015-07	-0.017	-0.109	0.083	884.759	0.760
month: 2015-08	-0.033	-0.088	0.023	1000.000	0.232
month: 2015-09	-0.008	-0.077	0.051	1000.000	0.800
month: 2015-10	-0.081	-0.130	-0.031	1187.884	0.002
month: 2014-11	-0.032	-0.071	0.003	1000.000	0.078
month: 2014-12	-0.019	-0.073	0.027	1000.000	0.426
Sex: Male	0.035	0.011	0.058	751.884	0.004
Age: Juvenile	0.017	-0.048	0.086	1223.846	0.670
Microbiota Richness	0.832	0.769	0.897	1000.000	0.001
Conspecific degree	0.071	-0.018	0.152	1160.988	0.108
AF-degree	-0.110	-0.205	-0.033	1096.161	0.012
Conspecific proximity sum	0.003	-0.067	0.058	1000.000	0.934
AF proximity sum	0.063	-0.018	0.142	1000.000	0.142
B) Effects of AF-degree on AS-taxa enrichment in Silwood					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.054	-0.018	0.140	1000.000	0.160
Sample read depth	-0.002	-0.091	0.080	884.599	0.956
month: 2015-02	-0.012	-0.076	0.061	1000.000	0.762
month: 2015-03	-0.014	-0.069	0.052	977.928	0.664
month: 2015-04	-0.023	-0.091	0.047	1277.727	0.538
month: 2015-05	0.075	-0.005	0.152	1000.000	0.058
month: 2015-06	-0.011	-0.071	0.049	1094.828	0.724
month: 2015-07	0.094	-0.035	0.210	1095.310	0.142
month: 2015-08	0.030	-0.040	0.098	1807.038	0.388
month: 2015-09	0.097	0.014	0.179	1096.007	0.022
month: 2015-10	0.055	-0.009	0.114	1000.000	0.088
month: 2014-11	-0.012	-0.055	0.033	1000.000	0.582
month: 2014-12	0.058	-0.002	0.125	1000.000	0.090
Sex: Male	-0.001	-0.032	0.029	1000.000	0.974
Age: Juvenile	0.047	-0.042	0.131	1000.000	0.290
Microbiota Shannon diversity	0.467	0.397	0.537	1000.000	0.001
Conspecific degree	0.019	-0.093	0.128	1000.000	0.732

AF-degree	-0.094	-0.205	0.020	1000.000	0.132
Conspecific proximity sum	0.073	-0.008	0.156	1000.000	0.086
AF proximity sum	0.028	-0.068	0.131	881.015	0.590

TABLE S5.8 **A) Effects of MG-degree on MG-taxa richness in Wytham**					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.127	0.024	0.243	1000.000	0.022
Sample read depth	-0.171	-0.306	-0.034	1000.000	0.026
month: 2018-12	-0.035	-0.254	0.176	895.102	0.750
month: 2019-01	0.038	-0.147	0.213	1000.000	0.714
month: 2019-02	0.061	-0.050	0.153	1000.000	0.232
month: 2019-03	0.014	-0.093	0.114	852.836	0.804
month: 2019-04	0.030	-0.084	0.134	1000.000	0.620
month: 2019-05	0.031	-0.163	0.262	882.555	0.778
month: 2019-06	-0.061	-0.179	0.064	1000.000	0.322
month: 2019-07	0.034	-0.071	0.151	1000.000	0.554
month: 2019-08	-0.037	-0.146	0.072	561.738	0.522
month: 2019-09	-0.069	-0.176	0.035	1000.000	0.190
month: 2019-10	-0.017	-0.115	0.081	1000.000	0.766
month: 2019-11	-0.042	-0.152	0.073	519.083	0.452
Sex: Male	-0.035	-0.067	0.000	1172.154	0.034
Age: Juvenile	-0.053	-0.151	0.051	888.732	0.290
Microbiota Richness	0.659	0.545	0.772	1000.000	0.001
Conspecific degree	-0.048	-0.151	0.051	1000.000	0.338
MG-degree	0.055	-0.059	0.178	1000.000	0.386
Conspecific proximity sum	-0.093	-0.262	0.039	1000.000	0.242
MG proximity sum	0.102	-0.047	0.229	1000.000	0.132
B) Effects of MG-degree on MG-taxa enrichment in Wytham					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.020	-0.037	0.072	1000.000	0.482
Sample read depth	-0.014	-0.065	0.041	1000.000	0.592

month: 2018-12	-0.050	-0.151	0.052	1000.000	0.332
month: 2019-01	-0.011	-0.097	0.074	1000.000	0.840
month: 2019-02	-0.010	-0.058	0.031	1000.000	0.690
month: 2019-03	-0.031	-0.080	0.014	1000.000	0.220
month: 2019-04	0.013	-0.039	0.061	1000.000	0.608
month: 2019-05	-0.039	-0.148	0.051	1000.000	0.442
month: 2019-06	-0.046	-0.102	0.013	1344.698	0.126
month: 2019-07	-0.021	-0.075	0.028	1431.393	0.416
month: 2019-08	-0.031	-0.078	0.016	1234.600	0.216
month: 2019-09	-0.040	-0.090	0.007	1000.000	0.110
month: 2019-10	-0.023	-0.070	0.020	1450.637	0.304
month: 2019-11	-0.029	-0.074	0.019	1303.991	0.236
Sex: Male	-0.005	-0.022	0.009	1000.000	0.528
Age: Juvenile	0.003	-0.046	0.053	1136.230	0.884
Microbiota Shannon diversity	0.081	0.047	0.117	901.386	0.001
Conspecific degree	-0.024	-0.069	0.020	1000.000	0.312
MG-degree	-0.002	-0.055	0.048	1000.000	0.922
Conspecific proximity sum	0.009	-0.059	0.078	1000.000	0.804
MG proximity sum	0.021	-0.046	0.078	1102.094	0.482

TABLE S5.9
A) Effects on Microbiota Shannon diversity in Wytham

	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.539	0.367	0.696	1000.000	0.001
Sample read depth	-0.082	-0.265	0.089	1000.000	0.352
month: 2018-12	-0.164	-0.471	0.154	871.434	0.340
month: 2019-01	-0.035	-0.308	0.238	1000.000	0.846
month: 2019-02	-0.041	-0.175	0.114	1000.000	0.582
month: 2019-03	-0.056	-0.229	0.080	1000.000	0.466
month: 2019-04	-0.035	-0.212	0.129	1000.000	0.666
month: 2019-05	0.101	-0.202	0.432	1000.000	0.564
month: 2019-06	0.074	-0.113	0.257	1256.919	0.432
month: 2019-07	0.046	-0.150	0.209	1000.000	0.610

month: 2019-08	-0.010	-0.176	0.146	1000.000	0.912
month: 2019-09	-0.003	-0.162	0.170	1000.000	0.970
month: 2019-10	-0.160	-0.303	0.012	1000.000	0.040
month: 2019-11	-0.121	-0.307	0.043	1000.000	0.166
Sex: Male	-0.009	-0.062	0.043	995.802	0.784
Age: Juvenile	0.028	-0.122	0.199	909.458	0.728
Full degree	0.086	-0.141	0.282	1000.000	0.420
Heterospecific degree	-0.001	-0.203	0.228	1000.000	0.968
Full density	0.055	-0.223	0.369	1000.000	0.732
Heterospecific density	-0.119	-0.422	0.222	1000.000	0.502
B) Effects on Microbiota richness in Wytham					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	0.308	0.192	0.423	1000.000	0.001
Sample read depth	0.612	0.441	0.787	85.369	0.001
month: 2018-12	-0.090	-0.316	0.112	1000.000	0.416
month: 2019-01	-0.051	-0.226	0.149	1000.000	0.584
month: 2019-02	0.025	-0.081	0.123	1000.000	0.616
month: 2019-03	-0.003	-0.107	0.100	1000.000	0.996
month: 2019-04	-0.023	-0.134	0.085	1000.000	0.700
month: 2019-05	0.167	-0.016	0.389	1228.848	0.100
month: 2019-06	0.050	-0.071	0.178	1000.000	0.420
month: 2019-07	0.072	-0.052	0.184	1000.000	0.214
month: 2019-08	0.011	-0.114	0.113	1000.000	0.808
month: 2019-09	0.018	-0.100	0.122	1000.000	0.744
month: 2019-10	-0.063	-0.179	0.032	1000.000	0.222
month: 2019-11	-0.031	-0.150	0.080	1000.000	0.628
Sex: Male	0.010	-0.025	0.045	1000.000	0.576
Age: Juvenile	-0.036	-0.139	0.069	801.506	0.512
Full degree	0.110	-0.021	0.249	1000.000	0.124
Heterospecific degree	-0.037	-0.180	0.099	1000.000	0.626
Full density	-0.137	-0.360	0.056	1000.000	0.232
Heterospecific density	0.061	-0.148	0.292	1000.000	0.582

TABLE S5.10 A) Effects on Microbiota Shannon diversity in Silwood					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	31.060	8.837	53.410	1000.000	0.008
Sample read depth	0.116	-0.076	0.287	603.064	0.240
month: 2018-12	7.236	-6.300	20.670	1387.549	0.276
month: 2019-01	3.437	-7.248	14.150	1000.000	0.522
month: 2019-02	7.536	-6.555	20.276	1000.000	0.250
month: 2019-03	7.445	-7.333	22.481	628.127	0.332
month: 2019-04	-0.793	-12.808	11.261	1000.000	0.916
month: 2019-05	-2.137	-24.185	22.172	1000.000	0.874
month: 2019-06	10.203	-2.892	24.269	1000.000	0.152
month: 2019-07	4.137	-11.618	21.603	452.439	0.602
month: 2019-08	5.904	-5.817	17.808	1000.000	0.314
month: 2019-09	-0.852	-10.969	7.668	882.204	0.878
month: 2019-10	6.335	-4.813	19.805	1199.460	0.306
month: 2019-11	1.413	-5.464	6.609	1000.000	0.612
Sex: Male	5.104	-12.716	23.072	1000.000	0.552
Age: Juvenile	-0.450	-1.337	0.437	786.204	0.330
Full degree	-0.109	-0.625	0.355	1000.000	0.668
Heterospecific degree	0.086	-0.408	0.645	1000.000	0.756
Full density	-0.027	-0.429	0.326	1000.000	0.902
Heterospecific density	-0.032	-0.416	0.335	1000.000	0.846
B) Effects on Microbiota richness in Silwood					
	post.mean	*l-95% CI*	*u-95% CI*	*eff.samp*	*pMCMC*
(Intercept)	21.739	2.665	42.365	1000.000	0.028
Sample read depth	0.438	0.276	0.600	490.882	0.001
month: 2018-12	13.247	2.021	25.775	1000.000	0.036
month: 2019-01	10.384	0.077	19.584	1000.000	0.034
month: 2019-02	8.969	-2.104	20.730	897.912	0.130
month: 2019-03	3.758	-10.235	17.942	1000.000	0.600
month: 2019-04	-9.412	-19.800	1.081	1000.000	0.076
month: 2019-05	-1.947	-22.009	18.557	1000.000	0.854
month: 2019-06	3.777	-8.942	15.502	1000.000	0.522

month: 2019-07	-9.190	-22.238	5.316	897.961	0.200
month: 2019-08	-4.439	-14.832	5.686	1000.000	0.392
month: 2019-09	0.396	-6.795	8.895	1000.000	0.948
month: 2019-10	11.166	0.963	21.078	1000.000	0.024
month: 2019-11	2.419	-2.611	7.268	1000.000	0.352
Sex: Male	6.770	-8.437	22.054	1000.000	0.396
Age: Juvenile	0.289	-0.467	1.056	1000.000	0.450
Full degree	0.007	-0.367	0.418	860.583	0.972
Heterospecific degree	0.039	-0.410	0.440	782.680	0.846
Full density	0.076	-0.251	0.388	1000.000	0.658
Heterospecific density	-0.115	-0.444	0.211	1000.000	0.516

Supplementary References

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6

General Discussion

6.1 The key findings of this thesis

Variation in the composition of the gut microbiota governs a range of physiological processes in the host, but the drivers of this variation in natural settings are poorly quantified. In this thesis I have shown that the composition of wild wood mouse microbiota is in many ways shaped by transmission of microbes through contact with other conspecifics as well as the broader environment and sympatric species therein. For example, the wood mouse gut was shown to host more microbes from their own local soil compared to other, more distant soils in the same woodland (Chapter 3), wood mouse pairs with stronger social associations had more similar microbiota than less socially associated mice independent of how much living space they shared (Chapter 2, Chapter 4) and social contacts and space sharing between individuals of wood mice and sympatric yellow-necked mice individuals spread gut microbes from one species to another (Chapter 5). Of all the studied types of contact with the external world, social behaviour among conspecifics has the strongest influence on gut microbiota composition, even though wood mice are not a highly social species. This finding was verified with multiple alternative statistical methods and replicated in two different mouse populations, both of which showed the same pattern of social association (spatiotemporal co-occurrence) predicting microbiota sharing to a much greater extent than expected through mere sharing of living space.

Microbiota transmission through social behavior has been shown to be an important force shaping microbiota of humans (Brito et al., 2019; Dill-McFarland et al., 2019), primates (Perofsky et al., 2017; Raulo, 2018; Tung et al., 2015; Wikberg et al., 2020) and other highly social mammals (Antwis et al., 2018; Leclaire et al., 2014; Theis et al., 2013). A parallel line of research has shown that transmission through contacts with soil and other aspects of the natural environment influence gut microbiota of humans (Lehtimäki et al., 2017; Ruokolainen et al., 2017), other primates (Grieneisen et al., 2019) and laboratory mice (Ottman et al., 2019, Liddicoat et al., 2020). The work presented in this thesis is the first thorough attempt to simultaneously assess the effect of both social and environmental transmission on gut microbiota in any wild mammalian system, and provides the first evidence for social transmission of the microbiota in a semi-social, non-group forming species. Microbiota homogenization in social groups

is by now a well-documented phenomenon in highly social mammals. For example, in my previous work I showed that social group membership explains 20% of variation in gut microbiota composition of wild red-bellied lemurs (Raulo et al., 2018), and similar evidence come from studies of other species of primates (Abondano et al., 2017; Bennett et al., 2016; Degnan et al., 2012; Grieneisen et al., 2017; Wikberg et al., 2020). However, the microbial transmission effects of social contacts within social groups are notoriously hard to distinguish from effects of convergent environmental influences, since animals in a social group often share the same space most of the time (Albery et al., 2021). The fact that wood mice have a non-modular social organization makes separating these effects easier, as individuals vary somewhat independently in their social association strength and spatial overlap, and on a continuous scale. As such, wood mice had strong social associations (were often observed together) with individuals who were not necessarily their nearest spatial neighbors. These social associations predicted microbiota similarity more strongly than measures of spatial proximity or space-sharing, such as overlap in their home ranges, implying that socially associated mice shared more microbes than mere neighbors.

The fact that social contacts predicted microbiota similarity over and above shared space and that social effect on microbiota depended on the sexes of the interacting pair implies that social behavior involving physical contacts, such as grooming or licking (observed in wood mice (Lambin, 1988), spreads gut microbiota between interacting individuals. Interestingly, this social signal in gut microbiota composition only involved a subset of the microbes present in the gut. Specifically, sharing of anaerobic microbes that cannot readily survive outside the host was predicted by social associations more strongly than sharing of aerotolerant and spore-forming microbes, which were more structurd by shared environment. This implies that social and environmental transmission pathways not only had different effects on overall microbiota composition, but seemed to transmit different sets of microbes.

6.2 Limitations of this work

The work presented in this thesis has some notable limitations. Many of the findings in this thesis were robustly replicated in the two separate study population studied, but not all. As study methods differed slightly between populations, it is impossible to say whether these inconsistencies arise from methodological or biological differences between the different studies. For example, I found inconsistent evidence over how social transmission affected the microbiota of pairs with different sex combinations: in Silwood, social associations predicted microbiota similarity only in pairs involving males (male-male, female-male), while in Wytham, microbiota seemed to be shared only among same-sex pairs (male-male, female-female) but not among opposite sex pairs (female-male). This could reflect behavioural differences between populations, caused for instance by the higher population density in Silwood, but this could also reflect methodological differences between logger data sets. Importantly, in Silwood, the loggers had a minor lure (peanut oil and a single peanut) while in Wytham they did not. It is possible that mice behave differently when they explore a location of interest (baited logger) compared to when they are randomly passing a less interesting logger (non-baited logger). For example, mice could be behaviong more territorially around a potential food source or drawn to explore the baited loggers from further away, even away from their natural home range lured by the smell of peanut oil. Such effects could explain the generally larger home range estimates based on logger data from Silwood (3366,9 m^2, sd= 1456,6 m^2) compared to Wytham (1909,0 m^2, sd=1661.2 m^2).

In addition to limitations created by inconsistent tracking methods, wood mice also have some clear limitations as a study species of social contact transmission. While the abundance and social structure of wood mice makes them a good species to disentangle social and spatial processes affectring microbiota, their actual behavioural interactions remain hard to observe and study directly. A lot of the inference of social interactions in this thesis is based on spatio-temporal co-occurrence, which is not as robust a measure of social relationship as measures based on active observations. Reflecting this, similar strengths of spatio-temnporal social association predicted microbiota sharing very differently across different sex combinations (Chapter 2 & 4), implying that our measure of social association encapsulates various

different types of social relationships on a more proximate scale. Wood mice are challenging to study behaviourally in the wild as they are nocturnal, fossorial, and small and consequently impossible to actively track. Currently, all insights into their behaviour are based on active (Wolton, 1985) or passive tracking data (as in this thesis or in Godsall et al., 2014) or observations of individuals kept in unnatural captive conditions (Zgrabczynska & Pilacinska, 2002). These give us an impressionistic idea of the social behaviour of this species, but inferring social contacts among cryptic species such as wood mice will remain vaguer than for instance primates that can be observed behaviong in their natural habitat. Arguably however, it is also important to study behaviour of the more cryptic species, since limiting behavioural studies only on species that are easy to observe will evidently bias research on animal behaviour towards speies with certain types of behavioural ecology. There are still improvements that could be made even into the passive tracking methods to infer more intimate types of social behaviour. For example, artificial nest boxes or more thorough tracking of natural burrows would tell us more about which mouse individuals may be nesting with each other.

The findings presented in this thesis are also based on phenomena happening on very fine spatial scale, and as such are insufficient to draw any definitive conclusions over how transmission patterns may vary across larger spatial scales. While the scale of the logger data used here may be sufficient to infer variations in social associations, it only captures vaery small spatial scales, the very extreme end of possible spatial distances that can influence microbiota variation. For more through assessment of spatial effects on microbiota composition, one would optimally compare microbiotas of hosts with a continuum of distances between them, from fine scale (e.g. among individual with overlapping ranges) to medium scale (e.g. among individuals of the same breeding population) tro larger scale (e.g. among individuals of different populations living far apart). Further, to separate pure spatial (dispersal limitation driven) and environmental (selective processes driven) effects of host location on gut microbiota, as was done for natural soil microbiotas in (Griffiths et al., 2011), one would need a larger number of different habitat types replicated many times across different locations. Also, to more reliably track the transmission of microbes from the environment to the host, finer methods for inferring sources and sinks in transmission pathways based on sequence data, such as SourceTracker (Knights et al., 2011), could be used. Notably, all these effects should be studied across multiple species with different spatial scales of movement.

While I found that social transmission effect on microbiota exceeded the effect of environmental transmission, this might be different for species whose movements happen in larger spatial scales, exposing them to more variable environmental microbiotas.

Many other more advanced mathematical methods for modelling transmission of single microbes exist within epidemiology and could be adapted to describe the flux of whole microbiota communities as well. My approach for modelling microbiota transmission is based on the idea that transmission is symmetrical between interacting individuals (which may well not be the case always, see for example (Song et al., 2013)) and that social relationships are relatively stable throughout an individual's lifetime. Thus, the transmission networks in this thesis are temporally simplified, as I have only used static social networks based on social associations occurring any time in the overlap of two individuals' lifetime to predict their microbiota sharing. Naturally, social contact networks and the microbiota transmission tied to them, are in fact much more dynamic than this, especially in animals that change social relationships throughout their life. For this reason, models with dynamic, instead of static, social networks (Newman, 2018) provide a promising avenue for future research in the study of social microbiota transmission and temporal dynamics therein, such as time lags between changing social contact patterns and changing microbiota compositions.

Finally, similarity of microbiota based on 16S rRNA profiles may not be the optimal molecular method for revealing transmission dynamics of the microbiota, as it has relatively low taxonomic resolution. Arguably, sharing a similar set of microbial 16S ASVs, or genera is a less robust indicator of transmission than sharing of the actual same bacterial strains. This is why microbiota data produced with methods with strain-specific resolution (Brito & Alm, 2016; Caro-Quintero & Ochman, 2015; Yassour et al., 2018) are more promising avenue for future microbiota transmission research, while 16S methods may for the time being remain the status quo in studies attempting to partition variation across various different forces shaping the microbiota, due to their low cost and consequent ease of application at a population-wide scale. For example, (Brito & Alm, 2016) showed that microbiota transmission signals are more readily revealed when tracking the sharing of microbial strains compared to higher level taxa.

6.3 The power of a network approach in metacommunity ecology

These findings highlight the power of a network approach in modeling microbiota transmission. In this thesis, considering microbiota as a metacommunity nested within the social contact network of their hosts has proven to be a fruitful framework to ask questions about this emergent microbiota variation. Instead of considering microbiota similarity of individuals belonging to a certain social group, using social networks as a template for microbiota metacommunity dynamics reveals the patterns of microbe sharing at the level of biological organisation where these transmission processes actually happen, that is between pairs of individuals. What is more, combining metacommunity theory (i.e. the idea that connectedness to other local communities affects the composition of an ecological community) with network theory (i.e. the idea of connectedness between things forming a step-wise network rather than a continuous space) provides powerful tools to model otherwise invisible processes of transmission. Here, I will summarise three ways in which transmission can shape microbiota metacommunity composition in ways that can be revealed by a network approach, that might remain invisible with other approaches. I will be drawing examples from the results in this thesis, and propose future lines of research based on these.

1) effect of indirect transmission on the microbiota,

2) effect of individual network position on emergent community properties of microbiotas, and

3) effect of whole-network structure on metacommunity properties

6.1

6.3.1 Effect of indirect transmission on the microbiota

Classic metacommunity theory generally assumes that the compositional similarity of two patches is affected by dispersal abilities of species from one patch to another, or dispersal limitation between patches, such that the further away/less connected patches are, the less likely the dispersal between them is, and thus the more dissimilar their composition (Leibold & Chase, 2018). In a microbial transmission network, connectedness between patches (hosts) is far more dynamic as hosts move in and out of contact. Furthermore, connectedness between patches in a microbial transmission network is not only captured by simple measures of pairwise distance or connectedness but also by indirect connections through

intermediate patches (Figure 1A). Here, direct links between patches are not only weaker the less connected they are, but in fact often nonexistent, in which case two distant patches can only affect each other indirectly, via intermediately-placed patches. This was illustrated by my finding that transmission signals between sympatric species of mice were enhanced by considering indirect connectedness between heterospecific pairs. Specifically, in Chapter 5 I found that wood mouse microbiota was most enriched by typical microbes of sympatric yellow-necked mice when they had many direct social connection links to yellow-necked mice but also when they had more indirect exposure to yellow-necked mice through other wood mice in their social network. Here, without a network approach in modeling an individual's position in its transmission landscape, the effect of indirect microbiota transmisison from yellow-necked mice to wood mice *through other wood mice* would have remained unnoticed. An interesting direction for future research will be to link these stepwise indirect transmission processes with within-host metacommunity dynamics and ask questions such as *do certain host physiologies impose selective filtering processes on their microbiota in a way that limits the flux of microbiota transmission through these hosts, creating barriers for direct and indirect microbiota transmission?*

6.3.2 Effect of individual network position on emergent community properties of microbiota

While microbiota as a whole is clearly influential over many aspects of the host's physiology, attempts at pinning these effects on specific bacterial species have largely failed. It seems that what really matters to the host is not the specific cocktail recipe of their microbiota or the presence of some important bacterial species, but rather emergent community properties of the microbiota as a whole, such as its stability, productivity or alpha diversity. In metacommunity ecology literature, these emergent community properties have been linked with connectedness between the sub-communities. For example, Mouquet & Loreau (2003) showed that local diversity on a habitat patch is expected to be highest at intermediate levels of connectedness – where low connectedness results in chance extinctions depleting the ecological diversity and overly high connectedness resulted in reduced diversity through weaker competitors being outcompeted from the system. These kind of connectedness landscapes can be defined with even more detail by using the rich set of "node metrics" from network theory developed to describe

different positions in a network. For instance, an individual's position in a social network can be summarised through measures of centrality (e.g. degree, eigenvector centrality; Newman, 2018) and these measures can then be linked to node-wise microbiota measures (e.g. alpha diversity) to ask questions about how certain positions in a transmission network shape emergent community composition of the microbiota (Figure 1B). For example, I found that certain positions in a social transmission network were more enrichning than others for the gut microbiota of wood mice. Specifically, mice with many direct connections to others had more microbial species residing in their gut compared to mice with few connections. However, the microbiota was also enriched by high "bridge propensity", a network centrality measure I developed to describe the relative distinctness of one's connection sources. This meant that even microbiota of mice with few connections could be enriched by their network position if these links were connecting very separate, otherwise disconnected parts of the network.

Some (inconsistent) evidence for individual variation in social connectedness affecting microbiota alpha diversity has been documented in wild primates (Perofsky et al., 2017; Raulo et al., 2018), but most studies have not considered the potential role of network node metrics other than simple degree in microbiota transmission, as has been done for other types of ecosystems. For example, across animal communities inhabiting brackish marsh ecosystems, parasite diversity within host species was predicted by host eigenvector centrality in their food web network (Anderson & Sukhdeo, 2011). The connectedness structures of dendritic river networks have also been shown to shape the local alpha diversity patterns of aquatic insect communities (Altermatt et al., 2013). Similar patterns of network centrality shaping emergent properties of a lower-level system are well-known also outside of ecology, in other complex systems, such as economic systems and information networks. For example, attitude diversity over climate change matters was positively correlated with degree in social communication network among American people (Leombruni, 2015). In addition to diversity, many aspects of systemic stability are influenced by the system's position in a network of influences. For instance, political ambivalence (a measure of opinion instability) among people belonging to student organisations was positively associated with their centrality in a communication network (Song & Eveland, 2015) and centrality of companies in financial networks was shown to be associated with their level and sensitivity to market variations (a measure of resilience; Bartesaghi et al., 2020)

The way in which transmission shapes microbiota composition seems to be mathematically more equivalent to processes of information transmission than the most common cases of pathogen transmission, mainly because the influential variation in microbiota arises through emergent community properties, whereas the influential variation in pathogen states is more simply described by presence or absence of a single microbe. In fact, some pathogen transmission studies, such as studies of viral load do use networks to model the accumulation rather than presence/absence of transmitting microbes (Goyal et al., 2020), and methodology from this line of research could be useful for microbiota studies as well. A no doubt fruitful direction for future research on microbiota transmission is linking network properties with emergent microbiota compositions in the nodes, and asking questions such *what kind of positions in such a network are expected to be most enriching, stabilizing or homogenizing. In other words, what kind of social personality gains the richest set of microbes, or ultimately has the most unique set of microbes across the population.* This kind of research will ultimately help link complex patterns of social behavior with physiologically influential variation the gut microbita of hosts, including humans.

6.3.3. Effect of whole-network structure on metacommunity properties

Properties of a whole metacommunity are influenced by network properties of connectedness between sub-communities. For example, research has shown that overall loss of connectedness between habitat patches, resulting from human-induced fragmentation of landscapes, is driving biodiversity loss in macro-ecological ecosystems (Hanski, 2011; Thompson et al., 2017). Similar links between microbiota and the host contact network structure likely play a role in shaping the global properties of the microbial metacommunity transmitting among hosts (Sarkar et al., 2020). The power of metacommunity theory in describing microbiota variation is that it allows modeling the processes shaping microbiota within an individual host through processes happening at the level of a population of interacting hosts (Miller et al., 2018; Miller & Bohannan, 2019; Robinson et al., 2019). While from the perspective of the host, its microbiota is a multivariate, plastic trait and important aspect of its phenotype, as a microbial community it does not clearly belong to any single host individual. Rather, its composition is clearly shaped by flux of transmitting microbes from one host to another and as such, individual variation in microbiota cannot

be explained by individual traits alone (Robinson et al., 2019). Considering the natural or social environment of hosts constitutes an important first step towards understanding microbiota variation that is not explained by host attributes. The second step can be to give this environment a deliberate shape as a network, which is a more complex and restrictive ecological space than the continuous geographic space where processes of spatial ecology are commonly modelled. Viewing hosts as cross-roads or knots in a network and the host-associated microbiotas as emergent communities accumulating in these specific positions in a network can give us tools to better explain microbiota variation between hosts and populations. This way the uniqueness, or individuality, of any given microbiota will not have to be explained by unique biology of the host alone, neither with a simple measure of its environment, but through a more realistic description of the shape of the surrounding tranmission network and the unique position of the host therein. Furthermore, viewing the microbiota as a metacommunity inhabiting a network of hosts will free us to consider the community ecology of microbiota *de novo*, without the pre-requisite of considering the sub-communities tied to a single host at a single time point as the primary unit in any explorations into microbiota dynamics. This way we can begin to understand, for instance, what other meaningful clusters than single hosts, such as those created by social coalitions between hosts, may exist in this microbial metacommunity (Figure 1C; Sarkar et al., 2020). A natural next step in microbiota transmission research will be to link the whole-network properties of the host contact network (e.g. social network or trophic network, or other ecological networks) with whole metacommunity properties (i.e. a population's microbiota) to ask questions such as *what kind of host population structures (e.g. whole network metrics like degree distribution, modularity) can maximise microbiota diversity within a population, and why this is?* This kind of research may help us better understand the correlations between loss of natural biodiversity or changes in human social culture (i.e. changes in connectedness among people and between people and environmental microbiota) and the observed loss of gut microbial diversity and subsequent autoimmune disease epidemics associated with modern western lifestyles (Brett Finlay et al., 2021; Browne et al., 2017b; Hanski et al., 2012; Vandegrift et al., 2016).

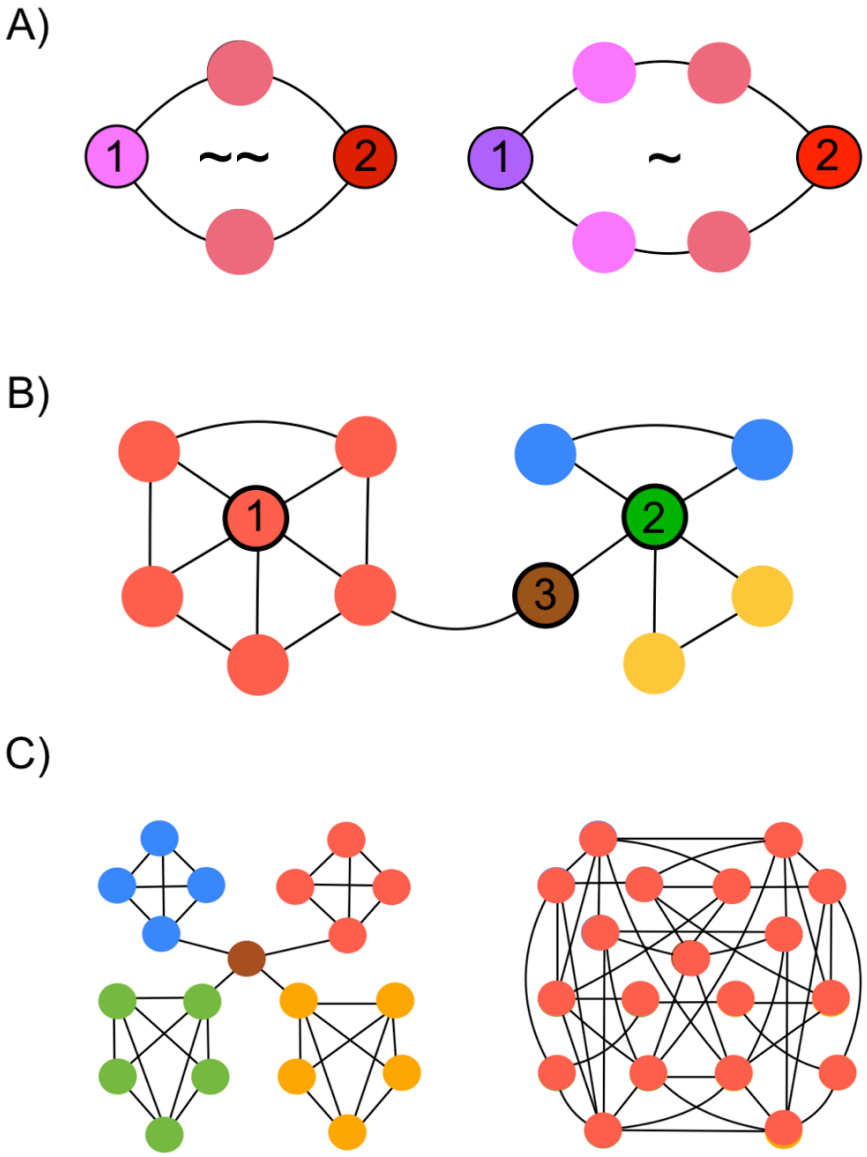

Figure 1. Emergent network phenomena that can influence compositional patterns in a metacommunity. A) ***Indirect transmission*** through intermediate nodes can homogenise community compositions, and the strength of this homogenisation decreases with a growing number of intermediate steps between nodes. Here, the compositions (colour) of node 1 and 2 influence each other indirectly by transmission through intermediate nodes. Consequently, their compositions are more similar to each other (~~) when the number of indirect steps between them is small and less similar (~) as the number of indirect steps increases. B) ***Different network positions*** can drive individual sub-communities to accumulate different emergent community compositions. Here, node 1 and node 2 have different centrality properties. While they both have a *degree* of 5 (five direct links), node 1's position in the network is described by high *clustering coefficient* (high level of interconnected connections) while node

2's position is best described by high *betweenness* (high levels of connections to otherwise disconnected parts of the network). These network processes affect the mixing of community compositions (colours) in a metacommunity, here described as more similar colours depicting more similar communities and compound colours (gree, brown) depicting more diverse communities than pure colours (red, yellow, blue). Specifically, as node 2 is exposed to transmission from more distinct sub-communities in a metacommunity, it gains elements of all of these distinct sources, leading to higher overall diversity (blue+yellow=green). Under this scenario, the most diverse community in this network accumulates in node 3 (brown) which connects to the most distinct sources, a position best described by high *bridge propensity* (high distinctness of connections relative to number of connections). C) ***Whole-network metrics*** may shape the global compositional patterns of a metacommunity. For example, compared to highly interconnected networks (right), more modular networks (left) may harbour higher global alpha diversity (gamma diversity) (more different colours) due to the enrichning effect of chance variation arising in separated local sub-communities combined with the rescuing effects of inter-node and inter-module connectedness on chance extinctions.

Notably, the research questions (*in underlined italics)* proposed in the last sections are not specific to microbiota science, but similar questions are long lasting open questions of theoretical metacommunity ecology and network sciences. As I have attempted to illustrate, questions about network metrics and emergent properties of complex systems have been asked and hypotheses made, in the study of information flow and economical market stability among other fields. Importantly, not only can microbiota research strongly benefit from such a metacommunity/network perspective but host-associated microbiotas in fact offer a great potential study system to address some of the open questions in metacommunity ecology more broadly, which are challenging to study in macroecological metacommunities such as oceanic islands.

6.4 Different contact landscapes, different transmission pathways

Considering microbiota in a metacommunity network has the power of translating host contact landscapes into eco-evolutionary landscapes for microbes residing inside hosts. Naturally, understanding the space in which microbial life happens can improve our predictions of the ecology and evolution of microbiotas. Compared to macroecological communities, the ecology of host-associated microbiota may happen in more complex space than the geographical space from which much of our current understanding of spatial ecology is derived. For instance, if the distribution of physical contacts between hosts (e.g. social contacts between conspecifics or reproductive contact between a mother and an offspring) define the structure of the metacommunity in which microbes reside, this means that the ecological and evolutionary space for microbiotas will be inherently nested inside the social networks or phylogenetic trees of their hosts (Figure 2). Importantly, within networks and trees distances operate in a different way from real geographic space (Newman, 2018). For example, connectedness between sub-communities in a social network is not a continuous measure like distance between macroecological communities, but rather a count of steps separating two nodes, possibly weighted with the strength of the connections (e.g. strength of social association) in each step. Lastly, and important difference between metacommunities in geographic space and host-associated living space is also the fact that the connectedness between and selective forces within are more dynamic: Specifically, unlike geographic distances, social contacts between hosts can rapidly change and unlike geographic locations, host physiology can evolve to adapt to or manipulate the microbial community for its own benefit (Miller & Bohannan, 2019).

Importantly, however, the shape of the ecological space in which microbiota live, assemble and evolve seems to be defined not only by the contact landscape of the host, but also by the varying phenotypes of the bacteria (Figure 2). In chapter 4, I found evidence that not all gut microbes seem to be transmitted through social networks – a subset of gut bacteria was specifically transmitted through the environment. In fact, microbial taxa transmitted through social contacts were remarkably non-overlapping with environmentally transmitted taxa. Supporting the idea that different taxonomic subsets of the microbiota

have adapted to different transmission pathways, I found that socially transmitted microbes were enriched in anaerobic lifestyles while environmentally transmitted microbes were enriched in aerobic and spore-forming lifestyles, as expected by their need to stay alive in an oxygenated environment outside the host. Experimental evolution research on zebrafish microbiota has demonstrated that when selection favors microbes living inside hosts (rather than the environment between hosts), microbes evolve to be better at transmitting between hosts (Robinson et al., 2018). This enhanced host-to-host transmission ability may lead to more aerotolerant lifestyles. For example, even strictly anaerobic human gut microbes were shown to stay viable in an aerobic environment of high tranmission potential (public toilets; Pausan et al., 2020), but importantly, microbes may gain enhanced host-to-host transmission ability also by specializing in transmitting through intimate social contact. In line with this, in the zebrafish example, while microbes evolved improved host-to-host transmission, they did not adapt to live in the media between hosts (water; Robinson et al., 2018). As a whole, the gut microbial community of a mammal will likely always be a mix of bacteria with different transmission ecologies, varying like a gradient between social transmission of strict anaerobes (Figure 2A), following the shape of the host social network, and environmental transmission of more aerotolerant taxa, following the shape of the geographical space and habitat variation around hosts (Figure2B).

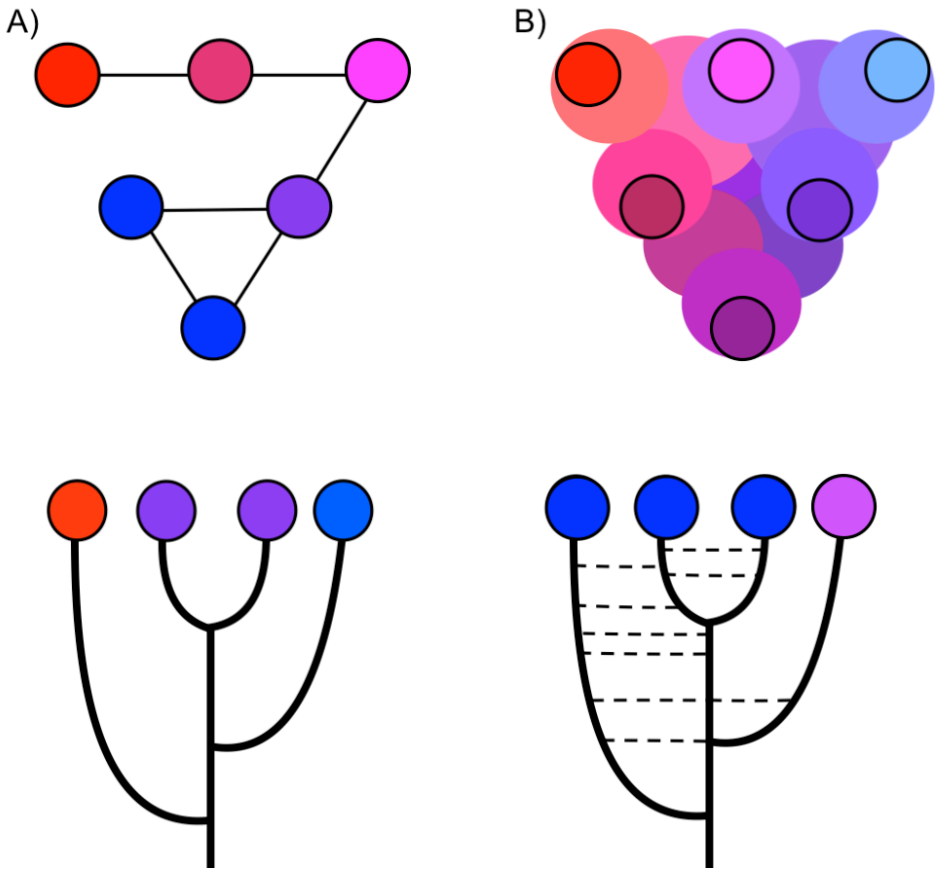

Figure 2. Shape of the ecological landscapes in which microbiota may live. A) Strictly anaerobic microbes, unable to persist outside their host, may have populations structured by transmission dynamics guided by the shape of the social networks (top) or phylogenetic trees (bottom) of their hosts. Hosts (circles) have more similar microbiota (more similar colour) to those hosts that they are most connected to (black lines), either socially (top) or phylogenetically (bottom). B) Aerotolerant or spore-forming microbes, capable of living outside as well as inside hosts, may have populations structured by transmission dynamics defined by actual space, i.e. geographic distances between hosts. Here, across a population inhabiting the same environment (top figure) hosts have more similar microbiota to those other hosts (circles), or environments between hosts (coloured areas), that they are most close to in space. Similarly, hosts may have more similar microbiota to other hosts with whom they share common ancestry, but also a common history of shared exposure to same environments and subsequent environmental microbial transmission (dashed lines).

Finally, considering these microbiota components with different transmission ecologies separately holds promise for enabling researchers to study patterns and processes of microbiota in more detail, targeting only the relevant part of the microbiota in their analyses. The mammalian gut microbiota harbours enormous amounts of compositional variation, and microbiota research consequently often ends up being a quest for digging relevant signals from vast multidimensional noise. When focusing on the subset of microbes that stably colonise individuals over long time periods, it may be useful to ignore as noise those microbes that seem to be transmitted indirectly via the environment. Similarly, studying the effects of environmental exposure on microbiota and immune development of human children or laboratory rodents (Lehtimäki et al., 2017; Ottman et al., 2019, Liddicoat et al., 2020) could be enhanced by limiting focus to only environmentally transmissible bacteria. Eventually, future research is also needed to assess the relationship between these different sub-parts of the microbiota with different transmission ecologies (aerotolerant and anaerobic), as they may have different relationships with, and different evolutionary optimums in relation to, the host organism. To date, microbiotas have been mostly studied as one, arguably noisy, entity, belonging to a single host, but it is most unlikely that such a complex system in a constant state of flux would be thoroughly described this way. Considering microbiotas as emergent communities living in a *network of hosts* as well as in a more *continuous landscape between the hosts* enables us to begin to understand not only the dynamics of microbiota across hosts but at what scale microbial ecological communities actually live: Are they ecologically bound to a host, a pair of hosts, a specific social group or a specific locality or environment? To what extent is their ecological space nested inside the phylogenetic trees and social networks of their hosts and to what extent it is shaped by geographical space? We may not yet have the language to describe the ecological phenomena or the coherent entities present in microbiota within and among host populations, but disentangling the microbiota members with different transmission pathways is a good start in drawing lines around new entities in this complex trait and unveiling new patterns for future research to study.

6.5 Microbial transmission as a beneficial force for the host

A great deal of discussion in this thesis has been dedicated to the effects of transmission processes on emergent microbiota compositions, yet the implications of microbiota composition for community properies such as stability and productivity, and ultimately for host health and fitness remain unclear (See Chapter 1: Box 1). What we do know, however, is that microbiota composition is in many ways influential to host health (Human-Microbiome-Project-Consortium, 2012; Round & Mazmanian, 2009) as well as evolutionary fitness (Suzuki, 2017), both in terms of survival (Salosensaari et al., 2021; Suzuki, 2017) and reproduction (Rowe et al., 2020). Linking specific microbial "cocktail recipes" with specific health/fitness outcomes is a field with a lot still to discover, but since variation in microbiota composition is to a great extent shaped by transmission, it appears that the contact landscape (=transmission landscape) of the hosts probably has important effects on their health and fitness through microbiota composition. Importantly, unlike pathogen transmission, microbiota transmission may greatly *benefit* host health (Browne et al., 2017). As such, this phenomenon is challenging the historical narratives of infectious agents causing disease while immune properties stay tied to single individuals as well as the idea of social transmission of microbes being primarily a negative, hindering, force in the evolution of social behaviour (Kappeler et al., 2015; Manlove et al., 2014; Udiani & Fefferman, 2020). For example, even very recent theoretical work suggested that simulated social networks maximized information transmission and minimized social microbial transmission were evolutionarily optimal social structures, without considering the importance of social transmission of beneficial microbiota or the risks of limiting it (Romano et al., 2020). Similarly, a recent meta-analysis on the health implications of living with relatives across numerous species was based on the hypothesis that microbial spread is the main down-side of social life style, and the benefits arise from other things, such as coopeartion (Bensch et al., 2021). Both of these paradigms view contact transmission as a challenge the host has to cope with, built on the basic idea of pathogens that transmit from one individual to another and defense systems that belong to each individual independently. While transmissible pathogens are an obvious threat, if host health (e.g. immunological resilience, metabolic capacity) is shaped by the gut microbiota, a physiological system

that is also transmissible, this means that to some extent positive aspects of health can also spread like epidemics (Browne et al., 2017; Vandegrift et al., 2016).

Consequently, recent research has suggested the idea that diminishing contact transmission of microbes could be a problematic part of ongoing human-induced biodiversity loss and may present unforeseen negative health consequences to humans (Brett Finlay et al., 2021; Browne et al., 2017; Haahtela, 2019; Hanski et al., 2012; Liddicoat et al., 2016; Ruokolainen et al., 2016). For example, the lack of contact with natural environments associated with industrialised human societies, and the consequent reduction in microbial exposures this entails, has been shown to negatively influence the immune development of children, increasing the prevalence of autoimmune diseases and allergies (Fyhrquist et al., 2014; Lehtimäki et al., 2017; Ruokolainen et al., 2015, 2017, 2020). Similar research assessing the health risks of decreasing transmission through social contact is currently lacking. However, scientists have suggested we might be underestimating the negative health consequences induced by diminishing social contact in modern human societies (Johnson, 2020; Walter & O'Mahony, 2019), that recent social isolation practises used to limit the spread of SARS-CoV-2 may have negative impacts through our microbiota (Brett Finlay et al., 2021; Domingues et al., 2020; Ghanemi et al., 2021), and that the whole concept of hygiene might need revising to include consideration of non-pathogenic symbiotic microbes (Vandegrift et al., 2016). While I write this, news reports from New Zealand describe rises in paediatric infections related to "immune debt" in children that have by now spent a large part of their childhood in lockdown social isolation (Cohen et al., 2021) and similar reports are coming from other countries as well. In a human society ridden not only with infectious epidemics, but also epidemics of mental health disorders, allergies and autoimmune disease, all of which are revealing to be influenced by microbiota composition, we need to view microbial transmission not only as spreading single pathogens of concern, but as a continuous flux of whole ecological communities of microbes that tie us together and into the ecosystem we live in. By revealing how microbiota transmit among hosts and influence their host's health and survival, we can better model the spread and epidemics of immune properties as well as non-infectioius diseases that are nonetheless microbially influenced, alongside those driven by pathogens.

6.6 Conclusion

Great effort in microbiota research has been dedicated to explaining individual differences in microbiota composition with individual variation in other traits, such as physiology, genotype or environmental influences like diet, but these attempts have typically only explained a minority of this variation. This may be because we are not paying enough attention to between-individual processes, such as transmission, in shaping microbiota compositions. This thesis has clearly illustrated how transmission processes can influence microbiota composition even more than individual attributes, and that social contacts networks can function as an influential transmission landscape for microbes even in a relatively non-social species. Taken together, these findings emphasize that if we want to understand how microbiota variation arises, we need to develop new means of considering the contact landscape of host individuals alongside their individualistic traits. I propose that combining tools from network theory and metacommunity theory, it will be possible to reveal more of the driving forces of microbiota variation by describing both the contact landscape and microbiota variation as dyadic traits, and by summarizing individual position in social contact networks through network metrics and linking these with emergent microbiota compositions that accumulate in those positions in a transmission network. This will be the first step towards better understanding of the role our microbiota plays in mediating how our relationships with each other and our natural environment shape our internal biology, health and well-being.

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Afterword

Reflections on holistic and reductionist perspectives in biology

I have grown to be a scientist alongside the young field of microbiota science, and I think the theme of this journey has been a process of revealing more and more complexity and describing more and more complex patterns of interconnectedness. For me personally, this has meant growing into more holistic, less reductionist philosophy over organic systems and re-considering the way we define individuality and other units of life. In defining units of ecological or evolutionary processes, current biology is still greatly influenced by last century's philosophies emphasizing separation instead of contact between forms of life, and I think that this limits our ability to perceive the more complex patterns of interconnectedness that exist in nature as well as negatively affecting our relationship with our ecosystem and each other. The philosophy of natural sciences during the twentieth century was greatly shaped by ideas of reductionism and general categorisation, or in summary, the ideas that nature consists of separate units or "things" (such as molecules, genes, cells, individuals, populations, species), which may have interactions with each other, or be nested inside each other, but are still best described as distinct entities. By extension, this narrative assumes that most of the important variaton in nature will be visible by comparing these "things" rather than focusing on their interactions. For example, for a long time, people believed that all microbes are either pathogenic or symbiotic, until it was understood that their pathogenesis is often context dependent. A more serious problem of reductionism in biology has been the urge to categorise units such as races, ecosystems or genotypes in a puritanist way, whereby any mixing of influences between these entities is considered "dirty" and harmful for their integrity. These ideas were abundant in early twentieth century genetics and microbiology, giving rise to the dark history of eugenics and "racial hygiene" (Rice, 1929) and some claim they still live on today in debates over conservation biology, especially on the matter of whether our images of the "natural state" of a given ecosystem are influenced by subjective ideas of what is pristine nature rather than being based on actual ecology in real-world landscapes (Kunz, 2016).

In biology, counter-arguments to reductionist perspectives have been abundant, well-debated and fruitful. For instance, so called process ontology for biology (Nicholson & Dupré, 2018) aims at viewing units of life as whole temporal processes, where what we perceive as individuals or species are just snapshots of their state in time. Process ontology has been an important framework for example for evolutionary developmental biology, emphasizing how evolutionary selective forces may work on

developmental pathways rather than genotypes or phenotypes (Müller, 2007). Another holistic movement in biology has been the so-called systems biology, focusing on mathematical exploration of living systems of interdependance (e.g. ecosystems, gene networks, phylogenies) instead of bottom-up modeling of phenomena created by smaller-scale units (e.g species, genes) (Kitano, 2002). Systems biology draws stongly from network theory and for example systems research on metabolic networks has provided powerful examples of how network-thinking can reveal dynamics in living systems that could not be perceived or analysed with reductionist thinking (Stelling et al., 2002). Another, influential discussion over reductionism in biology has been the debate over hierarchical vs. gene-based units of evolutionary selection. Here, the reductionist perspective states that evolutionary selection works on the lowest replicating level of complexity of living systems, that is genes, while the more holistic perspective claims that these "replicating units" need not to be the same as the "intearctors" that are factually selected and can also be higher-level units, such as individuals, or even groups (Gould, 2002; Hull, 1980). However, what are the criteria by which these lower level units can form any sort of coherent higher-level selection unit, is a hotly debated matter (Dawkins, 1976; Gould, 2002). Needless to say, my own research on microbiota transmission is very much following on the foot steps of the more holistic, network-oriented movements as I believe microbiota variation cannot be understood without understanding of *emergence* of variation through connectedness, which is an inherently holistic property of systems (Bedau & Humphreys, 2008). I am leaning towards a non-reductionist philosophy whereby the meaningful forms of life, worthy of study and curiosity, may be shapes that are invisible to the eye, such as the topology of a transmission network.

I believe that these new holistic perspectives on biology are not only powerful tools for studying variation in complex systems, but they have great potential in affecting our world view. On a closer look, in nature nothing is independent, everything is connected and dependent on something else. Without frameworks like the network theory, we would be left with thinking that as a wholly interconnected net, living systems are an un-characterisable mess, like a knotty bundle of yarn. But with the toolkit of network theory, we can begin to see more than a mess, distinguish the shapes and name the phenomena in this bundle of yarn. We can define individuality not just through some internal uniqueness but as a unique spot in cross-roads of interactions. Just like my microbiota may be a relfection of my social network, my genotype

reflects my pedigree, my ideas may be an emergent property of the information I have been exposed to and my resilience is built from the network of support around me. While I write this, I am in self-isolation after having hugged someone infected with SARS-CoV-2 virus. Yet, I hope these necessary periods of isolation will make it clear how dependant of each other we actually are. Understanding how everything that makes us unique is made of patterns of connectedness to others can help us see beyond last century's ideas of separation, segregation and hygiene – see that contacts with other humans, cultures, races, microbes and environments are a necessary source of resilience rather than a threat.

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"Doesn't each of our individualities begin right at the point where we own up to our extremely close connections and accept the inevitability of borrowing..."

- Seymour Glass (J.D Salinger)

The years I have spent doing this research have been filled with a strange collection of joyous moments, from nights spent listening to klezmer while trying to wind a copper coil with a certain shape of a magnetic field to late-night loitering in the lab, passionate discussions about network theory in various pubs with various fellow thinkers, and a well-established routine of running around Wytham Woods in search of mouse burrows. After all this I can whole-heartedly say that this has been a great privilege and the most fun I have ever had. I can hardly imagine life not filled with the constant humor, curiosity and wonder that has surrounded me in Oxford. My DPhil studies were not always as easy as one would hope, interrupted by the sudden closure of the Zoology department building, a snow storm that wiped out my study population of mice, a divorce, a global pandemic and a kidney operation, but looking back at these years now, I still see more joy and beauty than trouble and stress. No doubt this is largely because the privilege I have had in the insane amount of support and resources I have received, to do my research and to deal with life on the go.

For me this is a touching reminder of my own position in the network of influences, ideas and support, where my resilience or any unique perspective I end up holding has certainly arisen from a diverse set of social connections and supporting structures. I will try to acknowledge the various connections in my own network that has allowed, enabled and empowered me to try do what I most love to do in life, scientific research.

First of all, I want to thank the 513 wood mice in Wytham and Silwood that unwillingly participated in this research. I am sorry for interfering with their everyday life, hopefully the peanuts provided were a worthy compensation for the nuisance. I am grateful for the amazing insights this data collected from them has given us humans.

On the human front, I want to first thank my both parents for fostering critical thinking and wonder over the universe throughout my life. My mother Hanna Kirjavainen has always made it clear that what is normal might not be true and that it's worthy to fight against social pressure to stay true to one's self. She made sure I felt that there are no limits to what I can try and that I inherited a good, if slightly delirious, self-esteem by telling me at every turn how great I was in everything I tried. My father Erkki Raulo has really rooted natural sciences in my life from early age, taking me with to his biochemistry lab as well as to the tundra, bought me all books I ever wanted, hosted a chemistry club in our kitchen, arranged an aquarium for my pet leech, a terrarium for my pet snails, and brought me a rat pup from the lab to raise. Upon the tragic death of my baby flycatcher, whom I had raised from a nestling, father got me thinking whether it might have been the lack of maternal gut microbiota transmission that led to the disease that killed the bird. This was the first scientific hypothesis I ever made and curiously close to what I ended up studying later in life. I also want to thank my unusually large number of grandparents, Ansa and Heikki, Soili and Jyrki, Maija and Jaakko as they have been, and still are, an invaluable support during my life. Having not just a large family but in fact many large families has no doubt been the main source of strength as well as perspective in my life.

As I come from a not so wealthy background, I also want to thank the Finnish social support system for backing me up for such great extent that I have felt safe enough to try risky and even slightly crazy things. As a citizen and a scientist, I consider myself to be very much a product of this system, without which resources, including free university education and years' worth of subsidized psychotherapy, I would have never entered the academia or dared to try anything as risky as a scientific career. I am also fundamentally grateful to dear Juho Asikainen, whose fierce and continuous support over the first decade of my adult life I owe much of my self-esteem and mental health and whose totally unique perspective and clear mindedness has enabled me to let go of the suffocating grasp of social norms and inspire me to think outside of the box.

After trying out science for a few years, I think this might be a dream job for me and I am quite happy that I have, quite accidentally, ended up in a profession that lets me use my undying doubtfulness to

create something nice instead of just sabotaging thought constructions of others. For finding my way into a profession I enjoy I owe thanks to all my friends in Finland who spent a big part of their youth messing around with me and building up a lifestyle where curiosity and experimentation was valued over status, material and profit. Thanks to Ilona Sakaguchi and Janne Salo, the original nerdy Chemistry gang, with whom I did my first scientific experiments (a case-study on the neuro-development of my little sister), and to the rest of the Firma gang. It was amazing to have teenage friends with whom to spend time discussing theories of emotional intelligence, building toy railways and dropping water melons from high places while the rest of the cohort was worrying about makeup and power structures. Thank you also to Leena Pihlajamäki, whose kitchen was my safe space in years of growth and whose soul is still my safe space for obscure humor that no one else understands. A big thanks to Ezma Sihvola, my one and only fellow queen, my favorite composer and main partner in art, I hope to be able to make music with Ez until the day I die. Thanks to Lise Niska and Pia Rytilä for the countless philosophical discussions over hours spent fixing various broken trousers.

As said, I ended up in this career quite accidentally, following a vague string of intuition rather than a shiny vision or a dream. However, science has done a whole lot of good for me. It rescued me from excess self-centeredness that is my curse as an artist, filled my life with wonder over things I had no clue even existed and calmed down my restless spirit by sucking up my excess energy, and I am grateful for all this. I want to specifically thank the people that have un-knowingly pushed me towards this path of research. Importantly, the original research idea behind this whole thesis, the hypothesis that gut microbial populations may be structured by their host's social network, is not my own but something I picked up from the amazing brain of Avery Lane, a great friend, scientist and a poet, with whom I shared a tent in Madagascar for an unforgettable five months. The fact that we then ended up studying social microbiota transmission in lemurs was further made possible, unintentionally, by Sarah Zohdy, who donated a one-liter bottle of RNAlater for us "to use as you please", and consequently enabled us to collect the data for our first microbiome study. I hope Sarah knows how great of a fan of her research on parasite transmission I am, but I doubt she knows what long-lasting consequences giving us this one bottle of DNA preservative had for me as scientist. I want to thank my beloved friends Anna and Riikka Voutilainen, who encouraged me to take the Oxford offer and go when I was unsure and scared of leaving

my life in Finland, my friends, elderly grandparents and baby siblings behind. After years of living abroad I feel still just as close with these two, just as touched by Anna's restless and curious creativity and just as inspired by the crazy beautiful dragon spirit of Riikka's, and this is largely thanks to their heart-breaking commitment in loving friendship over whatever geographical scales.

I consider all my "own" ideas as mere emergent properties of the thoughts that I have been exposed to in my information network. Thus, in addition to the ones listed above, I want to thank all of the other amazing scientific thinkers that have strongly affected my own thinking in the process of growing up as a scientist. A great thanks to Scott Gilbert, whose thoughts on ecological developmental biology and host-microbe symbioses as processes of "becoming together" made me see the poetry in microbiomes, and whose general commitment to seeing beauty in the structures of life has really helped me, a child of an artist family, to find a comfortable philosophical niche within natural sciences. A lot of my excitement over the more holistic views of organic systems have been greatly influenced by him and people I know through him, such as Lynn Chiu, or John Dupre and Dan Nicholson, who earn a special thanks for their amazing conceptualizing of process ontology for biology. I am also forever grateful for my extremely wise, sharp and critical-minded masters-supervisors Lasse Ruokolainen and the late Ilkka Hanski. From Lasse I have learn a lot of my hands-on skills as a data scientist but he has also given me one of the best advice in dealing with science: "The most important tool of a scientist is the trash bin – if the bin is not full, it's either a sign that you do not have enough ideas or a sign that you are not willing enough to let go of your ideas". Needless to say, Ilkka Hanski, as one of the founders of metapopulation theory, has had an incomparable influence on my science and thinking ever since I was an undergraduate in his courses on fragmented landscapes and island biogeography. His ideas of metapopulation dynamics have been the very basis of my understanding of ecology in general. I am sure these ideas live on in microbiome science and the broader network of scientists he has influenced, just like the Glanville fritillary butterflies scattered across the Åland archipelago. A loving thanks also goes to my main comrades in Helsinki University, Matan Shenhav and Jani Anttila, who took me under their wing, taught me to code and to deal with critique, saved me from boredom, listened to me rambling over my ideas until I had reached something to write on an Oxford application and supported me in every possible way in my flamboyant quest of being an unfashionable nerd, a data scientist, an artist and a DIY-mystic at

the same time. On top of this, I owe many of my favorite thoughts and thinking tools to these guys, such as the ability to think about variation in the multidimensional ordination space (thanks to Jani) and the idea that the uniqueness of individuals can be seen as a unique position in a network (thanks to Matan). I also want to thank Brendan Bohannan and Karen Guillemin for hosting me on my visit to META-center, Oregon and for the very inspirational research on metacommunity dynamics of gut microbiota that has been foundational for my thinking and the theoretical framework in this thesis. Similarly, thanks to Matthew Leibold, Kevin Foster and Tim Barraclough for some very influential discussions over the years on theoretical assumptions in community ecology. Specifically, Matthew Leibold has helped me see the value of separating direct and indirect connectedness in metacommunity ecology and Tim Barraclough and Kevin Foster have helped me understand how the relationship between community stability and diversity depends on the nature of ecological interactions within the community. Lastly, I want to thank Renee Hoekzema, who is probably the broadest academic thinker I have known and to whom I owe a lot of my understanding over similarity and compatibility both in the mathematical and more personal sense (e.g. the idea that microbiomes can have more or less compatible compositions between hosts sharing bacteria through interaction). With Renee I have had many of the best scientific ideas in my life and losing her I have also lost a way of thinking that was unique to our discussions.

Working in Oxford University taught me, for the first time in my life, how much of a cooperative effort science actually is. Before moving to UK, I had felt that my research is my own business and only a burden to others, but in Oxford I have seen how great science only results from great cooperation and I want to thank all the amazing people that had made my research in Oxford possible. First huge thanks to my first friend in town, great supporter, landlord and inventor Curt Lamberth, without whose time and genius in building RFID tracking devices most of my research would have been impossible. I will forever warmly remember the countless Friday (and Saturday and Sunday and any other day) nights spent in Curt's backyard workshop winding copper coils to try create magnetic fields of different shape and oscillation frequency while blasting Amsterdam Klezmer Band and Hitch Hikers Guide to the Galaxy. Curt has a truly remarkable energy filled with fire, kindness and endless stamina (at least when sugar is provided). On top of spending considerable amounts of time building mouse-spying devices with me just for fun before we got funded, Curt also rescued me from a flooding apartment, gave me a house to live

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During my DPhil research I have probably wasted thousands of pounds due to my (unintentionally) bohemian style of laboratory work and all the fuck-ups that follow. Few things have made me as frustrated as contamination issues in the lab, and nothing has rescued me from this grumpiness as well as the emotional support of my lab-comrades. Big thanks to the ever so dynamic boys of the Physical Friendship Lab – Ash Sendell-Price, Michal Jezierski and Will Smith. Without you my epic lab failures would have been just as plentiful, but way more melancholic as I would not have gotten my own pony sticker in "The failure of the week" calendar every time. Thank you also to Jack Howe and Georgia Drew for support and jolly discussions and Kieran Bates for many great chats and accepting me to the "Wild Elegance" – research group.

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