Received: 24 October 2022 | Accepted: 5 May 2023

DOI: 10.1111/1365-2656.13961

Journal of Animal Ecology | BRITISH ECOLOGICAL SOCIETY

RESEARCH ARTICLE

Sexual transmission may drive pair similarity of the cloacal microbiome in a polyandrous species

Hanna Prüter[1] | Mark A. F. Gillingham[1,2] | Johannes Krietsch[1] | Sylvia Kuhn[1] | Bart Kempenaers[1]

[1]Department of Behavioural Ecology and Evolutionary Genetics, Max Planck Institute for Biological Intelligence, Seewiesen, Germany

[2]Biodiversity Research Institute (CSIC, Oviedo University, Principality of Asturias), University of Oviedo, Mieres, Spain

Correspondence
Hanna Prüter
Email: hanna.prueter@bi.mpg.de

Mark A. F. Gillingham
Email: mark.gillingham@bi.mpg.de

Bart Kempenaers
Email: bart.kempenaers@bi.mpg.de

Funding information
Max Planck Society

Handling Editor: Albert Phillimore

Abstract

1. All animals host a microbial community within and on their reproductive organs, known as the reproductive microbiome. In free-living birds, studies on the sexual transmission of bacteria have typically focused on a few pathogens instead of the bacterial community as a whole, despite a potential link to reproductive function. Theory predicts higher sexual transmission of the reproductive microbiome in females via the males' ejaculates and higher rates of transmission in promiscuous systems.
2. We studied the cloacal microbiome of breeding individuals of a socially polyandrous, sex-role-reversed shorebird, the red phalarope (*Phalaropus fulicarius*). We expected (i) higher microbial diversity in females compared to males; (ii) low compositional differentiation between sexes; (iii) lower variation in composition between individuals (i.e. microbiome dispersion) in females than in males; (iv) convergence in composition as the breeding season progresses as a consequence of sexual transmission and/or shared habitat use; and (v) higher similarity in microbial composition between social pair members than between two random opposite-sex individuals.
3. We found no or small between-sex differences in cloacal microbiome diversity/richness and composition. Dispersion of predicted functional pathways was lower in females than in males. As predicted, microbiome dispersion decreased with sampling date relative to clutch initiation of the social pair. Microbiome composition was significantly more similar among social pair members than among two random opposite-sex individuals. Pair membership explained 21.5% of the variation in taxonomic composition and 10.1% of functional profiles, whereas temporal and sex effects explained only 0.6%–1.6%. Consistent with evidence of functional convergence of reproductive microbiomes within pairs, some select taxa and predicted functional pathways were less variable between social pair members than between random opposite-sex individuals.

Hanna Prüter and Mark A. F. Gillingham contributed equally to the manuscript.

4. As predicted if sexual transmission of the reproductive microbiome is high, sex differences in microbiome composition were weak in a socially polyandrous system with frequent copulations. Moreover, high within-pair similarity in microbiome composition, particularly for a few taxa spanning the spectrum of the beneficial–pathogenic axis, demonstrates the link between mating behaviour and the reproductive microbiome. Our study is consistent with the hypothesis that sexual transmission plays an important role in driving reproductive microbiome ecology and evolution.

KEYWORDS
mating system, *Phalaropus fulicarius*, polyandrous, red phalarope, reproductive microbiome, sexual transmission, shorebird, social pairs

1 | INTRODUCTION

All vertebrates harbour trillions of microbes on their outer surfaces as well as inner mucous membranes, forming the so-called 'microbiome' (Berg et al., 2020). Several studies have shown that host health and behaviour depend on a well-balanced microbiome composition (Ezenwa et al., 2012; Hird, 2017; Parfrey et al., 2018) and that its function in metabolism and immune regulation is linked to host fitness (Gould et al., 2018; Shin et al., 2011). Thus, understanding mechanisms that drive community assembly of host microbiomes has attracted recent attention of both microbial and evolutionary ecologists (Costello et al., 2012; Coyte et al., 2021; DeLong, 2014; Verster & Borenstein, 2018).

Vertebrate organs are analogous to oceanic islands, whereby continuous events of microbial colonisation and extinction will shape community assembly as modelled by classical McArthur–Wilson island biogeography theory (Costello et al., 2012; DeLong, 2014; MacArthur & Wilson, 1967). These processes of colonisation and extinction are highly dynamic within a host's lifetime, because host microbial communities vary across space and time (e.g. Escallón et al., 2019; Gillingham et al., 2019; Hernandez et al., 2020; Skeen et al., 2021). Microbial colonisation success will depend on habitat suitability (i.e. the environment on or in the host's organs) and competition with already present taxa (known as priority effects; Costello et al., 2012; Coyte et al., 2021; DeLong, 2014; Verster & Borenstein, 2018). Social interactions that increase contact between hosts, such as mating behaviour, will also increase the potential for microbial transmission (i.e. colonisation; Sarkar et al., 2020).

An under-appreciated mechanism that may shift the microbial community assembly is reproductive transmission (Rowe et al., 2020). In many non-mammalian vertebrates, including birds, reptiles and amphibians, the cloaca is involved both in excretion (i.e. the distal part of the gut) and in gamete transfer. Thus, copulation may allow microorganisms present within reproductive organs (known as the 'reproductive microbiome') to colonise the reproductive and digestive tract, potentially impacting both reproductive function and gut homeostasis. While the interaction of the reproductive microbiome with fitness has traditionally been viewed through the lens of sexually transmitted pathogens (Lockhart et al., 1996; Price et al., 2010; Sheldon, 1993), which can have strong deleterious effects on fertility (van Dongen et al., 2019), considerably less attention has been given to the transmission of beneficial taxa (Rowe et al., 2020; Smith & Mueller, 2015). Yet, the transmission of beneficial taxa may improve reproductive outcomes. For example, in humans, vaginal eubiosis (a balanced microbiome) is associated with a dominance of *Lactobacillus*, which limits the growth of bacterial species, including pathogens, through the production of lactic acid (Tachedjian et al., 2017). Consequently, avoidance of mating partners transmitting pathogens, as well as mating with individuals transmitting beneficial microbes, will impact reproductive success and may even function as a driver of mating behaviour and sexual selection (Lombardo et al., 1999; Rowe et al., 2020; Sheldon, 1993; Smith & Mueller, 2015).

The spread of the reproductive microbiome within a population is likely driven by individuals with a high number of sexual partners (Ashby & Gupta, 2013), as shown in natural populations of common lizards (*Zootoca vivipara*; White et al., 2011) and tree swallows (*Tachycineta bicolor*; Dunn et al., 2009). The mating system is determined by the number of social and sexual partners of an individual. It has therefore been proposed that the reproductive microbiome's diversity and/or richness are linked to the mating system of the hosts, with high individual host diversity but low similarity between mating partners in the microbiome of non-monogamous compared to monogamous species (Rowe et al., 2020). Moreover, theory predicts that females will be more vulnerable to sexual transmission because of insemination of the male's ejaculate (Rowe et al., 2020). For instance, a study of socially monogamous captive zebra finches (*Taeniopygia guttata)* in which individuals were experimentally infected with *Bacillus licheniformes* showed that transmission of this bacterium was significantly higher from males to females than from females to males. This finding suggests bacterial transmission via the ejaculate and thus a crucial role of infected males in disease epidemiology in socially monogamous species (Kulkarni & Heeb, 2007). Similarly, in the socially monogamous rufous-collared sparrow (*Zonotrichia capensis*), temporal variation in cloacal microbiome composition was higher in males

during the breeding season (Escallón et al., 2019). In contrast, a study on socially monogamous, but highly promiscuous tree swallows found that the cloacal microbiome did not differ between the sexes and was not more similar for pair members than among two randomly chosen individuals of the population (Hernandez et al., 2020). However, sample sizes were relatively low (13 pairs) and the partners were not sampled at the same breeding stage, except for one pair, whose microbiomes were indeed more similar than expected by chance. Thus, shorter term convergence of the microbiome composition of social pairs during the stage of frequent copulation may have been overlooked.

Here, we report on a study of the role of sex and pair membership on the cloacal microbiome during the breeding season in the red phalarope (*Phalaropus fulicarius*), a socially polyandrous, sex-role-reversed species. Red phalaropes are highly pelagic, spending their entire life in marine habitats, except for a short time during breeding in the Arctic (Tracy et al., 2020). Several behavioural traits specific to the study species make this species particularly interesting to investigate the link between mating behaviour and the reproductive microbiome. First, we observed that both male and female red phalaropes copulate frequently as part of courtship, implying that both sexes might copulate with several potential partners before establishing a social pair bond. Second, both sexes engage in extra-pair copulations before and after egg-laying (Krietsch et al., 2022). Third, red phalaropes are non-territorial and can move large distances to visit different foraging sites and they potentially reproduce at several breeding sites within one season ('breeding site sampling', our unpublished data), as has been demonstrated for males of the polygynous pectoral sandpiper (*Calidris melanotos*; Kempenaers & Valcu, 2017), potentially increasing exposure to heterogeneous bacterial communities.

The main aim of our study is (1) to describe sex-differences in the taxonomic and in functional aspects of the cloacal microbiome in a population of red phalaropes and (2) to investigate whether pair members are more similar in their reproductive microbiome than expected by chance. Partially following the hypotheses outlined by Rowe et al. (2020) as well developing our own (Figure 1), we predict that in a socially polyandrous species: (i) microbial cloacal species diversity/richness will be higher in females than in males (Figure 1a, prediction 1), because (a) females potentially sample multiple breeding sites over a larger geographical area and (b) transfer of microorganisms from males to females (via ejaculates) is more likely than vice versa; (ii) as a consequence of sexual transmission of the microbiome, differentiation in cloacal microbiome composition between sexes will be low (Figure 1a, prediction 2); (iii) the variation in microbial composition between individuals (hereafter referred to as 'microbiome dispersion' but also known as 'multivariate dispersion', (Anderson, 2006; Anderson et al., 2006) and 'beta diversity dispersion'; see Zaneveld et al. (2017)) will be lower in females due to higher rates of sexual transmission through the males' ejaculate (Figure 1a, prediction 3); (iv) dispersion in microbial composition will decrease over the season due to sexual transmission and shared habitat use (Figure 1b); (v) the composition of the cloacal microbiome will be more similar in individuals that are socially paired (Figure 1c). Thus, for the latter, we tested whether beta diversity of social pairs is lower than that of two non-paired individuals sampled on the same day and around the same time (Figure 1c). We link taxonomic (amplicon sequencing variant; ASV) analyses with predicted MetaCyc functional pathways using the PICRUSt2 algorithm, an open-source database for metabolic pathways based on annotated genomes (Caspi et al., 2020; Douglas et al., 2020). Although this approach has its limitations, it does provide a powerful approach to infer hypothetical functional diversity of the microbiome (Djemiel et al., 2022).

2 | MATERIALS AND METHODS

2.1 | Study site and species

We studied a population of red phalaropes during the breeding season of 2019 in Utqiagvik (formerly Barrow), Alaska (71°18′ N, 156°44′ W). The first red phalaropes arrived when the tundra was still largely snow-covered and they started interacting and forming social pairs in small patches of open water. From the onset of pair formation until the start of egg laying, pair members copulated frequently. Typically, after the second egg was laid, the pair stopped copulating and some females started copulating with potentially new social partners (see Krietsch et al., 2022). Red phalaropes are highly mobile and individuals of both sexes—with unknown breeding history—arrived throughout the breeding season on the study site.

2.2 | Field procedures

Between 1 and 25 June, we captured 156 adult breeding red phalaropes (84 males, 72 females), using handheld mist nets. Individuals were caught either immediately after they arrived at the study site in feeding areas or near the nest. Each individual was banded with a U.S. Geological Survey metal band and a unique combination of colour bands (four colours), allowing individual identification during observations in the field. The sex of each captured individual was determined based on plumage characteristics (Tracy et al., 2020). In all cases, the assigned sex was confirmed with molecular methods (see Krietsch et al., 2022). We took a cloacal swab from all captured individuals, using a sterile cotton swab (DELTALAB). Swabs were immediately placed in an Eppendorf tube filled with RNA*later*® (Thermo Fisher). Within a few hours, samples were frozen at −20°C until further processing.

The 156 individuals belonged to a total of 97 nests. For 59 nests, we obtained cloacal microbiome samples from both partners, while for the remaining 38 nests, we only obtained data from one pair member (25 males, 13 females). A team of 2–10 people searched the study area to find nests, either by following males, females or pairs, or by flushing incubating males from the nest (accidentally or by rope dragging). For more detail on the nest searching and behavioural observations, see Krietsch et al. (2022).

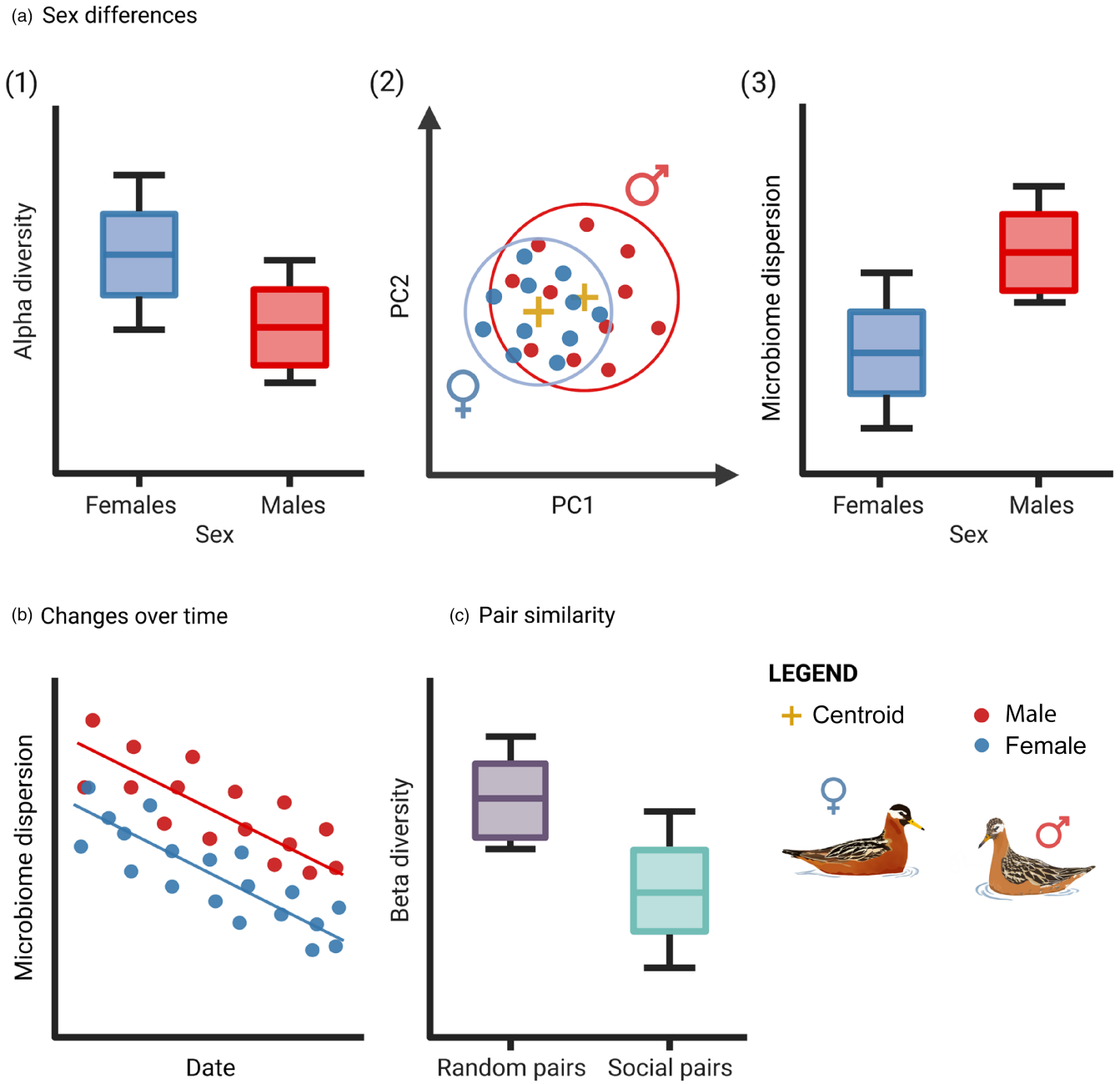

FIGURE 1 Predictions of how alpha diversity of the cloacal microbiome of Red Phalaropes varies in relation to (a) sex, (b) breeding season and (c) pair membership. (a) Sex differences in microbiome beta diversity. (1) If sexual transmission is higher in females via the males' ejaculates, a higher microbial alpha diversity in females than in males is predicted. (2) If sexual transmission is high at the population level due to promiscuity, weak differences in microbial composition between sexes are expected. PC1 and PC2 refer to the first and second principal component of an ordination analysis of the microbial composition of individuals using a beta diversity matrix (see Section 2). (3) If sexual transmission is higher in females than in males, microbiome dispersion (estimated as multivariate dispersion, Anderson, 2006; Anderson et al., 2006 and also known as beta diversity dispersion, Zaneveld et al., 2017; see Section 2) should be lower in females than in males. (b) A potential relationship between microbiome dispersion and time (in our case, sampling date relative to clutch initiation = 0; see Section 2). Microbiome dispersion should decrease over time (i.e. the population should become more homogeneous in microbiome composition) due to sexual transmission and shared habitat use. (c) Pair similarity in microbiome beta diversity. As a consequence of sexual transmission, microbiome composition should be more similar for social pairs than for random pairs (i.e. pairs of opposite sex individuals that do not breed together, but are sampled on the same day). Thus, beta diversity of social pairs should be smaller than that of random pairs (This figure was created with BioRender.com; the Red phalarope illustrations in this figure were made by Margherita Cragnolini).

We used the date of sampling relative to clutch initiation (clutch initiation date; zero is the day the first egg was laid, with negative and positive values indicating sampling dates before and after clutch initiation respectively). We predicted that sexual transmission may be an important factor driving cloacal microbiome composition between pairs. Thus, we used this metric as a temporal variable that

reflects the time since pair formation, assuming that date relative to clutch initiation was a good proxy for the number of days since pair formation. For example, we predict that a pair sampled 10 days before clutch initiation will have copulated much less with its mate compared to a pair sampled during egg-laying. Moreover, we also predicted that temporal effects on the cloacal microbiome will depend on the length of time an individual is exposed to the local environment. Given that there is individual variation in arrival date at the breeding site (Reynolds et al., 1986) and that birds that arrive later typically also initiate a clutch later, date of sampling relative to clutch initiation may better reflect the amount of time spent at the breeding site than the actual sampling date. Because the latter is rather uncertain, we repeated all analyses with the actual sampling date. Results were qualitatively similar (see Section 3; Tables S2 and S5). For nests that were found during laying (1–3 eggs), clutch initiation was determined by subtracting 1 day for each additional egg laid, assuming that one egg was laid per day. In nests that were found after clutch completion, we calculated clutch initiation date based on the flotation method that allows for the estimation of egg developmental stage (Liebezeit et al., 2007). For more details, see Krietsch et al. (2022).

2.3 | DNA isolation, amplification and sequencing

DNA extraction, PCRs, sample preparation and Illumina NGS Sequencing of the cloacal swabs were performed at Eurofins Genomic laboratory. DNA was extracted from all cloacal swab samples using a commercial extraction kit (Macherey Nagel, Nucleospin Food Kit). PCRs were carried out using primers that amplify a 443 bp sequence within the V3-V4 region of the 16S rRNA of a bacterial gene (357F-fw 5' TACGGGAGGCAGCAG 3′, 800R-rev 5' CCTAATCT ATGGGACC 3′; Kisand et al., 2002; Turner et al., 1999). Due to low sample amount and subsequent low DNA concentrations, PCRs were run with 35 cycles. Amplicons were then sequenced using Illumina MiSeq. All collected samples as well as four negative controls from the PCRs (blanks) were included. Sequences from the blanks were used to exclude buffer contaminants from the data in downstream analyses (see Section 2.4).

2.4 | Bioinformatics

To determine the microbiome composition, we used DADA2 (Callahan et al., 2016) in QIIME2 (Version 2019.7; Bolyen et al., 2019) to denoise the data from artefacts (e.g. chimeras) and resolve assigned sequence variants (ASV). We assigned ASVs to taxonomy using a classifier trained with our primers on the Silva database (v132; Pruesse et al., 2007) as a reference in QIIME2. Sequences not assigned to any bacterial phylum were filtered out within the QIIME2 downstream analysis. We routed the tree using an Archean sequence (accession number: KT433146.1) and aligned the fasta file using MAFFT (Katoh & Standley, 2013). The taxonomic name and branch of the route Archean sequence was removed before further analysis. The data and rooted tree were imported to R Version 4.2.2 (R Core Team, 2022) using the R package 'phyloseq' (McMurdie & Holmes, 2013). Four negative control samples were used to detect contaminants which were removed from samples using the R package 'decontam' (Davis et al., 2018) with a threshold of 0.5.

To predict functional profiles of the sequencing data (16S rRNA), we used the PICRUSt2 pipeline with the default parameters (Douglas et al., 2020). Enzyme classification numbers (ECs) and pathway abundances were assigned to the 16S rRNA sequences based on enzyme data from the open-source MetaCyc database. This database contains metabolic pathways as well as associated enzymes, metabolites and reactions which are based on organismal annotated genomes (Caspi et al., 2020). Information on the predicted functional pathways, which are not cited in particular in the text, arise from the MetaCyc website (Caspi et al., 2020).

2.5 | Statistical analysis

All analyses were run in R Version 4.2.2 (R Core Team, 2022). For all models, we checked for violation of model assumptions as suggested by Zuur et al. (2009) and Zuur and Ieno (2016). Additive models were used to test for nonlinear relationships, but if none were found, we reverted to linear models. We used the information-theoretic (IT) approach for model selection (Burnham & Anderson, 2002). We assessed all possible candidate models (i.e. possible hypotheses) by ranking them based on the model's Akaike information criterion for small sample sizes (AICc), AICc weight and adjusted-R^2 values using the R package MuMIn (Bartoń, 2022). We estimated effect sizes for linear continuous and categorical variables using Pearson *r* and Cohen's *D*, respectively, with associated 95% confidence intervals (Nakagawa & Cuthill, 2007).

2.5.1 | Alpha diversity

Alpha diversity describes the number of species within a certain habitat, which in our case is the number of bacterial ASVs or the number of predicted MetaCyc functional pathways in the cloaca. We used two measures of alpha diversity: (1) the unweighted observed species richness, which is the number of ASVs per sample (hereafter 'ASV species richness') and the number of predicted MetaCyc functional pathways per sample (hereafter 'pathway species richness') and (2) the Shannon index (hereafter 'ASV Shannon' and 'pathway Shannon'), which is species/pathway richness weighted for species/pathway abundance (Shannon & Weaver, 1949). We calculated species richness and Shannon diversity using the R package 'phyloseq' (McMurdie & Holmes, 2013).

First, we predicted that microbiome alpha diversity depends on an individual's sex and sampling date relative to clutch initiation. To model these effects, we fitted generalised linear mixed models (GLMMs) using the R package 'lme4' (Bates et al., 2015). For the

response variables ASV species richness, we used a GLMM with a Poisson distribution and added sex and sampling date relative to clutch initiation as fixed effects and the identity of the pair (hereafter 'pair ID') as a random factor. In addition, we also added individual sample ID as a random factor to control for overdispersion (Harrison, 2014), and sequencing depth (log transformed) as a fixed effect to control for the methodological fact that at higher sequencing depths, more ASVs will be detected. All continuous fixed effects were scaled. For the pathway species richness response variable, the same explanatory variables were used but the GLMM was fitted with a negative binomial distribution and pair ID was entered as the sole random factor. For the response variables ASV and pathway Shannon, we adopted the identical model approach, but using a GLMM with Gamma distribution and using pair ID as the sole random factor instead.

Second, we predicted that individuals with a higher microbiome diversity transmit more ASVs/pathways to their partner and vice versa. If so, the variation in alpha diversity will be smaller within social pairs than across pairs. To investigate this, we estimated the within-pair repeatability of alpha diversity based on the top-ranked GLMM models for both species richness and Shannon (Nakagawa & Cuthill, 2007) using the R package 'rpTR' (Stoffel et al., 2017). For this analysis, we used a dataset that only included nests where both partners had been sampled ($n = 56$ pairs).

2.5.2 | Beta diversity

Microbiome beta diversity is a measure of similarity or dissimilarity between two microbial communities (i.e. among-sample variation). Here, beta diversity is estimated to compare the cloacal microbiome composition (at the ASV and predicted MetaCyc functional pathway level) between individuals. Generally, microbiome data should be treated as compositional data because absolute bacterial loads are unknown, meaning that they should be analysed based on relative abundances (Gloor et al., 2017; Martino et al., 2019; Quinn et al., 2018, 2019). To estimate beta diversity, we therefore applied the Aitchinson's log-ratio approach (Aitchison, 1982), which is commonly used in microbiome studies (Gloor et al., 2017). The latter involves transforming the taxa counts within each sample with centred log ratios (clr) and using the Euclidean distance matrix across samples for ordination analyses (Gloor et al., 2017). The clr transformation is independent of absolute abundance and a clr value for a given taxon in an individual represents the abundance relative to the geometric mean of all taxa within a sample. Consequently, a sample with few reads but with an identical community to a sample with many reads will have an identical clr value. Thus, count normalisation is both unnecessary and not advised when using clr (Gloor et al., 2017).

First, we predicted that microbiome composition may differ according to an individual's sex and sampling date relative to clutch initiation. We applied redundancy analysis (RDA) with the Euclidean distance matrix to study the total amount of variation explained by sex and sampling date on beta diversity. For RDA analyses, we only included ASVs which occurred in more than 20 individual samples to avoid sparse and zero-inflated data due to rare ASVs, which can bias interpretations (Martino et al., 2019; Quinn et al., 2018, 2019). However, RDA analyses were repeated with prevalence thresholds of <2 and <77 (i.e. 50%), which gave quantitatively identical results (results not shown but see online script https://gillinghamlab.gitlab.io/mpi/red-phalarope-microbiome/). The latter is consistent with a previous study showing that varying prevalence thresholds (between 0% and 70%) generated similar trends in standardised alpha diversity and beta dissimilarity across eight gut and cloacal microbiome datasets (Risely et al., 2021). *p*-Values were estimated by permutation ($n = 9999$) and were reported alongside associated *F*-values and adjusted R^2 values.

Second, we hypothesised that after arrival at the breeding site, the variation in microbiome composition between individuals (i.e. microbiome dispersion, Zaneveld et al., 2017) first decreases and then stabilises. Microbiome dispersion is presumably highest just after arrival, because individuals likely differ in migration history and in exposure to diverse marine and tundra habitats. Microbiome dispersion should decrease as the breeding season progresses as the result of increased homogenisation due to copulation activity and similar environmental conditions, including foraging habitat. We further predicted that the rate of such homogenisation may differ between the sexes, because the females' cloacal microbiome is more exposed to sexual transmission. To test these predictions, we used the Aitchinson's log-ratio approach for compositional data on the full dataset (i.e. no prevalence threshold). Microbiome dispersion (also known as beta diversity dispersion, Zaneveld et al., 2017) was estimated as the multivariate dispersion, which is the average distance of each sample from the spatial median in a multivariate space (i.e. the median after reducing the original pairwise Euclidean distances to principal coordinates; Anderson, 2006; Anderson et al., 2006). The latter was calculated using the 'betadisper' function of the vegan package (Oksanen et al., 2022). We then tested the effect of sex, sampling date relative to clutch initiation and their interaction on microbiome dispersion using a linear mixed model (LMM) with pair ID as random factor. This test is a multivariate analogue of Levene's test for homogeneity of variances (Anderson et al., 2006).

Third, we predicted that microbiome composition will be more similar within social pairs than across pairs. If so, beta diversity should be smaller between social pair members than between two random opposite-sex individuals in the population (see Figure 1c). Almost all social pairs (44 of 59) were sampled on the same day and within a short period (mean: 26 min, range: 0–313 min). For 15 social pairs, the partners were captured on different days (median: 4 days apart, range: 1–9 days). To account for the effect of sampling date, we estimated beta diversity between random opposite-sex individuals that were sampled on the same day with the smallest time interval ($n = 104$) or 2 days apart ($n = 9$). Because the data were normally distributed, we used a paired *t*-test to assess whether beta diversity differed between social pairs and two random opposite-sex individuals sampled around the same time. Results were equivalent when excluding the latter nine individuals from the analysis (results not

shown but see online script https://gillinghamlab.gitlab.io/mpi/red-phalarope-microbiome/).

If microbiome convergence (transmission) between social pairs is gradual with time, we predict that social pairs that were sampled on a different day to have more similar microbiomes relative to opposite-sex individuals than social pairs that were sampled at the same time. Alternatively if microbiome convergence occurs instantly after copulation, then we predict that pairwise similarity in microbiome composition to be of a similar level of magnitude relative to random individuals regardless of whether social pairs were sampled on the same day or not. To test this, we repeated our analysis using only the 15 social pairs sampled on a different day and compared the effect size with an analysis using the remaining pairs that were sampled on the same day ($n=42$).

If social pairing (copulations) drive pair microbiome composition, then pair ID should be an important predictor of cloacal microbiome composition. We therefore repeated the RDA analysis described above on a dataset with the 59 pair IDs with both sexes sampled ($n=118$ individuals), but with pair ID as an explanatory variable in addition to the additive effect of sex and sampling date relative to clutch initiation. As above, we repeated these RDA analyses with two additional prevalence thresholds: <2 and <77 individuals (i.e. 50%).

2.5.3 | Relative abundance of specific ASVs and predicted functional pathways

We analysed the relative abundance of ASVs and predicted functional pathways using analysis of composition of microbiome (ANCOM; Mandal et al., 2015) on the same dataset as used for the RDA analyses of beta diversity (compositional data and only ASVs included that occur in at least 20 individuals). ANCOM generates a *W* score, which is the number of rejected null sub-hypotheses of the log ratios of ASVi/ASVj for a given predictor. Higher *W* scores indicate more support for an effect of the predictor on the abundance of the specific ASV. Moreover, ANCOM analysis controls for false discovery rates by applying a Benjamini–Hochberg procedure (Mandal et al., 2015). To illustrate the results, we present volcano plots of the ANCOM *W* score in relation to the LMM estimates controlling for the other covariates and the random factor pair ID. For this analysis, we used only those variables that showed a significant effect in the RDA beta diversity analyses (sex, sampling date relative to clutch initiation and nest ID). We first investigated differential relative abundance according to sex and sampling date relative to clutch initiation. We considered ASVs to be differentially abundant according to a given predictor if at least 70% of the subhypotheses were rejected ($W>0.7$) and if the 95% confidence interval of the LMM estimates did not overlap 0 (differential logs (clr)). Second, if sexual transmission of some cloacal bacteria is relevant in red phalaropes, the relative abundance of ASVs and predicted functional pathways will harmonise between sexual partners. To investigate whether variation in relative abundance of ASVs are more similar within pairs than across pairs (while accounting for the effect of sex and sampling date relative to clutch initiation), we also tested for an effect of nest ID. Here, thresholds were a *W* score of 0.7 and a significant *F*-value ($p<0.05$) for the random factor nest ID in the LMM models.

3 | RESULTS

The 156 cloacal samples from 84 male and 72 female red phalaropes contained a total of 6380 sequence variants (ASVs) belonging to seven phyla. In order of descending relative abundances, these ASVs belonged to the phyla Bacillota (formerly Firmicutes), Actinomycetota (formerly Actinobacteria), Fusobacteriota, Pseudomonadota (formerly Proteobacteria), Mycoplasmatota (formerly Tenericutes), Epsilonbacteraeota and Bacteroidota (formerly Bacteroidetes), which is a typical cloaca microbiome composition at the phylum level for birds and in particular for shorebirds (Grond et al., 2018; Figure S1). As reported in other avian microbiomes (Risely et al., 2021), most ASVs (93.6%) occurred only in a single individual.

3.1 | Effects of sex and sampling date relative to clutch initiation on the cloacal microbiome

We found no support for the hypothesis that sex and sampling date relative to clutch initiation predicted ASV species richness or Shannon diversity of the cloaca microbiome (Table S1). We did find an effect of the interaction between sex and sampling date on Shannon diversity of predicted functional pathways ($\Delta AIC=2.772$; Table S4; Figure 2a). However, the effects of sex and sampling date on Pathway Shannon diversity were weak. Pathway Shannon diversity tended to decrease with sampling date relative to clutch initiation in males (Pearson $r=-0.177$ [−0.377; 0.039]) but not in females (Pearson $r=0.134$ [−0.101; 0.354]). Overall, males also had a higher Shannon diversity of predicted functional pathways than females ($\Delta AIC=8.466$; Table S4; Figure 2b.; Cohen's D [±95% CI]$=0.187$ [−0.131; 0.505]). When repeating the analysis using actual sampling date instead of sampling date relative to clutch initiation, we found roughly equivalent results (Tables S2 and S5). However, we additionally found weak support that ASV Shannon diversity decreased with sampling date in males but not in females ($\Delta AIC=2.354$; Table S2; Figure S2; Pearson $r_{males}=-0.123$ [−0.329; 0.094]; Pearson $r_{females}=0.003$ [−0.229; 0.234]).

Sex was a significant predictor of cloacal microbiome beta diversity, although again the effects were small (Figure 3a,c; Tables 1 and 2; ASV RDA: $F_{1,\ 152}=2.929$, $p=0.002$; $R^2_{adj}=0.012$; Pathway RDA: $F_{1,152}=1.880$, $p=0.029$; $R^2_{adj}=0.006$). ANCOM analysis revealed that this effect was mainly driven by the higher relative abundance of an ASV belonging to the *Enterorhabdus* genus in females (Figure 3b) and by the higher relative abundance of the *CMP-legionaminate biosynthesis I* predicted pathway in males (Figure 3d). Sampling date relative to clutch initiation had a stronger effect on cloacal microbiome

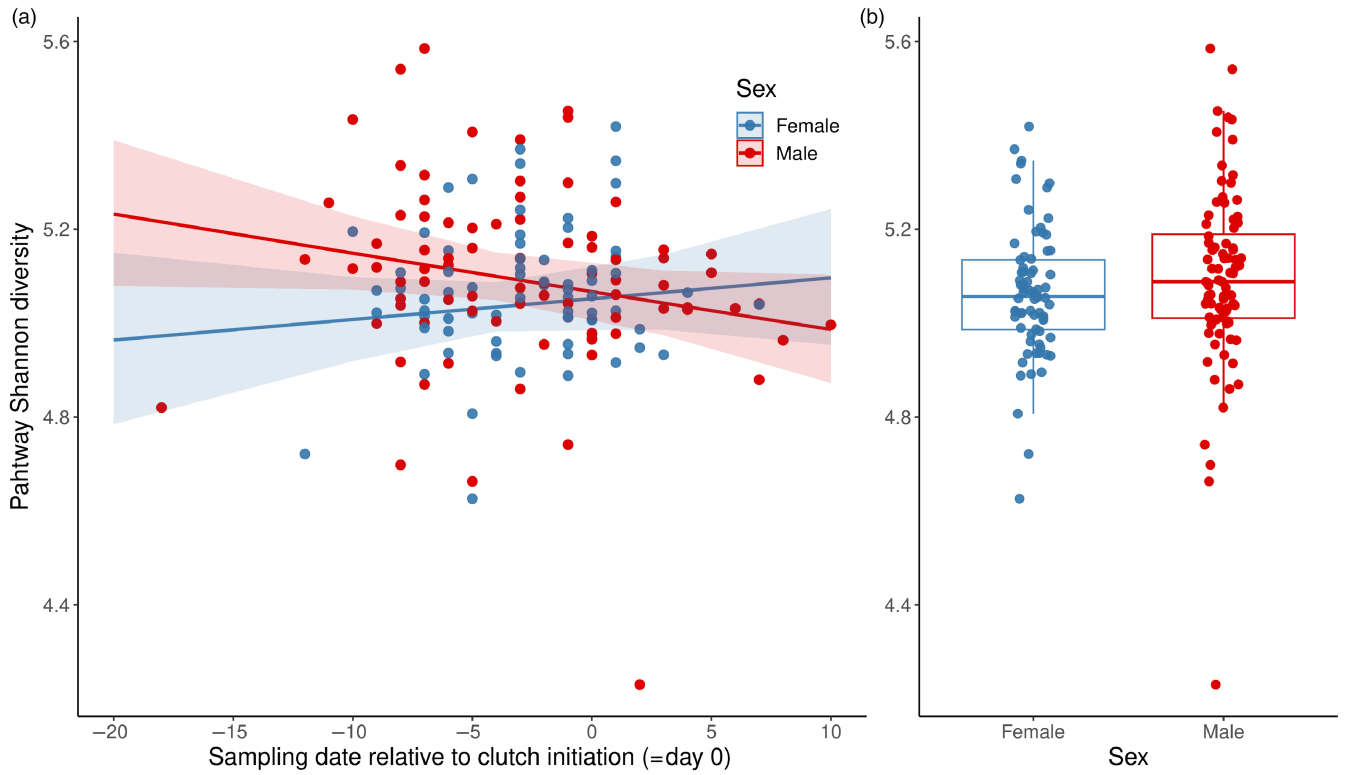

FIGURE 2 Pathway Shannon diversity as a function of sex and time. (a) Estimates and 95% confidence intervals based on a generalised linear mixed model (see Section 2). (b) Sex differences in diversity. Shown are boxplots and the raw data.

beta diversity (Tables 1 and 2; ASV RDA: $F_{1,\ 152}=3.542$, $p<0.001$, $R^2_{adj}=0.016$; Pathway RDA: $F=3.533$, $p<0.001$, $R^2_{adj}=0.016$), and this effect was independent of sex (Tables 1 and 2).

We found equivalent results when repeating the RDA analyses using a dataset with a single individual per nest to control for the potential effects of nest sampling pseudoreplication (Table S3 and S6). Using actual sampling date instead of date relative to clutch initiation also gave equivalent results but with slightly lower explanatory power (ASV RDA: $F_{1,\ 152}=3.077$, $p=0.002$; $R^2_{adj}=0.013$; Pathway RDA: $F_{1,\ 152}=2.155$, $p=0.017$; $R^2_{adj}=0.007$). ANCOM analyses indicated that two ASVs, belonging to the genera *Campylobacter* and *Tyzzerella* decreased in relative abundance over time of sampling relative to clutch initiation, while one ASV belonging to the *Cetobacterium* genus increased over time (Figure 4a). In total, 14 pathways increased in relative abundance over time of sampling relative to clutch initiation while three decreased (Figure 4b).

We found support for an effect of sampling date relative to clutch initiation on ASV microbiome dispersion from the population median (ΔAIC = 8.197; Table 3; Figure 5a), but there was no effect of sex (Table 3). Microbiome dispersion tended to decrease with time (Pearson $r=0.256$ [0.103; 0.397]; Figure 5a). Pathway microbiome dispersion decreased with sampling date relative to clutch initiation in both sexes (ΔAIC = 6.271; Figure 5c; Table 4; Pearson $r=-0.190$ [−0.337; −0.034]) and was overall lower for females than for males (ΔAIC = 2.814; Figure 5b; Table 4; Cohen's $D=0.349$ [0.030; 0.669]). When repeating the analysis using actual sampling date instead of sampling date relative to clutch initiation, results were qualitatively similar, but models had a weaker fit (Tables S7 and S8; Figure S3).

3.2 | Social pair similarity in cloacal microbiome composition

The within-pair repeatability in ASV and pathway alpha diversity was low (ASV species richness: $R=0.166$ [0; 0.396], $p=0.176$; ASV Shannon diversity: $R=0.204$ [0; 0.445], $p=0.052$; Pathway species richness: $R=0.153$ [0; 0.375], $p=0.117$; ASV Shannon diversity: $R=0.079$ [0; 0.316], $p=0.258$), suggesting that social pairing had a weak effect on individual microbial diversity.

ASV beta diversity was lower for social partners than across other opposite-sex individuals, indicating a higher similarity in cloacal microbiome assemblage within social pairs (Figure 6a; Cohen's D [±95% CI] = −0.332 [−0.596; −0.068]; paired t-test: $t_{112}=-3.439$; $p<0.001$). When controlling for the effects of sex and time, we found that pair ID was a strong predictor of ASV cloacal composition (ASV RDA: $F_{58,\ 118}=1.708$, $p<0.001$; $R^2_{adj}=0.261$; Figure S4A). Increasing the filtering threshold increased the amount of variation explained by pair ID from 21.5% to 29.4% (ASV RDA; prevalence threshold <2 individuals: $F_{58,\ 118}=1.550$, $p<0.001$; $R^2_{adj}=0.215$; prevalence threshold <50%: $F_{58,\ 118}=1.708$, $p<0.001$; $R^2_{adj}=0.294$). This suggests that similarity in composition within pairs was higher for core microbial communities (i.e. highly prevalent taxa) than for rarer taxa (i.e. potentially transient).

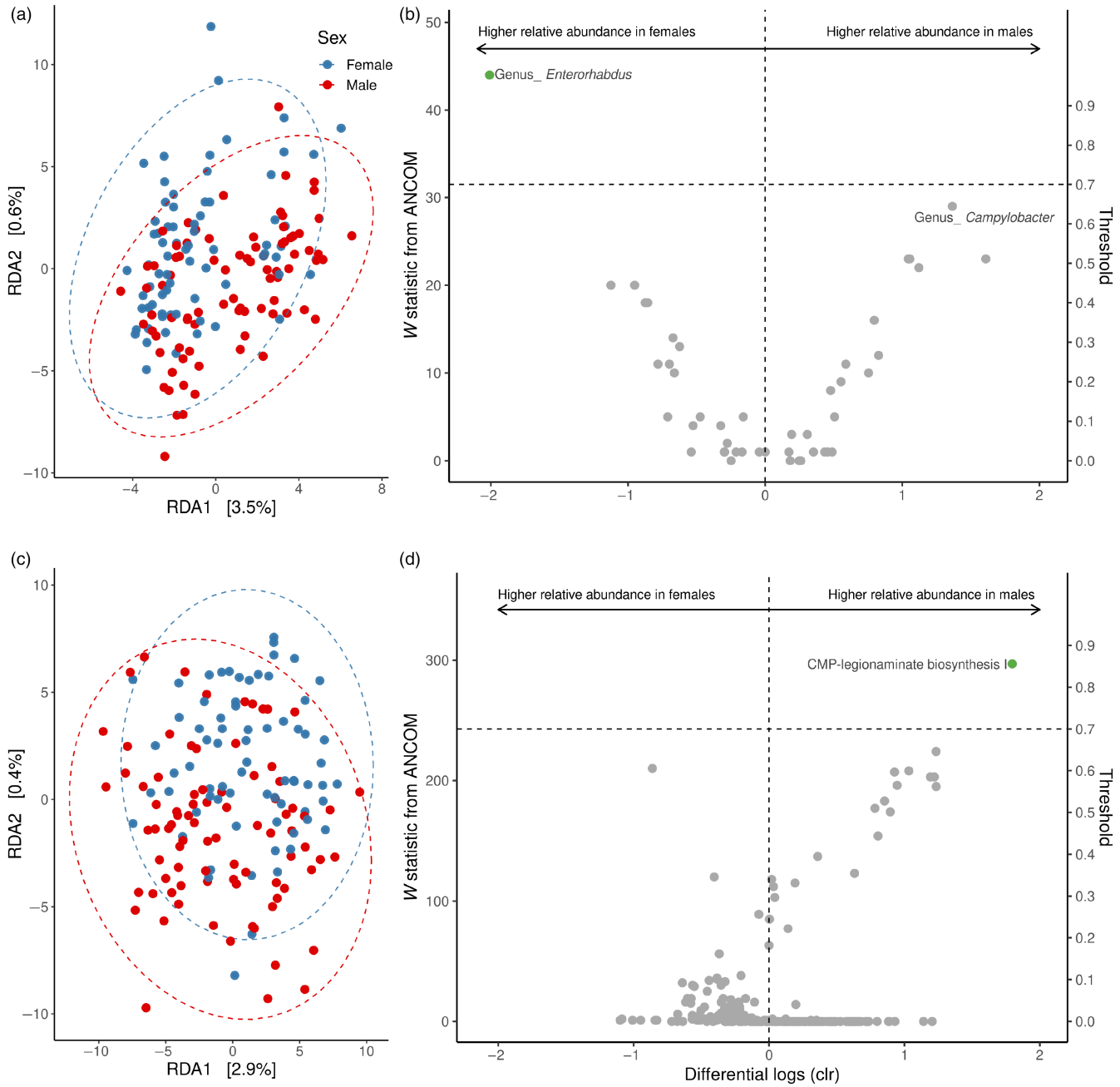

FIGURE 3 Redundancy analysis (RDA) ordination plot of amplicon sequencing variant (ASV) (a) and predicted MetaCyc functional pathway (c) of cloacal microbiome beta diversity as a function of the sex of red phalaropes sampled during the breeding season (see Section 2 for details). Ellipses depict 95% confidence intervals. The effect of sex on the relative abundance of particular ASVs (b) and of predicted MetaCyc functional pathways (d) is shown by volcano plots, based on an analysis of composition of microbiomes (ANCOM; see Section 2 for details) according to differential log estimates of centred logged ratios (clr) from linear mixed models. Dots represent individual ASVs or predicted pathways. Those that are above the ANCOM *W* threshold of 0.7 are coloured green.

ANCOM analyses revealed that 10 ASVs had larger variation across social pairs than within (Figure 6b), suggesting higher transmission between pairs for these taxa. The effect of social pairing on the cloacal microbiome assemblage was weaker and marginally not statistically supported for predicted pathway beta diversity (Figure 6c; Cohen's *D* [±95% CI] = −0.199 [−0.461; 0.064]; paired *t*-test: $t_{112} = -1.779$; $p = 0.078$), suggesting that the effect of social pairing on the assemblage of cloacal microbes may be dampened by functional redundancy between different taxa. Nonetheless, pair ID was a strong predictor of variation of predicted pathways' cloacal composition (Pathway RDA: $F_{58,\ 118} = 1.231$, $p = 0.002$; $R^2_{adj} = 0.104$; Figure S4B) and four predicted pathways showed larger variation between random opposite-sex individuals than between social pair members (Figure 6d). As for ASVs above, increasing the prevalence filtering threshold of predicted pathways increased the amount of variation explained by pair ID from 10.1% to 13.4% (Pathway RDA;

TABLE 1 Analysis of variation in cloacal microbiome amplicon sequence variant beta diversity in red phalaropes during the breeding season. Shown are changes in fit if an explanatory variable is dropped from the full redundancy analysis model. See Section 2 for further details. Date refer to the sampling date relative to clutch initiation (=day 0).

Dropped variable	df	*F*	*p*	R^2_{adj}
Date	1	3.542	<0.001	0.016
Sex	1	2.929	0.002	0.012
Date × sex	1	0.785	0.697	0.001

TABLE 2 Analysis of variation in cloacal microbiome predicted functional pathway beta diversity in red phalaropes during the breeding season. Shown are changes in fit if an explanatory variable is dropped from the full redundancy analysis model. See Section 2 for further details. Date refer to the sampling date relative to clutch initiation (=day 0).

Dropped variable	df	*F*	*p*	R^2_{adj}
Date	1	3.533	<0.001	0.016
Sex	1	1.880	0.029	0.006
Date × sex	1	0.897	0.522	<0.001

prevalence threshold <2 individuals: $F_{58,\ 118}=1.224$, $p=0.002$; $R^2_{adj}=0.101$; prevalence threshold <50%: $F_{58,\ 118}=1.313$, $p=0.002$; $R^2_{adj}=0.134$). This result is consistent with social pairing also affecting common predicted functional pathways.

We found an equivalent effect size when repeating the analysis using a subset of the data including only pairs that were sampled on different days (ASVs: Cohen's *D* [±95% CI] = −0.390 [−0.916; 0.136]; paired *t*-test: $t_{28}=-1.813$; $p=0.081$; Pathways: Cohen's *D* [±95% CI] = −0.217 [−0.742; 0.309]; paired *t*-test: $t_{28}=-1.094$; $p=0.321$) and when including pairs that were sampled on the same day (ASVs: Cohen's *D* [±95% CI] = −0.315 [−0.620; −0.010]; paired *t*-test: $t_{83}=-2.907$; $p=0.005$; Pathways: Cohen's *D* [±95% CI] = −0.279 [−0.496; 0.287]; paired *t*-test: $t_{28}=-1.459$; $p=0.114$).

4 | DISCUSSION

While theory predicts higher microbial transmission in promiscuous species and from males to females (Rowe et al., 2020), evidence that the reproductive microbiome is sexually transmitted in wild populations remains scarce. To address this issue, we studied the reproductive microbiome of a socially polyandrous shorebird species, the red phalarope, during the breeding season. We found that differences in the reproductive microbiome between breeding females and males are small, but that females showed more homogeneous functional profiles compared to males. Regardless of the sex, microbial composition became more similar among individuals as the season progressed. Finally, we found higher similarity in bacterial composition in the reproductive microbiome of social pairs than in random pairs. Social pair membership explained 21.5% of the variation of cloacal ASV composition. This effect was driven by a few select taxa with more similar relative abundance within pairs than across random pairs. Combined, these results suggest sexual transmission of the reproductive microbiome, particularly in females. We discuss the implications of these findings in terms of reproductive transmission and its interaction with mating behaviour below.

4.1 | Sex differences in the cloacal microbiome

Rowe et al. (2020) predicted that promiscuity should promote reproductive microbial diversity. Thus, we predicted that in our socially polyandrous system, females should have higher cloacal microbial diversity and richness (Figure 1a; prediction 1). However, as reported for monogamous birds (Kreisinger et al., 2015), males and females of the red phalarope did not differ substantially in alpha diversity of the cloacal microbiome, estimated as species richness and by Shannon index. Consistent with our predictions, we found that the evenness of predicted functional pathways (based on the MetaCyc database) tended to increase as the season progressed in females, in contrast to males for which it tended to decrease. Nonetheless, overall the effects were weak and, contrary to predictions, females tended to have lower pathway evenness than males. While repeated sampling within a breeding season of both males and females could lead to different conclusions, our current results are inconsistent with the prediction that high promiscuity increased reproductive microbial diversity. These results may be explained if most of the sampled females did not (yet) copulate with multiple males. Almost all samples were taken before or at the start of laying with the first known social partner (i.e. in the study site). Moreover, compared to many other socially monogamous birds, the level of extra-pair paternity is relatively low (11% of broods contained extra-pair sired offspring, Krietsch et al., 2022). Another explanation for this result is that cloacal microbiomes are already at full carrying capacity and that potential shifts in microbial composition that results from copulation do not affect diversity.

We additionally predicted that in a polyandrous system with frequent copulation during courtship, differences in microbiome composition between sexes should be low as a consequence of high sexual transmission of the reproductive microbiome. Overall, the effect of sex on composition was weak and largely driven by a single ASV belonging to the *Enterorhabdus* genus which was in higher relative abundance in females. Nonetheless, the weak differences between sexes did appear to have a knock-on effect on predicted function, since the functional pathway *CMP-legionaminate biosynthesis I* had a higher relative abundance in males compared to females. This pathway describes the biosynthesis of 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-nonulosonic acid, a sialic acid of α-keto sugars. Sialic acids are important mediators for cellular interactions in prokaryotes and eukaryotes. These molecules are involved in immune evasion and host cell invasion of pathogens and thus influence the virulence of pathogenic microorganisms. Indeed,

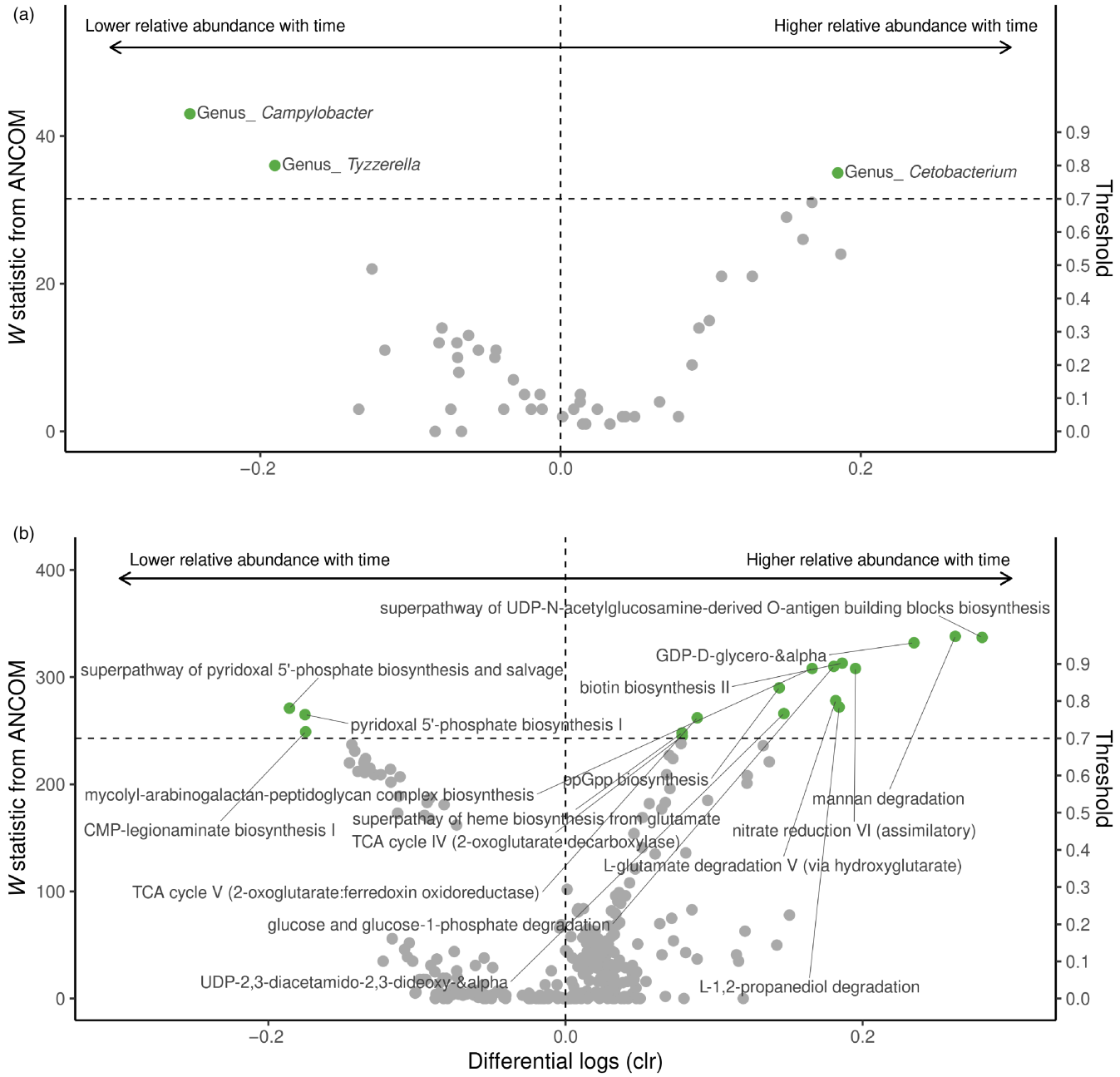

FIGURE 4 Effect of sampling date relative to clutch initiation on the relative abundance of amplicon sequencing variants (ASVs) (a) and of predicted MetaCyc functional pathways (b) is shown by volcano plots, based on an analysis of composition of microbiomes (ANCOM; see Section 2 for details) according to differential log estimates of centred logged ratios (clr) from linear mixed models. Dots represent individual ASVs or predicted pathways. Those that are above the ANCOM *W* threshold of 0.7 are coloured green.

the *CMP-legionaminate biosynthesis I* pathway has been shown to be an important pathway for *Campylobacter* sp. such as the pathogenic *C. jejuni* (Schoenhofen et al., 2009). An ASV of *Campylobacter sp.* showed higher relative abundance in males, but the effect remained just below the 0.7 threshold in the ANCOM analysis (Figure 3b). The more abundant predicted *CMP-legionaminate biosynthesis I* pathway in males could be linked to a higher abundance of virulent *Campylobacter* taxa in males compared to females.

We predicted that microbiome dispersion will be lower in females due to higher rates of sexual transmission (Figure 1a, prediction 3). While we found no evidence for this at the taxonomic level, at the functional level, microbiome dispersion was lower in females than in males regardless of sampling date (relative to clutch initiation). This result is consistent with high sexual transmission eroding among individual variation in microbial composition and its associated predicted functional pathways in females. Knock-on fitness implications remain unclear, but if sexual transmission of detrimental taxa is more likely from males to females and there are sex-specific reproductive costs, then sex differences in the transmission of the reproductive microbiome

TABLE 3 Candidate linear mixed model of the effect of sex, sampling date relative to clutch initiation (Date) and their interaction on amplicon sequence variant microbiome dispersion from the population median. The inclusion of a term in a model is denoted as '+'. Model rank, degrees of freedom (df), log-likelihood (LogLik), Akaike information criterion for small sample sizes (AICc) and AICc weight (ω) are shown. Effect sizes are given for variables in the top-ranked model.

Model rank	Date	Sex	Date × sex	df	LogLik	AICc	ΔAICc	ω
1	+			4	−533.523	1075.312	0.000	0.520
2	+	+		5	−532.857	1076.115	0.803	0.348
3	+	+	+	6	−532.857	1078.277	2.966	0.118
4				3	−538.676	1083.509	8.197	0.009
5		+		4	−538.029	1084.323	9.012	0.006
Effect size [95% CI]	$r = -0.256$ [−0.397; −0.103]							

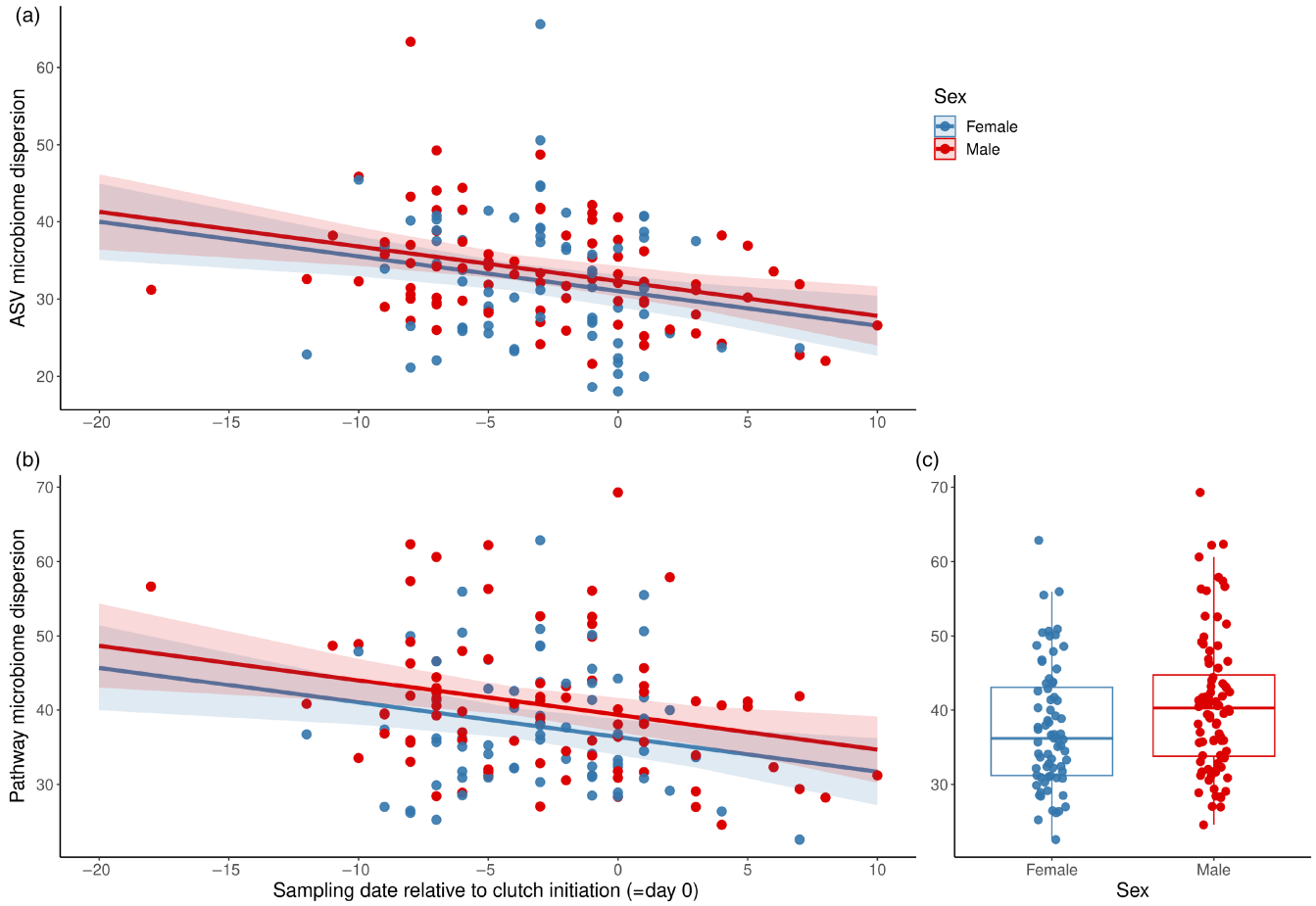

FIGURE 5 The effect of sex and sampling date relative to clutch initiation on amplicon sequencing variant (ASV) microbiome dispersion (a) and on predicted pathway microbiome dispersion (b, c). Shown are the fits from linear mixed models along with 95% confidence intervals (a, b) and boxplots (with raw data) as a function of sex (c).

may drive the evolution of sexually antagonistic strategies (Rowe et al., 2020). Empirical evidence of the interaction between the male's ejaculate and the female's reproductive microbiome comes from studies demonstrating antimicrobial properties of seminal fluid and immunity-related proteins (see Rowe et al., 2020; Smith & Mueller, 2015 and references therein). Such antimicrobial properties may protect male sperm from the harmful effects of bacteria on motility, but may also be detrimental to beneficial taxa in the female reproductive tract. In species with a cloaca, such as birds, disruption of microbial communities from male ejaculates may further extend into the female's digestive tract (i.e. the gut). This would generate sexual conflict where males would be under selective pressure to inseminate seminal fluid with protective antimicrobial properties that counteract female mechanisms to

TABLE 4 Candidate linear mixed model of the effect of sex, sampling date relative to clutch initiation (Date) and their interaction term on predicted functional pathway microbiome dispersion from the population median. The inclusion of a term in a model is denoted as '+'. Model rank, degrees of freedom (df), log-likelihood (LogLik), Akaike information criterion for small sizes (AICc) and AICc weight (ω) are shown. Effect sizes are given for variables in the top-ranked model.

Model rank	Date	Sex	Date × sex	df	LogLik	AICc	ΔAICc	ω
1	+	+		5	−555.428	1121.256	0.000	0.56
2	+	+	+	6	−554.953	1122.470	1.214	0.304
3	+			4	−558.140	1124.546	3.289	0.108
4		+		4	−559.631	1127.527	6.271	0.024
5				3	−562.213	1130.583	9.327	0.005
Effect size [95% CI]	$r = -0.220$ [−0.364; −0.065]	Cohen's $D = 0.349$ [0.030; 0.669]						

maintain reproductive microbial homeostasis, while females will be under selection to counteract the harmful effects of ejaculate antimicrobial properties (Rowe et al., 2011, 2020). For instance, upregulation of the female's immune system post-mating appears to be widespread (Morrow & Innocenti, 2012) and one potential mechanism may include the selective sculpting of protective taxa against pathogens by the immune response (Hooper et al., 2012; Montero et al., 2021; Stagaman et al., 2017). However, evidence for such mechanisms in birds remains scarce and experimental studies are needed to elucidate if sexual transmission of microbiomes results in sex-specific fitness outcomes.

4.2 | Temporal effects on the cloacal microbiome

The host–microbiome should be viewed as the outcome of a constant interaction between host control and colonisation from the environment. The effect of the environment is known to dominate cloacal microbiome composition in early host development, but diminishes in adults because host control starts to play a greater role in maintaining homeostasis (Carranco et al., 2022; Chen et al., 2020; Grond et al., 2017; Teyssier et al., 2018; Videvall et al., 2019; White et al., 2011). Nonetheless, the cloacal microbiome is known to be temporarily dynamic in breeding adults within a season (Escallón et al., 2019; Hernandez et al., 2021). We predicted that dispersion in microbiome composition will decrease over the season due to sexual transmission and shared habitat use driving microbiome homogenization (Figure 1b, scenario 2). We sampled individuals between their arrival at the breeding site after migration and up to 10 days after clutch initiation. Microbiome dispersion in cloacal microbiome composition should be at its highest immediately after arrival at the breeding site, because individuals differ in migration history and in exposure to diverse marine and tundra habitats. Variation should then homogenise as the season progresses and individuals acclimatise to a similar environment (e.g. homogenization of bacteria associated with convergence in diet) and as a consequence of increased transmission through copulation (Escallón et al., 2019; Hernandez et al., 2021).

Our study adds to an increasing number of studies that demonstrate temporal shifts in cloacal microbiome composition within a breeding season (Escallón et al., 2019; Hernandez et al., 2021). As predicted, we found that in both sexes, the composition of the cloacal microbiome became less dispersed as the season progressed (both at the ASV level and at the level of predicted functional pathways), suggesting temporal convergence in the cloacal microbiome. Moreover, regardless of the sex, 17 predicted functional pathways but only three ASVs shifted in relative abundance over time, indicating a general shift of function after migration which is driven by a variety of bacterial groups. While the within-season effect in our study was quite small and only explained 1.6% of the variation, the comparatively large number of affected predicted functional pathways does suggest that these shifts may have important functional consequences. The impact of these temporal shifts on host fitness and adaptation remains unclear.

4.3 | Similarity within social pairs

Our final prediction was that the cloacal microbiome will be more similar in individuals that are socially paired than among random opposite-sex individuals in the population (Figure 1c). Here, we show that social pair members of the polyandrous red phalarope were significantly more similar in cloacal microbiome composition at the ASV level than randomly chosen opposite-sex individuals sampled at the same time. This finding contradicts the general expectation that socially polyandrous species should show low microbiome similarity between mating partners as a consequence of promiscuity driving high transmission of reproductive microbiomes across social pairs (Rowe et al., 2020), but fits with the idea that most of the sampled females may only have copulated with their social mate (see above). Comparing pair similarity between species of different mating systems is needed to put our results into a broader context.

Three non-mutually exclusive mechanisms may drive higher within-pair similarity in the reproductive microbiome: (i) within-pair convergence of diet-associated microbiota (e.g. convergence of habitat use for feeding; Bodawatta et al., 2021; Dion-Phénix et al., 2021;

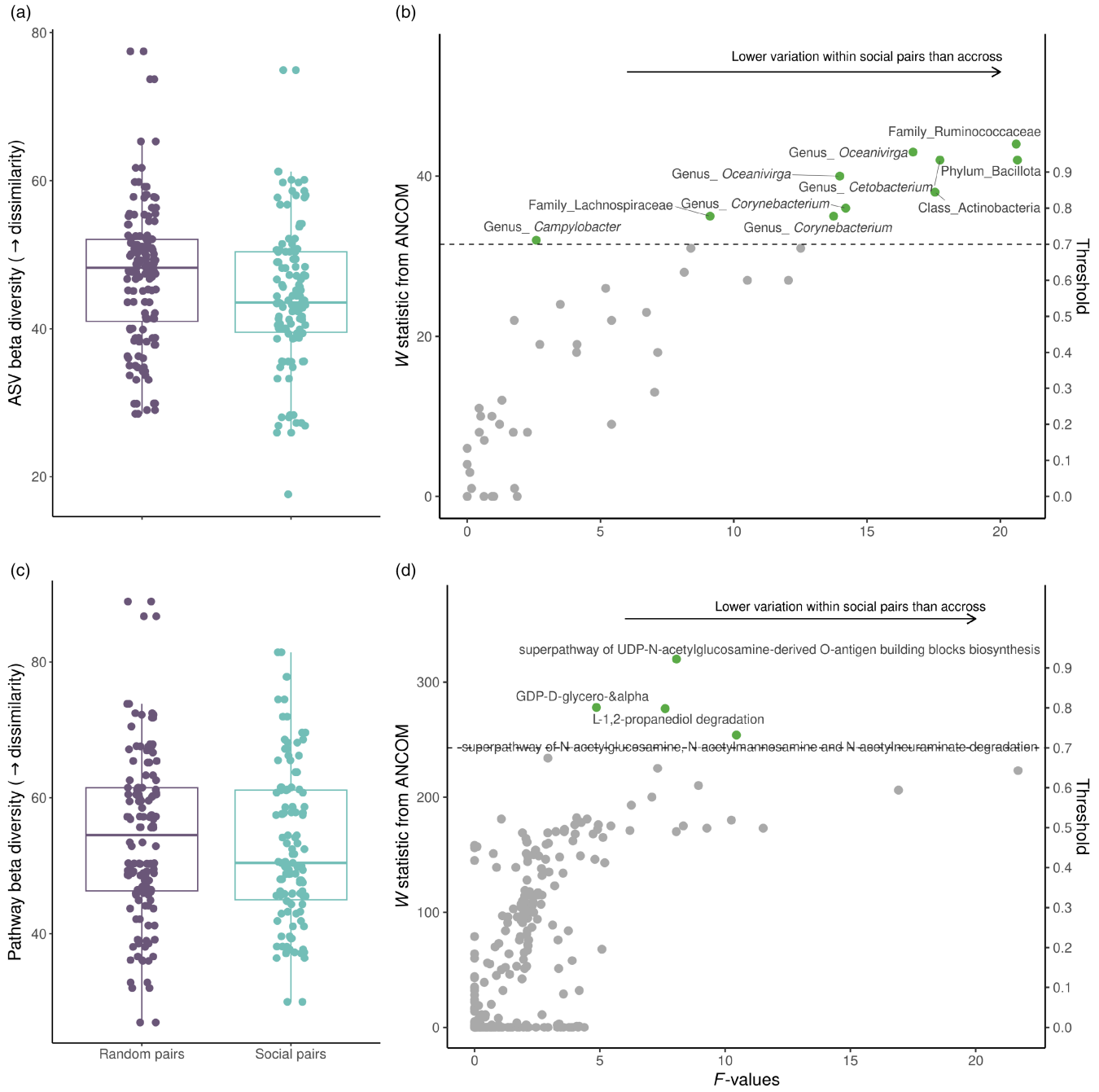

FIGURE 6 Comparison of beta diversity of the cloacal microbiome between random pairs and social pairs (see Section 2 for details). For the 'random pairs', two opposite-sex individuals were chosen that were sampled on the same day and at the closest possible time interval (see Section 2). Beta diversity in amplicon sequence variants (ASVs) (a) and in predicted MetaCyc functional pathways (c) for both groups are shown as individual data points and in boxplots (with raw data). The effect of social pair identity on relative abundance of ASVs (b) and predicted MetaCyc functional pathways (d) is shown in volcano plots based on analysis of composition of microbiomes (ANCOM; see Section 2 for details) according to the *F*-values of centred logged ratios (clr) from linear mixed models. Dots represent individual ASVs or predicted pathways. Those that are above the ANCOM *W* threshold of 0.7 are named.

Teyssier et al., 2020); (ii) assortative mate choice for similar microbiome composition or (iii) sexual transmission of cloacal microbes (Lombardo et al., 1996; Stewart & Rambo, 2000; van Dongen et al., 2019; White et al., 2010, 2011). Members of social pairs that were sampled on different days showed comparable levels of microbiome similarity to social pairs that were sampled at the same time. Moreover, the effect of social pair membership on cloacal bacterial composition was much larger (21.5% of taxonomic cloacal variation was explained by pair ID) than the overall temporal effects (only 1.6% of the variation explained). Combined, these results indicate that social pair members were either already similar prior to pairing or became more similar in their cloacal microbiome immediately

after pairing, a scenario more consistent with rapid sexual transmission as a result of frequent copulations during pair formation than with gradual diet convergence.

If reproductive transmission during copulation is an important mechanism driving pair microbiome similarity, then we can expect the potency of sexual transmission to vary among members of the bacterial community (for instance higher for ejaculate-linked bacteria than for bacteria from the upper gastrointestinal tract). One strong line of evidence is that we observed that some specific strains (a total of 10 ASVs) had lower variation in abundance in social pairs than in random pairs. One of these belongs to the genus *Corynebacterium* (Actinomycetota) which includes several pathogenic species that can be sexually transmitted and have been linked to infertility in mammals and birds (Hartigan, 1980; Riegel et al., 1995; van Dongen et al., 2019). In addition, we also identified higher repeatability in abundance within pairs of taxa associated with host metabolism, such as from the Lachnospiraceae family (Gosalbes et al., 2011; Kittelmann et al., 2013). Increasing the prevalence filtering threshold of analysed ASVs from <2 individuals to <50%, increased the amount of variation explained by pair ID from 21.5% to 29.4%. This suggests that sexual transmission may have affected core microbial communities (i.e. highly prevalent taxa) more than rarer and potentially transient taxa. Overall, our results are consistent with sexual transmission being an important mechanism of host microbial colonisation, whereby these taxa may span the spectrum of the beneficial-pathogenic axis.

The effect of within-pair similarity of predicted functional profiles of the reproductive microbiome was much weaker than the effect observed at the taxonomic level, suggesting that high functional redundancy between taxa dampens the functional effects of sexual transmission. Nonetheless, we found that 10.1% of variation in predicted functional profiles was explained by social pair membership. Four predicted functional pathways were significantly less variable between social pair members than between random pairs of opposite-sex individuals, consistent with some functional convergence of the cloacal microbiome of mating partners as a consequence of reproductive transmission. It remains unclear whether and how these predicted functions would be linked to reproductive outcomes and fitness. Indeed, while predicted functional databases have greatly improved in the last decade, inferences based on PICRUSt2 and metagenomics are limited by uncertain annotations as well as unknown gene transcription or translation (Djemiel et al., 2022). Moreover, annotation of reference genomes may not be representative of the taxa in non-model host microbiomes (Djemiel et al., 2022). Therefore, the predicted MetaCyc functional pathways identified in this study should only be treated as hypotheses that merit further investigation.

5 | CONCLUSION

Given that sexual transmission appears to shift reproductive microbiomes, it seems highly likely that host mating behaviour will influence the ecology and evolution of the reproductive microbiome, with higher rates of sexual transmission (i.e. bacterial colonisation) predicted in species with higher rates of promiscuity. However, evidence that the reproductive microbiome influences sexual selection is still lacking. Longitudinal and experimental studies which combine microbiome sequencing with metabolome approaches, under controlled conditions, are needed to further decipher the functional consequences of microbial sexual transmission on reproductive outcomes. Such studies could elucidate potential sex-specific effects, which would give scope for the reproductive microbiome to act as a driver for sexually antagonistic strategies (Rowe et al., 2011; Smith & Mueller, 2015). Our study demonstrates the link between social pairing and the reproductive microbiome, providing a baseline for future research at the interface between the reproductive microbiome and sexual selection.

AUTHOR CONTRIBUTIONS

Bart Kempenaers and Hanna Prüter conceived the study with the help of Mark A. F. Gillingham; Johannes Krietsch and Bart Kempenaers collected the samples in the field; Johannes Krietsch managed the database, Sylvia Kuhn organised the lab work; Mark A. F. Gillingham and Hanna Prüter analysed the data with input from Bart Kempenaers and Johannes Krietsch; Hanna Prüter, Mark A. F. Gillingham and Bart Kempenaers wrote the paper with input from all authors.

ACKNOWLEDGEMENTS

We thank Mihai Valcu, Margherita Cragnolini, Eunbi Kwon, Kim Teltscher, Anne Cillard, Alice Pintaric, Fenja Squirrell, Kristina Beck and Andrea Wittenzellner for help in the field, and Margherita Cragnolini for the red phalarope illustrations in Figure 1 and the graphical abstract. We are grateful to the state and federal committees that reviewed and approved permits for this study, and to the Utqiaġvik Iñupiat Corporation for logistic support and access to their lands. We are also grateful to B. Karina Montero and two anonymous reviewers for comments on earlier versions of this manuscript. Open Access funding enabled and organized by Projekt DEAL.

FUNDING INFORMATION

This work was funded by the Max Planck Society (to B.K.).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interest.

DATA AVAILABILITY STATEMENT

The QIIME2, PICRUST2 and R scripts as well as raw data are available at GitLab: https://gitlab.com/gillinghamlab/mpi/red-phalarope-microbiome. Data available from the Dryad Digital Repository https://doi.org/10.5281/zenodo.7928430 (Prüter et al., 2023). The individual 16S rRNA gene sequences of this Targeted Locus Study project have been deposited at DDBJ/EMBL/GenBank under the accession KFVW00000000. The version described in this paper is the first version, KFVW01000000 (BioProject: PRJNA879073).

ETHICS STATEMENT

All procedures were approved by the US Geological Survey Bird Banding Laboratory (permit numbers 23520), the Alaska Department of Fish and Game (permit number 19–143), the US Fish and Wildlife Service (permit number MB210494-0) and the North Slope Borough and the Ukpeaġvik Iñupiat Corporation.

ORCID

Hanna Prüter https://orcid.org/0000-0002-1844-4481
Mark A. F. Gillingham https://orcid.org/0000-0002-7935-9539
Johannes Krietsch https://orcid.org/0000-0002-8080-1734
Sylvia Kuhn https://orcid.org/0000-0002-8728-0337
Bart Kempenaers https://orcid.org/0000-0002-7505-5458

REFERENCES

Aitchison, J. (1982). The statistical analysis of compositional data. *Journal of the Royal Statistical Society. Series B (Methodological)*, *44*(2), 139–177.

Anderson, M. J. (2006). Distance-based tests for homogeneity of multivariate dispersions. *Biometrics*, *62*(1), 245–253. https://doi.org/10.1111/j.1541-0420.2005.00440.x

Anderson, M. J., Ellingsen, K. E., & McArdle, B. H. (2006). Multivariate dispersion as a measure of beta diversity. *Ecology Letters*, *9*(6), 683–693. https://doi.org/10.1111/j.1461-0248.2006.00926.x

Ashby, B., & Gupta, S. (2013). Sexually transmitted infections in polygamous mating systems. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1613), 20120048. https://doi.org/10.1098/rstb.2012.0048

Bartoń, K. (2022). *MuMIn: Multi-model inference*. https://CRAN.R-project.org/package=MuMIn

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G. H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J. A., Maguin, E., Mauchline, T., McClure, R., … Schloter, M. (2020). Microbiome definition re-visited: Old concepts and new challenges. *Microbiome*, *8*(1), 103. https://doi.org/10.1186/s40168-020-00875-0

Bodawatta, K. H., Freiberga, I., Puzejova, K., Sam, K., Poulsen, M., & Jønsson, K. A. (2021). Flexibility and resilience of great tit (*Parus major*) gut microbiomes to changing diets. *Animal Microbiome*, *3*(1), 20. https://doi.org/10.1186/s42523-021-00076-6

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 8. https://doi.org/10.1038/s41587-019-0209-9

Burnham, K. P., & Anderson, D. R. (2002). *Model selection and multimodel inference: A practical information-theoretic approach* (2nd ed.). Springer.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 7. https://doi.org/10.1038/nmeth.3869

Carranco, A. S., Romo, D., de Lourdes Torres, M., Wilhelm, K., Sommer, S., & Gillingham, M. A. F. (2022). Egg microbiota is the starting point of hatchling gut microbiota in the endangered yellow-spotted Amazon river turtle. *Molecular Ecology*, *31*(14), 3917–3933. https://doi.org/10.1111/mec.16548

Caspi, R., Billington, R., Keseler, I. M., Kothari, A., Krummenacker, M., Midford, P. E., Ong, W. K., Paley, S., Subhraveti, P., & Karp, P. D. (2020). The MetaCyc database of metabolic pathways and enzymes—A 2019 update. *Nucleic Acids Research*, *48*(D1), D445–D453. https://doi.org/10.1093/nar/gkz862

Chen, C.-Y., Chen, C.-K., Chen, Y.-Y., Fang, A., Shaw, G. T.-W., Hung, C.-M., & Wang, D. (2020). Maternal gut microbes shape the early-life assembly of gut microbiota in passerine chicks via nests. *Microbiome*, *8*(1), 129. https://doi.org/10.1186/s40168-020-00896-9

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M., & Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. *Science*, *336*(6086), 1255–1262. https://doi.org/10.1126/science.1224203

Coyte, K. Z., Rao, C., Rakoff-Nahoum, S., & Foster, K. R. (2021). Ecological rules for the assembly of microbiome communities. *PLoS Biology*, *19*(2), e3001116. https://doi.org/10.1371/journal.pbio.3001116

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., & Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*, *6*(1), 226. https://doi.org/10.1186/s40168-018-0605-2

DeLong, E. F. (2014). Alien invasions and gut "island biogeography". *Cell*, *159*(2), 233–235. https://doi.org/10.1016/j.cell.2014.09.043

Dion-Phénix, H., Charmantier, A., de Franceschi, C., Bourret, G., Kembel, S. W., & Réale, D. (2021). Bacterial microbiota similarity between predators and prey in a blue tit trophic network. *The ISME Journal*, *15*(4), 4. https://doi.org/10.1038/s41396-020-00836-3

Djemiel, C., Maron, P.-A., Terrat, S., Dequiedt, S., Cottin, A., & Ranjard, L. (2022). Inferring microbiota functions from taxonomic genes: A review. *GigaScience*, *11*, giab090. https://doi.org/10.1093/gigascience/giab090

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., Huttenhower, C., & Langille, M. G. I. (2020). PICRUSt2 for prediction of metagenome functions. *Nature Biotechnology*, *38*(6), 6. https://doi.org/10.1038/s41587-020-0548-6

Dunn, P. O., Lifjeld, J. T., & Whittingham, L. A. (2009). Multiple paternity and offspring quality in tree swallows. *Behavioral Ecology and Sociobiology*, *63*(6), 911–922.

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

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M., & Xavier, J. B. (2012). Animal behavior and the microbiome. *Science*, *338*(6104), 198–199. https://doi.org/10.1126/science.1227412

Gillingham, M. A. F., Béchet, A., Cézilly, F., Wilhelm, K., Rendón-Martos, M., Borghesi, F., Nissardi, S., Baccetti, N., Azafzaf, H., Menke, S., Kayser, Y., & Sommer, S. (2019). Offspring microbiomes differ across breeding sites in a Panmictic species. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00035

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., & Egozcue, J. J. (2017). Microbiome datasets are compositional: And this is not optional. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.02224

Gosalbes, M. J., Durbán, A., Pignatelli, M., Abellan, J. J., Jiménez-Hernández, N., Pérez-Cobas, A. E., Latorre, A., & Moya, A. (2011). Metatranscriptomic approach to analyze the functional human gut microbiota. *PLoS One*, *6*(3), e17447. https://doi.org/10.1371/journal.pone.0017447

Gould, A. L., Zhang, V., Lamberti, L., Jones, E. W., Obadia, B., Korasidis, N., Gavryushkin, A., Carlson, J. M., Beerenwinkel, N., & Ludington, W. B. (2018). Microbiome interactions shape host fitness. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(51), E11951–E11960. https://doi.org/10.1073/pnas.1809349115

Grond, K., Lanctot, R. B., Jumpponen, A., & Sandercock, B. K. (2017). Recruitment and establishment of the gut microbiome in arctic shorebirds. *FEMS Microbiology Ecology*, *93*(12), fix142. https://doi.org/10.1093/femsec/fix142

Grond, K., Sandercock, B. K., Jumpponen, A., & Zeglin, L. H. (2018). The avian gut microbiota: Community, physiology and function in wild birds. *Journal of Avian Biology*, *49*(11), e01788. https://doi.org/10.1111/jav.01788

Harrison, X. A. (2014). Using observation-level random effects to model overdispersion in count data in ecology and evolution. *PeerJ*, *2*, e616. https://doi.org/10.7717/peerj.616

Hartigan, P. J. (1980). Fertility management in the dairy herd: The need to control bacterial contamination of the environment. *Irish Veterinary Journal*, *34*(4), 43–48.

Hernandez, J., Escallón, C., Medina, D., Vernasco, B. J., Walke, J. B., Belden, L. K., & Moore, I. T. (2020). Cloacal bacterial communities of tree swallows (*Tachycineta bicolor*): Similarity within a population, but not between pair-bonded social partners. *PLoS One*, *15*(2), e0228982. https://doi.org/10.1371/journal.pone.0228982

Hernandez, J., Hucul, C., Reasor, E., Smith, T., McGlothlin, J. W., Haak, D. C., Belden, L. K., & Moore, I. T. (2021). Assessing age, breeding stage, and mating activity as drivers of variation in the reproductive microbiome of female tree swallows. *Ecology and Evolution*, *11*(16), 11398–11413. https://doi.org/10.1002/ece3.7929

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

Hooper, L. V., Littman, D. R., & Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science*, *336*(6086), 1268–1273. https://doi.org/10.1126/science.1223490

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Kempenaers, B., & Valcu, M. (2017). Breeding site sampling across the Arctic by individual males of a polygynous shorebird. *Nature*, *541*, 7638. https://doi.org/10.1038/nature20813

Kisand, V., Cuadros, R., & Wikner, J. (2002). Phylogeny of culturable estuarine bacteria catabolizing riverine organic matter in the northern Baltic Sea. *Applied and Environmental Microbiology*, *68*(1), 379–388. https://doi.org/10.1128/AEM.68.1.379-388.2002

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I., & Janssen, P. H. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS One*, *8*(2), e47879. https://doi.org/10.1371/journal.pone.0047879

Kreisinger, J., Čížková, D., Kropáčková, L., & Albrecht, T. (2015). Cloacal microbiome structure in a long-distance migratory bird assessed using deep 16sRNA pyrosequencing. *PLoS One*, *10*(9), e0137401. https://doi.org/10.1371/journal.pone.0137401

Krietsch, J., Cragnolini, M., Kuhn, S., Lanctot, R. B., Saalfeld, S. T., Valcu, M., & Kempenaers, B. (2022). Extrapair paternity in a sequentially polyandrous shorebird: Limited evidence for the sperm storage hypothesis. *Animal Behaviour*, *183*, 77–92. https://doi.org/10.1016/j.anbehav.2021.10.021

Kulkarni, S., & Heeb, P. (2007). Social and sexual behaviours aid transmission of bacteria in birds. *Behavioural Processes*, *74*(1), 88–92. https://doi.org/10.1016/j.beproc.2006.10.005

Liebezeit, J. R., Smith, P. A., Lanctot, R. B., Schekkerman, H., Tulp, I., Kendall, S. J., Tracy, D. M., Rodrigues, R. J., Meltofte, H., Robinson, J. A., Gratto-Trevor, C., Mccaffery, B. J., Morse, J., & Zack, S. W. (2007). Assessing the development of shorebird eggs using the flotation method: Species–Specific and generalized regression models. *Condor*, *109*(1), 32–47. https://doi.org/10.1650/0010-5422(2007)109[32:ATDOSE]2.0.CO;2

Lockhart, A. B., Thrall, P. H., & Antonovics, J. (1996). Sexually transmitted diseases in animals: Ecological and evolutionary implications. *Biological Reviews*, *71*(3), 415–471. https://doi.org/10.1111/j.1469-185X.1996.tb01281.x

Lombardo, M. P., Thorpe, P. A., Cichewicz, R., Henshaw, M., Millard, C., Steen, C., & Zeller, T. K. (1996). Communities of cloacal bacteria in tree swallow families. *The Condor*, *98*(1), 167–172. https://doi.org/10.2307/1369521

Lombardo, M. P., Thorpe, P. A., & Power, H. W. (1999). The beneficial sexually transmitted microbe hypothesis of avian copulation. *Behavioral Ecology*, *10*(3), 333–337. https://doi.org/10.1093/beheco/10.3.333

MacArthur, R. H., & Wilson, E. O. (1967). *The theory of island biogeography (REV-revised)*. Princeton University Press. https://www.jstor.org/stable/j.ctt19cc1t2

Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R., & Peddada, S. D. (2015). Analysis of composition of microbiomes: A novel method for studying microbial composition. *Microbial Ecology in Health and Disease*, *26*(1), 27663. https://doi.org/10.3402/mehd.v26.27663

Martino, C., Morton, J. T., Marotz, C. A., Thompson, L. R., Tripathi, A., Knight, R., & Zengler, K. (2019). A novel sparse compositional technique reveals microbial perturbations. *MSystems*, *4*(1), e00016-19. https://doi.org/10.1128/mSystems.00016-19

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Montero, B. K., Wasimuddin, Schwensow, N., Gillingham, M. A. F., Ratovonamana, Y. R., Rakotondranary, S. J., Corman, V., Drosten, C., Ganzhorn, J. U., & Sommer, S. (2021). Evidence of MHC class I and II influencing viral and helminth infection via the microbiome in a non-human primate. *PLoS Pathogens*, *17*(11), e1009675. https://doi.org/10.1371/journal.ppat.1009675

Morrow, E. H., & Innocenti, P. (2012). Female postmating immune responses, immune system evolution and immunogenic males. *Biological Reviews*, *87*(3), 631–638. https://doi.org/10.1111/j.1469-185X.2011.00214.x

Nakagawa, S., & Cuthill, I. C. (2007). Effect size, confidence interval and statistical significance: A practical guide for biologists. *Biological Reviews*, *82*(4), 591–605. https://doi.org/10.1111/j.1469-185X.2007.00027.x

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., Caceres, M. D., Durand, S., … Weedon, J. (2022). *vegan: Community ecology package*. https://CRAN.R-project.org/package=vegan

Parfrey, L. W., Moreau, C. S., & Russell, J. A. (2018). Introduction: The host-associated microbiome: Pattern, process and function. *Molecular Ecology*, *27*(8), 1749–1765. https://doi.org/10.1111/mec.14706

Price, L. B., Liu, C. M., Johnson, K. E., Aziz, M., Lau, M. K., Bowers, J., Ravel, J., Keim, P. S., Serwadda, D., Wawer, M. J., & Gray, R. H. (2010). The effects of circumcision on the penis microbiome. *PLoS One*, *5*(1), e8422. https://doi.org/10.1371/journal.pone.0008422

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., & Glöckner, F. O. (2007). SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research*, *35*(21), 7188–7196. https://doi.org/10.1093/nar/gkm864

Prüter, H., Gillingham, M., Krietsch, J., Kuhn, S., & Kempenaers, B. (2023). Data from: Sexual transmission may drive pair similarity

of the cloacal microbiome in a polyandrous species. *Dryad Digital Repository*. https://doi.org/10.5281/zenodo.7928430

Quinn, T. P., Erb, I., Gloor, G., Notredame, C., Richardson, M. F., & Crowley, T. M. (2019). A field guide for the compositional analysis of any-omics data. *GigaScience*, *8*(9). https://doi.org/10.1093/gigascience/giz107

Quinn, T. P., Erb, I., Richardson, M. F., & Crowley, T. M. (2018). Understanding sequencing data as compositions: An outlook and review. *Bioinformatics*, *34*(16), 2870–2878. https://doi.org/10.1093/bioinformatics/bty175

R Core Team. (2022). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Reynolds, J. D., Colwell, M. A., & Cooke, F. (1986). Sexual selection and spring arrival times of red-necked and Wilson's phalaropes. *Behavioral Ecology and Sociobiology*, *18*(4), 303–310. https://doi.org/10.1007/BF00300008

Riegel, P., Ruimy, R., de Briel, D., Prévost, G., Jehl, F., Bimet, F., Christen, R., & Monteil, H. (1995). *Corynebacterium seminale* sp. Nov., a new species associated with genital infections in male patients. *Journal of Clinical Microbiology*, *33*(9), 2244–2249. https://doi.org/10.1128/jcm.33.9.2244-2249.1995

Risely, A., Gillingham, M. A. F., Béchet, A., Brändel, S., Heni, A. C., Heurich, M., Menke, S., Manser, M. B., Tschapka, M., Wasimuddin, & Sommer, S. (2021). Phylogeny- and abundance-based metrics allow for the consistent comparison of core gut microbiome diversity indices across host species. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.659918

Rowe, M., Czirják, G. Á., McGraw, K. J., & Giraudeau, M. (2011). Sexual ornamentation reflects antibacterial activity of ejaculates in mallards. *Biology Letters*, *7*(5), 740–742. https://doi.org/10.1098/rsbl.2011.0276

Rowe, M., Veerus, L., Trosvik, P., Buckling, A., & Pizzari, T. (2020). The reproductive microbiome: An emerging driver of sexual selection, sexual conflict, mating systems, and reproductive isolation. *Trends in Ecology & Evolution*, *35*(3), 220–234. https://doi.org/10.1016/j.tree.2019.11.004

Sarkar, A., Harty, S., Johnson, K. V.-A., Moeller, A. H., Archie, E. A., Schell, L. D., Carmody, R. N., Clutton-Brock, T. H., Dunbar, R. I. M., & Burnet, P. W. J. (2020). Microbial transmission in animal social networks and the social microbiome. *Nature Ecology & Evolution*, *4*(8), 8. https://doi.org/10.1038/s41559-020-1220-8

Schoenhofen, I. C., Vinogradov, E., Whitfield, D. M., Brisson, J.-R., & Logan, S. M. (2009). The CMP-legionaminic acid pathway in campylobacter: Biosynthesis involving novel GDP-linked precursors. *Glycobiology*, *19*(7), 715–725. https://doi.org/10.1093/glycob/cwp039

Shannon, C. E., & Weaver, W. (1949). *The mathematical theory of communication*. University of Illinois Press.

Sheldon, B. C. (1993). Sexually transmitted disease in birds: Occurrence and evolutionary significance. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *339*(1290), 491–497. https://doi.org/10.1098/rstb.1993.0044

Shin, S. C., Kim, S.-H., You, H., Kim, B., Kim, A. C., Lee, K.-A., Yoon, J.-H., Ryu, J.-H., & Lee, W.-J. (2011). Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science*, *334*(6056), 670–674. https://doi.org/10.1126/science.1212782

Skeen, H. R., Cooper, N. W., Hackett, S. J., Bates, J. M., & Marra, P. P. (2021). Repeated sampling of individuals reveals impact of tropical and temperate habitats on microbiota of a migratory bird. *Molecular Ecology*, *30*(22), 5900–5916. https://doi.org/10.1111/mec.16170

Smith, C. C., & Mueller, U. G. (2015). Sexual transmission of beneficial microbes. *Trends in Ecology & Evolution*, *30*(8), 438–440. https://doi.org/10.1016/j.tree.2015.05.006

Stagaman, K., Burns, A. R., Guillemin, K., & Bohannan, B. J. (2017). The role of adaptive immunity as an ecological filter on the gut microbiota in zebrafish. *The ISME Journal*, *11*(7), 7–1639. https://doi.org/10.1038/ismej.2017.28

Stewart, R., & Rambo, T. B. (2000). Cloacal microbes in house sparrows. *The Condor*, *102*(3), 679–684. https://doi.org/10.1093/condor/102.3.679

Stoffel, M. A., Nakagawa, S., & Schielzeth, H. (2017). rptR: Repeatability estimation and variance decomposition by generalized linear mixed-effects models. *Methods in Ecology and Evolution*, *8*(11), 1639–1644. https://doi.org/10.1111/2041-210X.12797

Tachedjian, G., Aldunate, M., Bradshaw, C. S., & Cone, R. A. (2017). The role of lactic acid production by probiotic lactobacillus species in vaginal health. *Research in Microbiology*, *168*(9), 782–792. https://doi.org/10.1016/j.resmic.2017.04.001

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

Teyssier, A., Matthysen, E., Hudin, N. S., de Neve, L., White, J., & Lens, L. (2020). Diet contributes to urban-induced alterations in gut microbiota: Experimental evidence from a wild passerine. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1920), 20192182. https://doi.org/10.1098/rspb.2019.2182

Tracy, D. M., Schamel, D., & Dale, J. (2020). Red phalarope (*Phalaropus fulicarius*), version 1.0. In *Birds of the World*. https://doi.org/10.2173/bow.redpha1.01

Turner, S., Pryer, K. M., Miao, V. P. W., & Palmer, J. D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *Journal of Eukaryotic Microbiology*, *46*(4), 327–338. https://doi.org/10.1111/j.1550-7408.1999.tb04612.x

van Dongen, W. F. D., White, J., Brandl, H. B., Leclaire, S., Hatch, S. A., Danchin, É., & Wagner, R. H. (2019). Experimental evidence of a sexually transmitted infection in a wild vertebrate, the black-legged kittiwake (*Rissa tridactyla*). *Biological Journal of the Linnean Society*, *127*(2), 292–298. https://doi.org/10.1093/biolinnean/blz009

Verster, A. J., & Borenstein, E. (2018). Competitive lottery-based assembly of selected clades in the human gut microbiome. *Microbiome*, *6*(1), 186. https://doi.org/10.1186/s40168-018-0571-8

Videvall, E., Song, S. J., Bensch, H. M., Strandh, M., Engelbrecht, A., Serfontein, N., Hellgren, O., Olivier, A., Cloete, S., Knight, R., & Cornwallis, C. K. (2019). Major shifts in gut microbiota during development and its relationship to growth in ostriches. *Molecular Ecology*, *28*(10), 2653–2667. https://doi.org/10.1111/mec.15087

White, J., Mirleau, P., Danchin, E., Mulard, H., Hatch, S. A., Heeb, P., & Wagner, R. H. (2010). Sexually transmitted bacteria affect female cloacal assemblages in a wild bird. *Ecology Letters*, *13*(12), 1515–1524. https://doi.org/10.1111/j.1461-0248.2010.01542.x

White, J., Richard, M., Massot, M., & Meylan, S. (2011). Cloacal bacterial diversity increases with multiple mates: Evidence of sexual transmission in female common lizards. *PLoS One*, *6*(7), e22339. https://doi.org/10.1371/journal.pone.0022339

Zaneveld, J. R., McMinds, R., & Vega Thurber, R. (2017). Stress and stability: Applying the Anna Karenina principle to animal microbiomes. *Nature Microbiology*, *2*(9), 9. https://doi.org/10.1038/nmicrobiol.2017.121

Zuur, A., Ieno, E. N., Walker, N., Saveiliev, A. A., & Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R*. Springer.

Zuur, A. F., & Ieno, E. N. (2016). A protocol for conducting and presenting results of regression-type analyses. *Methods in Ecology and Evolution*, *7*(6), 636–645. https://doi.org/10.1111/2041-210X.12577

SUPPORTING INFORMATION

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

Figure S1. Relative abundances of bacteria grouped by phylum from the cloaca of 84 male and 72 female red phalaropes that nested in the study area.

Figure S2. The effect of sex and sampling date on amplicon sequence variant Shannon diversity.

Figure S3. Comparison of beta diversity of the cloacal microbiome between random and social pairs.

Figure S4. Redundancy analysis ordination plot of amplicon sequence variant (A) and predicted MetaCyc functional pathway (B) beta diversity of the cloacal microbiome as a function of social pair ID.

Table S1. Candidate generalised linear mixed models of the effect of sequencing depth, sex and sampling date relative to clutch initiation (Date) on amplicon sequencing variant (ASV) species richness and on ASV Shannon diversity.

Table S2. Candidate generalised linear mixed models of the effect of sequencing depth, sex and sampling date (Date) on amplicon sequencing variant (ASV) species richness and on ASV Shannon diversity.

Table S3. Changes in fit if an explanatory variable is dropped from the full redundancy analysis model of cloacal microbiome amplicon sequence variant beta diversity of red phalaropes, based on a reduced dataset with a single individual per pair.

Table S4. Candidate linear mixed models of the effect of sampling date relative to clutch initiation date (Date) on amplicon sequence variant beta diversity within a social pair (i.e. within social pair microbiome similarity as a function of time).

Table S5. Candidate generalised linear mixed models of the effect of sequencing depth, sex and sampling date relative to clutch initiation date (Date) on the number of pathways and on pathway Shannon diversity.

Table S6. Candidate generalised linear mixed models of the effect of sequencing depth, sex and sampling date (Date) on the number of pathways and on pathway Shannon diversity.

Table S7. Changes in fit if an explanatory variable is dropped from the full redundancy analysis model of cloacal microbiome pathway beta diversity of red phalaropes, based on a reduced dataset with a single individual per pair.

Table S8. Candidate linear mixed models of the effect of sampling date relative to clutch initiation (Date) on pathway beta diversity within a social pair (i.e. within social pair microbiome similarity as a function of time).

Table S9. Candidate linear mixed model of the effect of sex, sampling date (Date) and their interaction on amplicon sequence variant beta diversity divergence from the population median.

Table S10. Candidate linear mixed model of the effect of sex, sampling date (Date) and their interaction term on functional pathway beta diversity divergence from the population median.

How to cite this article: Prüter, H., Gillingham, M. A. F., Krietsch, J., Kuhn, S., & Kempenaers, B. (2023). Sexual transmission may drive pair similarity of the cloacal microbiome in a polyandrous species. *Journal of Animal Ecology*, *92*, 1639–1657. https://doi.org/10.1111/1365-2656.13961